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Quantification of bovine leukemia virus proviral DNA using a low-cost real-time polymerase chain reaction

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ABSTRACT

The detection of bovine leukemia virus (BLV) proviral DNA is an important tool to address whether an animal is infected with BLV. Compared with serological assays, real-time PCR accounts for greater sensitivity and can serve as a confirmatory test for the clarification of inconclusive or discordant serological test results. However, the high cost related to real-time PCR assays has limited their systematic inclusion in BLV surveillance and eradication programs. The aim of the present study was to validate a low-cost quantitative real-time PCR. Interestingly, by using SYBR Green detection dye, we were able to reduce the cost of a single reaction by a factor of 5 compared with most common assays based on the use of fluorogenic probes (i.e., TaqMan technology). This approach allowed a highly sensitive and specific detection and quantification of BLV proviral DNA from purified peripheral blood leukocytes and a milk matrix. Due to its simplicity and low cost, our in-house BLV SYBR quantitative real-time PCR might be used either as a screening or as a confirmatory test in BLV control programs.
Key words: bovine leukemia virus, proviral DNA, real-time polymerase chain reaction, epidemiology

INTRODUCTION

Bovine leukemia virus (**BLV**) is a deltaretrovirus from the *Orthoretrovirinae* subfamily and *Retroviridae* family. The BLV causes a persistent infection in cattle, and in most cases this infection is asymptomatic (Ghysdael et al., 1984). In one-third of infected animals the infection progresses to a state of persistent lymphocytosis, and in 1 to 10% of infected cattle it develops into lymphosarcoma (Ghysdael et al., 1984). Bovine leukemia virus is distributed worldwide with the exception of Western Europe and Oceania. This virus was first found in Argentina in 1973; since then, BLV rapidly spread throughout the region (Ciprian, 1973; Trono et al., 2001). A recent study highlighted that the average within-herd prevalence of BLV in the main productive area of Argentina was 80% (Gutiérrez et al., 2012). High prevalence of BLV is associated with a significant economic impact on the dairy industry due to trade restrictions, replacement cost, reduced milk production, immunosuppression, and increased susceptibility to pneumonia, diarrhea, mastitis, and so on (Trainin et al., 1996; Bartlett et al., 2014; Frie and Coussens, 2015).

Classic BLV eradication programs rely on the correct identification and segregation or elimination of BLV-infected animals. Serologic assays for the detection of BLV-specific antibodies in sera and milk have been widely used as screening tests due to their high sensitivity and ability to test a large number of samples at a very low cost. In this regard, the agar gel immunodiffusion and ELISA are both cited as prescribed tests (OIE, 2012). However, serologic tests might fail to detect BLV-specific antibodies in samples from animals that have been recently infected with BLV due to the low level of BLV-specific antibodies present in these particular samples (Eaves et al., 1994). Furthermore, the inclusion of highly sensitive screening assays might increase the efficiency of a particular eradication program, especially for herds with a low within-herd prevalence. For that reason, the development and validation of more sensitive assays for BLV testing in cattle are necessary.

Nucleic acid amplification tests, such as PCR, allow a rapid and highly sensitive detection of BLV proviral DNA, even during recent infection. Compared with serological assays, quantitative real-time PCR (**qPCR**) not only allows the detection of BLV infection but also allows the estimation of BLV proviral load, which directly correlates with the disease stage and the risk of disease transmission (Yuan et al., 2015; Juliarena et al., 2016). Additionally, qPCR assays can serve as confirmatory tests for the clarification of inconclusive and discordant serological test results (Rola-Łuszczak et al.,

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Table 1. Bovine leukemia virus (BLV) quantitative real-time PCR feasibility and reproducibility using a reference panel

Sample ID (BLV status)[1]	BLV copies,[2] no./μg of DNA	Ct[3] mean	SD	CV, %	Replicates, no.	Positive replicates, no.
A (–)	NA[4]	0	0.0	NA	8	0
B (–)	NA	0	0.0	NA	8	0
C (+)	45,000	22.74	0.28	1.23	8	8
D (+)	10,000	23.61	0.09	0.40	8	8
E (+)	8,000	24.25	0.08	0.35	8	8
F (+)	2,000	26.39	0.10	0.37	8	8
G (+)	1,000	28.55	0.19	0.66	8	8
H (+)	500	28.88	0.32	1.10	8	8
I (+)	500	29.00	0.41	1.40	8	8
J (+)	400	29.02	0.14	0.47	8	8
K (+)	400	29.92	0.22	0.72	8	8
L (+)	10	30.71	0.67	2.18	8	8
M (+)	100	31.05	0.44	1.41	8	8
N (+)	50	31.64	0.71	2.25	8	8

[1]– = negative; + = positive.

[2]BLV copies per microgram of DNA as estimated in the reference laboratory (National Veterinary Research Institute, Pulawy, Poland).

[3]Cycle threshold.

[4]Not applicable.

2013). Several qPCR assays have been developed for BLV proviral DNA detection (Lew et al., 2004; Heenemann et al., 2012; Rola-Łuszczak et al., 2013). Previous studies comparing *pol*, *gag*, and *env* genes reported that *pol* was the most suitable gene to target for detection purposes because it provided the most sensitive assays (Rola-Łuszczak et al., 2013). This might be in part due to a higher sequence conservation of *pol* among strains from different geographical areas. Importantly, most of these assays are based on the use of fluorogenic probes, making them very expensive for use as screening tests in BLV eradication programs.

The aim of the present study was to validate a low-cost qPCR for the detection of BLV proviral DNA. For that purpose we used an international panel of BLV proviral DNA samples and a set of field samples obtained from the main productive area of Argentina. Overall, the assay allowed a highly sensitive and specific detection of BLV proviral DNA from purified peripheral blood leukocytes (**PBL**) and a milk matrix.

MATERIALS AND METHODS

Reference Samples

Bovine leukemia virus DNA reference samples were obtained from 12 serologically positive and 2 serologically negative cattle from 7 countries (Ukraine, Russia, Moldova, Croatia, Japan, Argentina, and Poland). The number of BLV proviral DNA copies in each of these samples was determined by qPCR in 5 laboratories worldwide: (1) the Molecular and Cellular Biology Laboratory of Gemboux, University of Liege, Liege, Belgium; (2) the Institute of Virology, Center for Infectious Diseases, University of Leipzig, Leipzig, Germany; (3) the Department of Veterinary Medicine, Iwate University, Iwate, Japan; (4) the National Veterinary Research Institute (**NVRI**), Pulawy, Poland; and (5) the Animal and Plant Health Agency, Weybridge, United Kingdom. Three laboratories at the Animal and Plant Health Agency, NVRI, and Leipzig University act as the World Organization of Animal Health reference laboratories for enzootic bovine leukemia. The average number of BLV copies in these samples ranged from 10 to 50,000/μg of total DNA (Table 1). The reference panel was kindly provided by Jacek Kuzmak (NVRI, Poland).

Field Samples Collection and DNA Extraction

Field samples were obtained from a dairy farm located in Rafaela, Santa Fe, Argentina (31°16S, 61°29W). This region belongs to the main dairy-producing area of the country, with an average within-herd prevalence of BLV of 80%. The farm comprised a milking herd (n = 330 Holstein cows), 64 dry cows, 94 heifers (>1 yr) and 123 calves (<1 yr). The BLV serological status of all animals was determined by ELISA at the time that the study was initiated. Consistent with other farms located in the same region, the individual cow prevalence for this particular farm was close to 90%. For this study, a total of 67 animals (>1 yr) were randomly selected based on their serological status (24 BLV sero-negative and 43 BLV sero-positive animals). Sero-conversion