Inter-laboratory comparison of six real time polymerase chain reaction assays for detection of bovine leukemia virus proviral DNA

Running title: First international ring-trial of qPCR for BLV

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Abstract

Real-time polymerase chain reaction (qPCR) is increasingly being used for detection of bovine leukemia virus (BLV) proviral DNA. Nevertheless, quality control for validation and standardization of such tests are currently lacking. Therefore, the present study was initiated by three OIE reference laboratories and three collaborating laboratories to measure inter-laboratory variability of six, already developed and available, BLV qPCR assays. For that purpose, an international panel of 58 DNA samples reflecting the dynamic range of the majority of the assays was distributed to 6 testing centers. Based on qualitative results, the overall agreement amongst all six laboratories was moderate. However, significant variability in BLV proviral DNA copy number measurement was observed amongst different laboratories. Quantitative PCR assays, even when performed by experienced staff, can yield large variability in BLV proviral DNA copy number without harmonization. Further standardization of different factors (i.e. utilization of unified protocols and unique calibrators) should increase inter-laboratory agreement.

Keywords: Bovine Leukemia Virus (BLV); Proviral DNA; Inter-laboratory ring-trial; Real-time polymerase chain reaction (qPCR)

Introduction

Bovine leukemia virus (BLV) is a Deltaretrovirus in the Orthoretrovirinae subfamily, Retroviridae family and is the etiologic agent of Enzootic Bovine Leucosis (EBL) (1). BLV in cattle causes a persistent infection, which in most cases remains clinically silent; however, in one third of infected animals progress to a state of persistent lymphocytosis and 1 to 10% of infected cattle develop lymphosarcoma (2). BLV causes 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 other diseases (3).

BLV is distributed worldwide, with high prevalence reported in North and South America, Asia and Eastern Europe. The disease has been eradicated from Western Europe, Scandinavia and Oceania due to the implementation of official programs, based on the detection and sacrifice of infected animals, thus providing a trading advantage.

Serologic assays have been widely used as screening tests for BLV due to their ability to test a large number of samples at a very low-cost and high sensitivity (4). However, there are multiple scenarios where direct detection of the antigen is necessary e.g. screening calves with maternal antibodies, hyper-acute infection or animals not generating a persistent antibody response. In this regard, nucleic acid amplification tests, such as polymerase chain reaction (PCR), play an increasing role in the detection of BLV proviral DNA. Moreover, the use of quantitative PCR (qPCR) assays has the added benefit of the generation of quantitative results (5-7). Considering that BLV proviral load directly correlates with the risk of transmission (8, 9), this feature of qPCR is important for developing rational segregation programs, based on minimizing the risk of transmission. In addition, the high sensitivity characteristic of qPCR is crucial, as BLV may be present in very low quantities in some infected, seronegative and asymptomatic animals that are responsible for the recurrence of infection in disease free-herds (10, 11).

In recent years, several molecular tests based on real-time technology have been developed and used for detection of BLV proviral DNA in naturally infected animals (5-7, 12, 13). Nevertheless, quality control for validation and standardization of such tests are currently lacking. Evaluation of overall inter-laboratory variability in methods and results is necessary for the future implementation of an appropriate harmonization scheme (14, 15). Furthermore, harmonization and standardization of BLV qPCR assays are essential to be able to compare

Journal of Clinical Microbiology

data from international surveillance programs (14-17). Therefore, a study was initiated by three OIE reference laboratories and three collaborating laboratories to assess inter-laboratory variability of six, already developed and available real-time PCR assays for detection of BLV proviral DNA.

Material and Methods

Participants

The six laboratories that took part in the study were at (i) the National Agricultural Technology Institute (INTA), Buenos Aires, Argentina (ARG); (ii) the Molecular and Cellular Biology Laboratory of Gembloux Agro-Bio Tech, Gembloux, Belgium (BE); (iii) the Institute of Virology, Center for Infectious Diseases, University of Leipzig, Leipzig, Germany (GER); (iv) the Department of Veterinary Medicine, Iwate University, Iwate, Japan (JPN); (v) the National Veterinary Research Institute (NVRI), Pulawy, Poland (PL) and (vi) the Animal and Plant Health Agency (APHA), Weybridge, United Kingdom (UK). Three laboratories at APHA, NVRI and University of Leipzig are acting as OIE reference laboratories for EBL. The six participating laboratories were termed: ARG, BE, GER, JPN, PL and UK respectively.

Samples collection and DNA extraction

Blood samples were taken from a total of 56 cattle, serologically positive for BLV infection. The animals came from seven countries (n): Ukraine (8), Russia (10), Moldova (9), Croatia (1), Japan (4), Argentina (5) and Poland (19). The serological testing and sample processing were conducted by the laboratories from where the samples - originated. At the NVRI, peripheral blood leukocytes (PBLs) were isolated by centrifugation at 1,500 g for 25 min and erythrocytes were hemolysed by osmotic shock with H_2O and 4.5% NaCl. After two

washes in PBS, the supernatant was discarded and the cell pellet was used for extraction of genomic DNA. PBLs isolated from cattle from Ukraine, Russia, Moldova were sent to NVRI as a dry pellets. Genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen), following manufacturer's recommendations. The genomic DNA from Argentinian samples was extracted from peripheral blood mononuclear cells (PBMCs) using High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. Samples from Japan were preprocessed as previously described by Somura and colleagues (18); briefly, DNeasy Blood and Tissue Kit was used to purify genomic DNA from blood. The DNA samples from Argentina and Japan were then sent to NVRI in the form of DNA solutions. One DNA sample from Croatia was kindly supplied by Dr D. Balic (Veterinary Institute, Vinkovci,). Additionally, one DNA sample extracted from FLK-BLV cells and one sample extracted from PBLs of serologically negative cattle were included as positive and negative controls, respectively. DNA concentration in all samples was calculated using nanophotometer (Implen). Each sample was divided into seven identical aliquots containing 1.5 µg of DNA. Seven identical sets of these samples were prepared and distributed to participating laboratories. The samples were stored and shipped refrigerated at 4^0C.

Examination of DNA quality/stability

Since different extraction and purification methods were employed for the preparation of the DNA samples it was necessary to test the quality of the DNA. For that purpose, one complete set of samples (n=58) was tested by qPCR to detect H3 histone family 3A (H3F3A) housekeeping gene copy number (19). Results were expressed as number of H3F3A gene copy per 500 ng of DNA, present in each sample. To determine whether any outlier values of H3F3A copy numbers in these samples, Grubbs' test was performed. In order to test the

stability of DNA, samples were stored at +4^{0}C and retested by qPCR to detect H3F3A copy numbers, 21 days after the original measurement. T-test was used to compare the mean values between the two groups (time 0 and time 21days at +4^{0}C).

Sample distribution

All samples were coded (numbers 1-58) in order that the testing was performed blind. The samples were air-shipped with a cold pack. All the samples arrived at their destination within 2 to 5 days.

Description of BLV real time PCR (qPCR) protocols used by participant laboratories

All participating laboratories performed the qPCR using a variety of different equipment, reagents and reaction conditions, that have been previously set-up, validated and evaluated and are currently used as working protocols. The specific features of each of these protocols are described below and summarized in **Table 1**. All laboratories applied standard procedures for avoiding false positive results as a consequence of DNA contamination, such as the use of separate rooms for preparing reactions, adding the samples and performing the amplification reaction. Five out of six BLV qPCRs used *pol* gene as target sequence for amplification, while qPCR JPN amplified *tax* gene.

BLV qPCR (ARG)

PCR reactions for BLV contained Fast Start Universal SYBR Green Master Mix (Roche), 800 nM of forward and reverse primers (Fw: 5'–CCTCAATTCCCTTTAAACTA-3'; Rv: 5'–GTACCGGGAAGACTGGATTA–3' (7) and 100 ng of purified DNA as template. Real-time PCR was performed on the ABI 7500 machine (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 95°C for 10 min, followed by 40 cycles at 95°C

for 15 s, 55°C for 15 s and 60°C for 1 min. After completing the reaction, the specificity of the amplicons was checked by analyzing the individual dissociation curves. As standard, a plasmid pBLV1 containing BLV *pol* fragment was used (kindly provided by Dr. J. Kuzmak, NVRI). Ten-fold dilutions of this standard were made from 5 x 10^{6} copies μl^{-1} to 5 copy μl^{-1}. High and weak positive controls, as well as two negative controls, were included in each tested plate.

BLV qPCR (BE)

BLV sequences were PCR amplified using *pol* gene sequence-specific primers Fw: 5'-GAAACTCCAGAGCAATGGCATAA-3' and Rv: 5'-GGTTCGGCCATCGAGACA-3'. As reference for quantification, *β-actin* was amplified with oligonucleotides Fw: 5'-TCCCTGGAGAAGAGCTACGA-3' and Rv: 5'-GGCAGACTTAGCCTCCAGTG-3' (20). DNA was amplified in a Roche light cycler using MESA green master mix (Eurogentec). The thermal protocol was initiated by a 5 min denaturation step at 95°C, followed by 45 cycles (15 s at 95°C, 20 s at 60°C, 40 s at 72°C) and terminated by a melting curve. PCR efficiencies were calculated for each sample using 100ng, 33ng and 11ng of DNA. Standard curves were generated using PCR4topo vectors (Life Technologies) containing the corresponding *pol* or *actin* amplicon. Proviral load was calculated, as an average of the three dilutions, from the number of proviral copies divided by half of the number of Actin copies and expressed as number of proviral copies per 100 of PBMCs

BLV qPCR (GER)

The BLV real-time PCR was performed as previously published (5) with the following details: the PCR was performed in a 20 µl reaction volume using the TaqMan universal PCR master mix including AmpliTaq Gold DNA Polymerase, AMPErase UNG,

deoxyribonucleotide triphosphate with 2′-deoxyuridine 5′-triphosphate, and passive reference dye (5-carboxy-X-rhodamine [ROX]). The reaction mix included 10 µl of universal PCR master mix, 10 pmol/µl of each oligo (*pol*-specific primers, *pol*-sp probe and internal control probe), and 50 ng of the sample DNA. If necessary, distilled H2O was added until 20 µl reaction volume was reached. A control plasmid containing *pol* amplicon (112-bp) was diluted from 4.68 x 10^{6} to 4.68 x 10^{-1} to generate a standard curve. The reaction was carried out using the Rotor-Gene Q (QIAGEN) with the following temperature profile: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 58°C. The fluorescence data were collected during the annealing step for the BLV *pol* probe in the green channel and for the BLV control probe in the yellow channel.

BLV qPCR (JPN)

The BLV proviral load was measured using a Cycleave PCR bovine leukemia virus detection kit (TaKaRa, Shiga, Japan) and an ABI Step One plus (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Cycleave BLV qPCR is a commercially available method designed to amplify the BLV *tax* region of BLV genome.

BLV qPCR (PL)

The BLV qPCR was performed as was previously published (7). Quantitative TaqMan® PCR was carried out in 25 µl PCR mixture containing 12.5 µl of 2× QuantiTect Multiplex PCR NOROX Master Mix (Qiagen), 0.4 µM of each of the primers and 0.2 µM of the specific BLV probe and 500 ng of extracted genomic DNA. The amplification was performed in the Rotor-Gene Q System (Qiagen) using an initial denaturation step and polymerase activation at 95°C for 15 min, followed by 50 cycles of 94°C for 60 seconds and 60°C for 60 seconds. All samples were amplified in duplicate. As standard, the pBLV1

plasmid containing BLV 120 bp *pol* fragment was used. Ten-fold dilutions of this standard were made from 1 x 10^6 copies per µl to 1 x 10^0 copy per µl, and used to estimate the BLV copy numbers.

BLV qPCR (UK)

APHA´s protocol was performed as at the NVRI (7) except for the use of 5x Quantifast Master Mix (Qiagen), instead of 2× QuantiTect Multiplex PCR NOROX Master Mix (Qiagen). Ten-fold dilutions of pBLV1 standard were made from 1 x 10^6 copies per µl to 1 x 10^0 copy per µl, and used to estimate the BLV copy numbers.

Analysis of BLV *pol* and *tax* sequences targeted by particular qPCR assays

In order to assess full length *pol* and *tax* sequence variability amongst BLV genotypes, all BLV whole genome sequences (n=74) available in public repositories (https://www.ncbi.nlm.nih.gov/genbank/) were retrieved. Thirty eight of these full genome sequences had already been assigned to *env*-based BLV genotypes (i.e, G1, G2, G4, G6, G9 and G10) and the remaining were of unknown genotypes. In order to represent all known genotypes (i.e., G1-G10), complete Env sequences belonging to each missing genotype (G3, G5, G7 and G8) were considered (n=12). Thus, an *env*-based ML phylogenetic tree (**Figure S1**) was constructed in order to assign genotypes to the not-assigned BLV genomes. Sequences were aligned using the MAFFT program (21). The model selection for the best fit the sequence data was performed through the SMS software (22). A ML tree employing Akaike information criteria (AIC)-based HKY85 + I model, as implemented in the PhyML program (23), was built. As a measure of the branches robustness an approximate Likelihood Ratio Test (aLRT) was calculated. From this analysis, a total of 74 *pol* and *tax* full length sequences from 7-assigned BLV genotypes (G1, G2, G3, G4, G6, G9 and G10) were used for

multiple sequence alignment (MSA) according to the MAFFT program. Measures of nucleotide diversity (π) and nucleotide differences per site (dps) were calculated using MEGA7 software (24) as $\pi = \sum_{ij} x_i x_j \pi_{ij} = 2 * \sum_{i=2}^{n} \sum_{j=1}^{i-1} x_i x_j \pi_{ij}$; where x_i and x_j are the frequencies of the ith and jth sequences, π_{ij} is the number of nucleotide differences per nucleotide site between the ith and jth sequences, and n is the absolute number of sequences in study. Shannon information entropy (H) *per site* was calculated using SHiAT v1.1 (25) where: $H(X) = -\sum_{i=1}^{n} p(x_i)\, log_m p(x_i)$; $p(x_i)$ = observed probability (frequency) of nucleotide x_i for all possible i and m = 5 (A, C, G, T and gaps) is the number of possible information states.

Results

Examination of the quality and stability of DNA samples

In order to test the quality of DNA samples the *H3F3A* gene copy number was calculated per 500 ng of DNA at the NVRI and then the respective values were tested by Grubs' test. Results of all 58 DNA samples followed a normal distribution, with a mean value of 46,923 copies (95% CI = 42,858 – 52,984); min value=1,552; max value=87,669 (**Table S1**). Despite this single very low value, no outlier was found for any samples (p value <0.05). Therefore, it can be assumed that the DNA quality was acceptable for all samples present in the panel.

Next, DNA stability was assessed by retesting the *H3F3A* gene copy number in all samples after 21 days at $+4^0$C. T-test was used to compare the mean values between the two groups (time 0 and time 1:21days later) and no significant difference was observed at the 5% level (p=0.18) **(Figure 1).** Therefore it appears that sample storage and shipping did not influence the DNA stability and further testing during the inter-laboratory trial.

Detection of BLV proviral DNA by different qPCR assays

A total of 58 DNA samples, including a positive (sample #53) and a negative (sample #36) control were tested independently by six qPCR methods (**Table 2**). Except for the negative and the positive (FLK cell line) controls all samples showed detectable levels of BLV specific antibodies (BLV-Abs) by ELISA. Both the positive and negative controls were assessed adequately by all six tests. Considering all of the 56 DNA samples from BLV seropositive animals, 47, 45, 49, 32, 53 and 56 samples were detected as positive, when ARG, BE, GER, JPN, PL and UK qPCRs were applied, respectively. Based on these observations, the most sensitive method was the qPCR UK, and the one with the lowest sensitivity was the qPCR JPN. Thirty three out of 58 samples were identified correctly by all qPCRs. In contrast, the other 25 samples gave discordant results. Comparison of qualitative results (positive vs. negative) from all six laboratories revealed 80% overall agreement and a kappa value of 0.342 (Cohen's kappa method adapted by Fleiss). The level of agreement among the results from the six laboratories is represented in the **Table 3**. The highest agreement was seen between two laboratories (PL and UK; 94.8% agreement and 0.549 Cohen´s kappa) which shared similar protocols and targeted the same region of BLV Pol.

Analysis of BLV *pol* and *tax* sequences targeted by particular qPCR assays

Due to differences in performance observed for inter *pol*-based qPCR assays (ARG/PL/UK/GER/BE) and considering that the *tax*-based qPCR (JPN) assay showed the lowest sensitivity and the poorest agreement compared to the other assays, the degree of sequence variability between *pol* and *tax* gene was addressed. From the MSA for *pol* and *tax*, the average number of nucleotide difference per site (dps) was calculated. The dps value for *tax* was lower than *pol* (*tax* 11 dps vs. *pol* 512 dps). In addition, we observed that *tax* nucleotide diversity (π) was lower compared with *pol* [π_{tax} 0.017 (SD: 0.002) vs. π_{pol} 0.211

(SD: 0.02)]. From this analysis, *tax* sequences appeared to be less variable than *pol* sequences. In addition, we performed a Shannon entropy-based per site variability profile of *pol* and *tax* genes (**Figure 2**). This analysis showed a marked region of variability toward the 3´ region of *pol*. Interestingly, we noted that BE qPCR primers targeted this particular region of *pol*; whereas ARG, GER, PL and UK primers targeted the most conserved region (**Figure 2; Fig S2.a-c**). In comparison to *pol*, the profile observed for the *tax* gene was homogeneous along the whole sequence.

Quantitation of BLV proviral DNA by different qPCR assays

In order to analyze whether the range of copy number detected by each qPCR were comparable to each other, the Kruskal–Wallis one-way analysis of variance was used **(Figure 3)**. Independent grouping variable revealed that there were significant differences among the distributions of copy number of proviral DNA when tested by the various qPCRs ($p < 0.0001$). These results showed that the ability of particular qPCR to quantify the copy number of proviral DNA was different. The correlation between copy numbers detected by each qPCRs was calculated. Despite of statistically significant differences found in distribution of copy numbers, a moderate correlation was found (Kendall tau= 0.514, $p<0.001$) between particular qPCRs.

Since inter-laboratory variation may be influenced by the number of BLV proviral copies present in each sample, we compared the average number of BLV copies between the group of samples that gave concordant (Group I; n=33) and discordant results (Group II; n=25). The mean number of copies in group I was 14,634 (0.0-69,491) while in group II was 902 (0.2-19,766) and this difference was statistically significant (t-Student test, $p<0.001$) (**Figure 4**).

Discussion

Classic BLV eradication programs consist in the correct identification and segregation/elimination of BLV infected animals. In this regard, the detection of BLV-Abs (mainly directed to gp51 BLV glycoprotein) in blood serum is the most common indicator of BLV infection; the agar gel immunodiffusion and ELISA are both cited by the OIE as prescribed tests (4). As the eradication program progresses and the prevalence decreases, the inclusion of more sensitive assays might increase the program efficiency. Compared with serological assays, qPCR assays have the potential of detecting BLV DNA even during recent BLV infection, when animals might present transient or very low level of BLV-Abs (2, 8, 11, 26, 27). Additionally, qPCR assays can serve as confirmatory tests for the clarification of inconclusive and discordant serological test results (7). Moreover, qPCR not only allows the detection of BLV infection, but also the estimation of BLV proviral load, which directly correlates with the risk of disease transmission (28, 29). This feature of qPCR is important to perform a more rational segregation of animals, based on the risk of transmission.

In order to compare different qPCR methods from six laboratories distributed worldwide and evaluate the inter-laboratory variability, an international panel of reference positive samples was produced. The amounts of BLV DNA in these samples were representative of the different BLV proviral loads found in field samples (from 1 to more than 100,000 copies of BLV proviral DNA). In addition, it covered the dynamic range of the majority of the assays used in this study. A single negative sample was included in the panel. The addition of further negative samples may have allowed comparison of specificity amongst the different tests; however, as described in the Materials and Methods each assay had already been validated at its originating institute. In addition, appropriate measures were taken to avoid contamination and false-positive results. Therefore, for purposes of this study the inclusion of a single negative sample was deemed sufficient.

Journal of Clinical Microbiology

The comparison of qualitative qPCR results among all six different laboratories (raters) revealed an overall observed agreement of 80%, indicating a strong inter-rater reliability (30). However, considering a kappa value of 0.342, that observation might be overestimated by chance. The kappa statistic was originally described by Cohen to take account for the possibility of guessing; however, it might lower the estimate of agreement excessively (30). Altogether, by adjusting the overall level of agreement using the kappa statistic we observed an expected overall agreement of 70%. From all six laboratories, the higher level of concordance was observed between PL and UK (94.8%; kappa= 0.549). This result was expected since both laboratories shared the same target sequence and a similar reaction protocol, based on TaqMan® technology. Another laboratory (ARG) also targeted the same region of BLV genome; however, the qPCR ARG was based on SYBR green ® dye technology. It has been reported that the use of different reagents and reaction conditions might account for increased variability of qPCR results (14, 15) which may explain for the variation in results as observed in this study

The diagnostic sensitivity (DxSn) was estimated for each particular qPCR assay. From a total of 57 positive samples that constituted the panel, 56 (98%), 53 (93%), 49 (86%), 47 (82%), 45 (79%) and 32 (56%) samples were scored as positive by UK, PL, GER, ARG, BE and JPN, respectively. The qPCR UK and qPCR JPN were the methods with the highest and the lowest sensitivity, respectively. Interestingly, qPCR JPN was the only method that targeted BLV *tax* region instead of *pol*. In this regard, previous studies comparing *pol*, *gag* and *env* genes reported that *pol* gene was the most suitable region to target for diagnostic purposes since it provided the most sensitive assays (5-8, 12, 13, 31). This might be in part due to a higher sequence conservation of *pol* among strains from different geographical areas. However, none of these studies compared *pol* with *tax* genes. In this study we found that *tax* variability and nucleotide diversity was lower when compared with *pol*. A deeper analysis

Journal of Clinical Microbiology

focused on the specific region of *tax* targeted by qPCR JPN would have helped to better understand our observations; however, as the qPCR JPN is a commercial kit, the oligo sequences were not available. Hence, the reason that the *tax* region showed a lower sensitivity compared with *pol* region could not be elucidated. Another possibility is that temperature variation during long-distance shipping might have led to the degradation of DNA in the samples, particularly effecting those with lower concentration. Consequently, the degradation of target DNA might have limited the detection of BLV DNA by qPCR.

Since we only had partial information regarding the genotypes of the sample panel, we considered all *pol* sequences available from BLV full length genomes in order to visualize how sequence variability would impact the annealing of the different primers used in each of the *pol* qPCRs. The variability profile analysis of the *pol* gene showed that ARG, POL, UK and GER oligos were located in the less variable region, whereas BE oligos aligned to a region of high variability. This is in agreement with the level of sensitivity observed for each of the *pol* based qPCR assays in this study (UK>PL>GER>ARG>BE).

Since the detectability of each assay and consequently, the level of agreement among assays might be also influenced by the number of BLV proviral copies present in each sample (8), we compared the level of proviral DNA from samples that gave concordant and discordant results. This analysis showed that samples that gave discordant results had significantly lower number of BLV copies compared to the samples that gave concordant results. Related to this observation, the high proportion (31%) of samples with low BLV proviral loads (< 20 copies 100 ng^{-1}) might have accounted for the level of disagreement among the different assays, but as such type of samples (low proviral loads) are frequently observed in surveillance programs, both from hyper-acute and chronic asymptomatic infection they were included in the panel.

Journal of Clinical Microbiology

When the number of copies of proviral DNA detected by each assay were compared, we observed that the ability to quantify proviral DNA among the six laboratories was different. However, despite of statistically significant differences in the distribution of copy numbers, a moderate correlation among different assays was found. It has been demonstrated that protocol variations can increase inter-laboratory variability considerably (14-17). In this regard, the use of different reagents (i.e. master mix, oligos, etc.) and calibrators (standard curve) have been acknowledged as major contributors for this variability. In this study, except for PL and UK which shared most of the reagents and protocols, each laboratory performed particular qPCR protocols. The variation in the methods used by different laboratories in this study might have generated inconsistencies regarding the estimation of the number of copies of BLV proviral DNA among them.

As BLV is a global problem, harmonization across diagnostic laboratories worldwide is a key requirement and studies such as this are the first step towards the ability to compare surveillance data globally. To this end, this first inter-laboratory qPCR trial has highlighted the issues described above, some of which need to be further explored and addressed. As can be seen from the results additional efforts are required to ensure all laboratories are able detect the lower viral load samples. By sharing protocols the factors which may lead to the variation in detection may be elucidated. To an extent the unification of protocols and standards is an option but the laboratory set up and availability of resources and reagents at that testing laboratory must be kept in mind. Whilst it may not be feasible for all laboratories to follow identical protocols, small adjustments that fit with existing methodology may improve detection capabilities, e.g. addition of a probe to an existing SYBR assay. In order to monitor detection capabilities it is important that this is a continuous rather that one-time effort. To support this objective future attempts will be made to obtain more geographically diverse DNA panels for testing and to extend the number of laboratories included in the trial.

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533

534 **Table 1.** Primers and probes used in this study

PCR assay	Target gene	Primers & probe position*	Sequence 5´-3´	Amplicon size	Reference
ARG	*Pol*	Fw 2321-2340	CCTCAATTCCCTTTAAACTA	120	(7)
		Rv 2421-2440	GTACCGGGAAGACTGGATTA		
		Probe	n/a		
BE	*Pol*	Fw 3994-4016	GAAACTCCAGAGCAATGGCATAA	67	(20)
		Rv 4043-4060	GGTTCGGCCATCGAGACA		
		Probe	n/a		
GER	*Pol*	Fw 3298-3318	CCCTGGCCTACTTTCAGACC	114	(5)
		Rv 3390-3411	CTTGGCATAAGAGCTTAAGGCC		
		Probe 3322-3345	TTGACTGACAACCAAGCCTCACCT		
JPN	*Tax*	N/A	N/A	N/A	N/A
PL	*Pol*	Fw 2321-2340	CCTCAATTCCCTTTAAACTA	120	(7)
		Rv 2421-2440	GTACCGGGAAGACTGGATTA		
		Probe 2341-2360	GAACGCCTCCAGGCCCTTCA		
UK	*Pol*	Fw 2321-2340	CCTCAATTCCCTTTAAACTA	120	(7)
		Rv 2421-2440	GTACCGGGAAGACTGGATTA		
		Probe 2341-2360	GAACGCCTCCAGGCCCTTCA		

535 N/A: no data available

536 *Nucleotide positions according to GenBank accession no.K02120.1

537

538

539 **Table 2.** Results of qPCR analysis on 58 DNA samples used in this study

ID	Origin	Genotype	ELISA	ARG *(pol)*	BE *(pol)*	GER *(pol)*	JPN *(tax)*	PL *(pol)*	UK *(pol)*
1	Moldova	4	+	2 666.4	45 500	33 377.44	NEG	26 253	10 800
2	Russia	4	+	2 027.9	37 500	18 981.07	172.2	19 717.4	4 500
3	Russia	4	+	301.3	2 770	1 631.99	0.4	2 254.7	444
4	Poland	4	+	3 697.2	31 300	20 626.58	243.3	18 976.1	6 170
5	Poland	4	+	111.7	1 030	1 359.96	NEG	608.4	225
6	Moldova	7	+	67.2	776	1 361.42	NEG	801.9	230
7	Moldova	7	+	5	12.1	NEG	NEG	7.3	2
8	Russia	7	+	8 975	91 700	116 114.22	0.5	47 375.5	25 000
9	Russia	7	+	11 948.5	93 900	156 664.48	0.3	48 499	14 200
10	Poland	7	+	1 714.1	18 000	19 729.44	0.4	9 817.7	3 680
11	Poland	7	+	3 111.7	16 300	3 568.71	0.4	7 373.6	3 950
12	Croatia	8	+	1 001	2 770	4 703.54	118.8	2 354.5	768
13	Poland	8	+	10 784	10 400	23 085.46	355.4	5 944.9	2 810
14	Poland	8	+	6 239	12 500	43 294.68	615.0	10 938.2	5 220
15	Argentina	1	+	6 549.7	44 900	51 693.00	4 892.4	13 455.4	8 800
16	Argentina	1	+	16.8	1 960	2 274.46	284.3	110.9	309
17	Japan	1	+	2 264.3	7 328.5	7 310.87	1 117.3	5 711.2	535
18	Japan	1	+	2 615.7	10 285.7	11 541.46	1 662.6	6 429.6	744
19	Japan	1	+	1 228.6	2 685.7	3 707.16	537.1	2 459.7	403.4
20	Japan	1	+	2 051.3	1 442.8	9 552.46	1 102.3	6 721.6	633
21	Argentina	2	+	9 385	33 800	10 127.31	7 183.9	21 341.1	10 100
22	Argentina	2	+	10.1	99.7	24.31	10.6	53.7	24
23	Argentina	2	+	3 240.9	27 100	5 607.81	3 229.3	9 925.6	6 580
24	Moldova	Ns	+	NEG	NEG	1.71	NEG	NEG	0.5
25	Moldova	Ns	+	NEG	NEG	NEG	NEG	0.9	0.5
26	Moldova	Ns	+	5	NEG	29.14	NEG	9	8.3
27	Moldova	Ns	+	NEG	424	NEG	NEG	NEG	1.3
28	Moldova	Ns	+	NEG	12.9	10.39	NEG	NEG	0.5
29	Moldova	7	+	12 228.3	94 500	67 968.93	2.7	41 253.9	17 330
30	Russia	Ns	+	NEG	NEG	0.8	NEG	0.9	1.2
31	Russia	Ns	+	NEG	NEG	NEG	NEG	NEG	NEG
32	Russia	Ns	+	5	NEG	NEG	NEG	1.6	1.6
33	Russia	7	+	NEG	NEG	11.76	NEG	8.1	4.2
34	Russia	4	+	5	NEG	13.08	NEG	9.1	9.2
35	Russia	Ns	+	6 609	87 800	253 658.03	0.7	42 000.9	26 880
36	Poland	Neg control	-	NEG	NEG	NEG	NEG	NEG	NEG
37	Poland	Ns	+	5	NEG	10.52	NEG	13.3	2.1

38	Poland	Ns	+	NEG	NEG	NEG	NEG	3.5	8.0
39	Poland	Ns	+	5.5	NEG	5.95	NEG	6.8	1.5
40	Poland	Ns	+	9.2	NEG	211.10	NEG	11.7	3.3
41	Poland	Ns	+	5	23.1	NEG	NEG	43	2.9
42	Poland	Ns	+	NEG	14.5	5.64	NEG	15.7	1.7
43	Poland	Ns	+	NEG	12.2	NEG	NEG	11.1	2.3
44	Poland	7	+	1 486	5 020	5047.76	0.1	4 488.6	1 399.4
45	Ukraine	Ns	+	178	3 190	9 527.11	24.2	5 416.2	1 337.6
46	Ukraine	Ns	+	5	78.8	100.13	NEG	91.3	17.1
47	Ukraine	Ns	+	18.5	541	613.39	10.3	690.1	150.1
48	Ukraine	Ns	+	19.66	300	921.89	8.6	702.7	120.5
49	Ukraine	Ns	+	532	58 200	147 263.11	38.6	36 005.9	13 650
50	Ukraine	Ns	+	4 959	31 600	68 628.13	24.3	55 435.8	6 888
51	Ukraine	Ns	+	623	26 800	141 701.70	258.4	8 232.8	10 080
52	Ukraine	Ns	+	1 555	27 300	156 518.46	139.4	7 477.5	9 950
53	Na	Pos Control (BLV.FLK)	Na	44 997	282 000	725 329.97	16 312.6	91 585.7	85 300
54	Poland	Ns	+	19	13.6	158.67	NEG	10.8	38.46
55	Poland	Ns	+	5	151	474.54	1.7	42.7	59.28
56	Poland	Ns	+	73	380	1 023.20	1.1	63.8	123.68
57	Poland	Ns	+	61	503	284.03	NEG	65.9	128.56
58	Poland	7	+	88	1 520	59.74	NEG	259.2	254.8

Values represent BLV DNA copies per 100 ng of total DNA

NEG – negative results

Ns – not sequenced

Na – not applicable

Table 3. Assessment of qPCR results by pairwise comparisons. Kappa values are presented in the upper half of the table and the percentage of agreement are in the lower half.

	ARG	BE	GER	JPN	PL	UK
ARG	-	0.476	0.518	0.447	0.575	0.265
BE	82.8%	-	0.332	0.525	0.239	0.220
GER	86.2%	79.3%	-	0.369	0.357	0.326
JPN	74.1%	77.6%	70.7%	-	0.208	0.084
PL	89.7%	79.3%	86.2%	63.8%	-	0.549
UK	84.5%	81.0%	87.9%	58.6%	94.8%	-

549 **Figure legends**

550 **Figure 1. Assessment of the stability of DNA samples**. Copy number of housekeeping
551 H3F3A gene in 58 DNA samples, stored at 4^0C and tested twice at 21 days interval. T-test
552 was used to compare the mean values between the two groups ($p = 0.18$).

553

554 **Figure 2. Sequence variability measured as *per site* Entropy. A.** *p*ol gene multiple
555 alignment. qPCR fragment location within *pol* gene region for ARG/PL/UK; GER and BE
556 qPCR assays. **B.** *tax* gene multiple alignment.

557

558 **Figure 3. Comparison of detection of BLV proviral DNA copy numbers among six**
559 **testing centers**. Box plot of data from the Kruskal-Wallis ANOVA by Ranks test. The DNA
560 copy number for 56 samples, determined independently by each of the six qPCRs, were used
561 to the variance analysis. In this analysis a positive control (no 53) and negative control (no 36)
562 were excluded

563

564 **Figure 4. Impact of BLV proviral copy numbers on the level of agreement.** T-test was
565 performed in order to compare the number of BLV proviral copies in two groups of samples:
566 i) blue-33 samples with fully concordant results by all six qPCRs and (ii) red-25 samples with
567 discordant results by different qPCRs ($p < 0.001$).

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Figure 1.

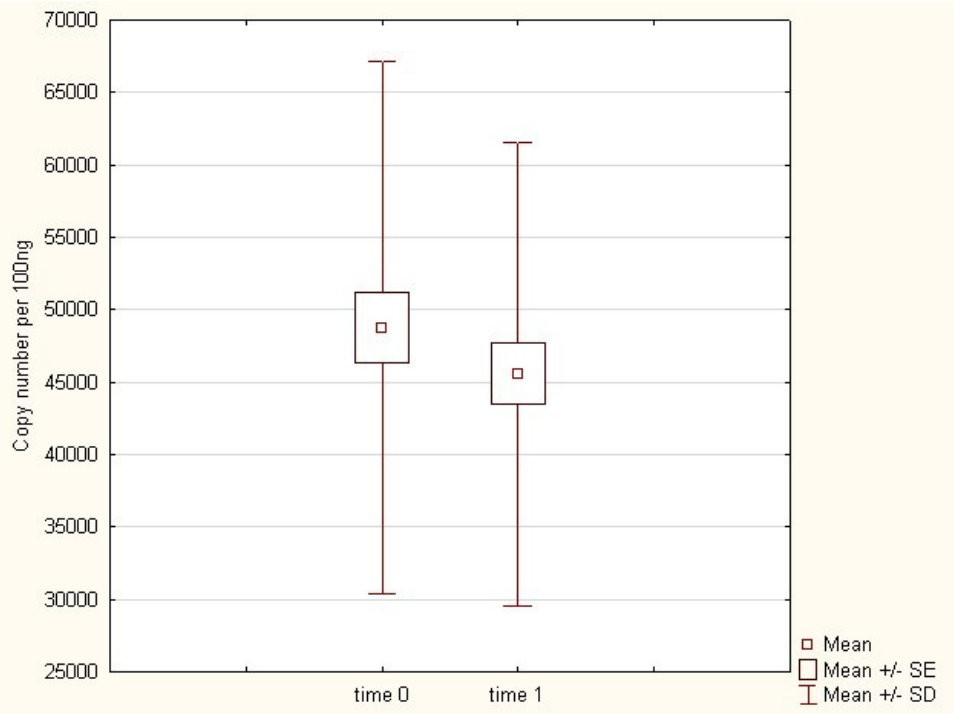

Figure 2.

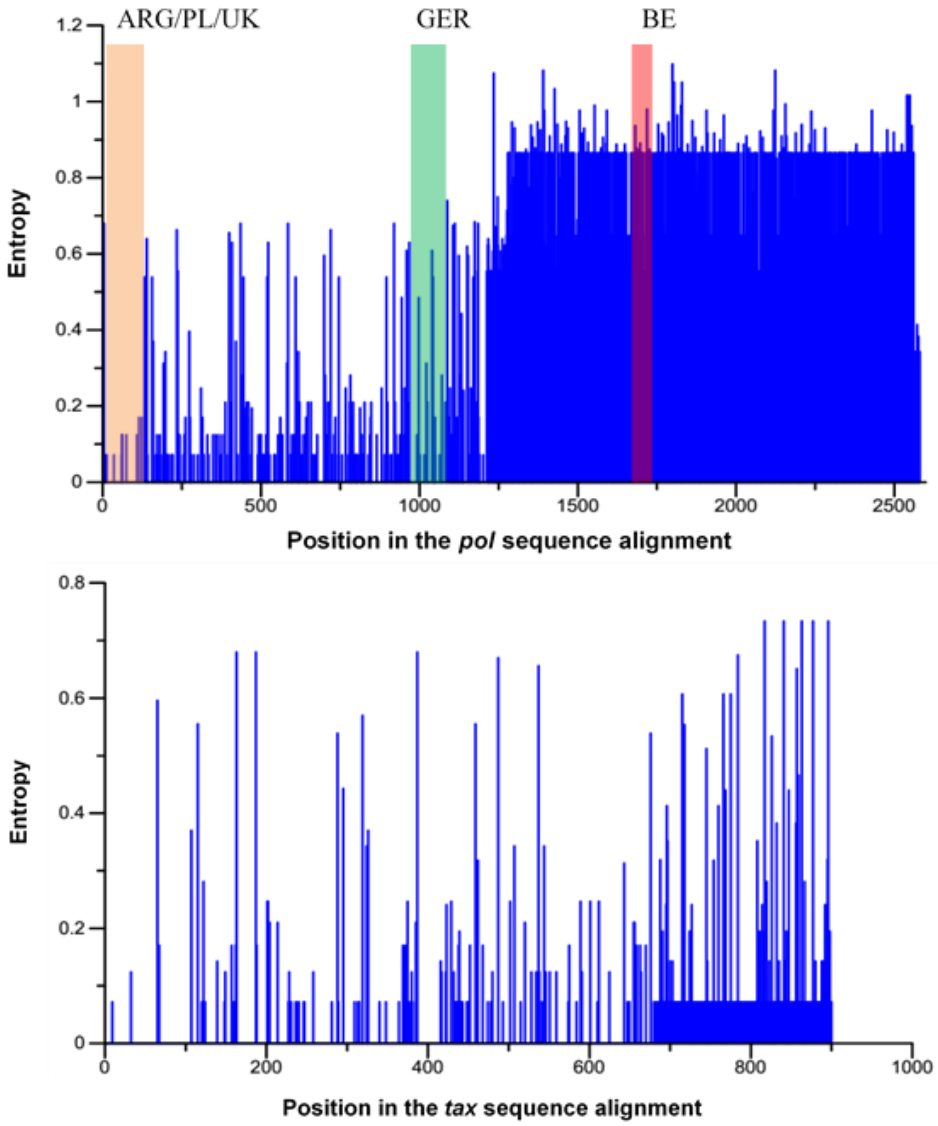

Figure 3.

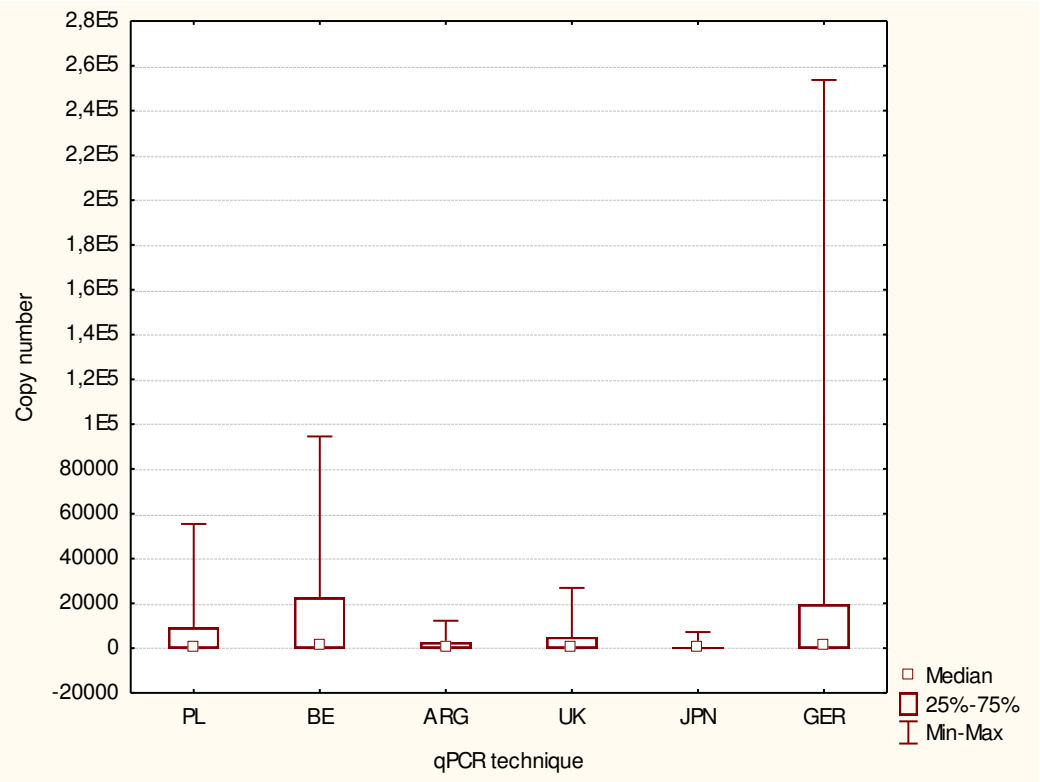

Figure 4.