diagnostyka laboratoryjna Journal of Laboratory Diagnostics
Diagn Lab. 2018; 54(2): 85-90
ISSN 0867-4043

Praca oryginalna • Original Article

Influence of the 1st International WHO EBV Standard on quantitation of Epstein-Barr virus viral load in serum samples

Wpływ zastosowania międzynarodowego wzorca WHO na wyniki oznaczeń ilościowych DNA wirusa Epsteina-Barr w próbkach surowicy

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Abstract

Objectives: The aim of this study was to evaluate the impact of normalization with 1st International WHO Standard for Epstein-Barr Virus (EBV) on EBV DNA quantitation in 90 clinical serum samples obtained from hematopoietic stem cells transplant recipients.

Methods: EBV DNA loads (EDLs) obtained with the use of six different commercially available and in-house developed assays, including various automated DNA extraction systems, real-time PCR tests and cyclers were compared, both before and after recalculation with conversion factors obtained with 1st International WHO Standard for Epstein-Barr Virus

Results: None of six methods was able to detect EBV DNA in all 80 serum samples identified previously as positive but the most effective method turned out to be combination of MagnaPure, LC2.0 and EBV QK (sensitivity 90%). Conversion factors for compared assays, obtained with the WHO standards ranged from 0.998 (MagnaPure/LC2.0/EBV QK) to 1.138 (MagnaPure/LC2.0/*In house*). In two out of four comparisons, differences in the average EDLs, initially significant, have changed to statistically not significant after conversion to IU/mL.

Conclusions: Positive impact of EDLs standardization was shown, resulting in lower discrepancies between average values obtained with various methods. Method-to-method variability was lower for samples with a higher EDLs (>3.5 log), regardless the units used. Results showed the advantage of certain commercial methods over "in-house" method.

Streszczenie

Cel pracy: Ocena wpływu wzorca międzynarodowego WHO/NIBSC dla wirusa Epsteina-Barr (EBV) na wyniki oznaczeń ilościowych DNA EBV przeprowadzonych w 90 próbkach surowicy uzyskanych od chorych poddanych przeszczepieniu komórek krwiotwórczych

Metody: Oceniono wpływ konwersji z wykorzystaniem międzynarodowego wzorca EBV na wyniki oznaczeń ilościowych DNA EBV uzyskane przy użyciu sześciu różnych układów diagnostycznych, obejmujących testy PCR z detekcją w czasie rzeczywistym, zarówno dostępne komercyjnie, jak również metodę opracowaną samodzielnie, z wykorzystaniem dwóch zautomatyzowanych systemów izolacji DNA.

Wyniki: Żadna z sześciu metod nie wykryła DNA EBV we wszystkich 80 próbkach surowicy zidentyfikowanych wcześniej jako pozytywne. Najbardziej efektywne było połączenie MagnaPure, LC2.0 i EBV QK (czułość 90%). Współczynniki konwersji z kopii/mL na IU/mL dla porównywanych testów, uzyskane na podstawie standardu WHO, wynosiły od 0,998 (MagnaPure/LC2.0/EBV QK) do 1,188 (MagnaPure/LC2.0/*In house*). W dwóch z czterech porównanych układów diagnostycznych różnice w średnich liczbach kopii DNA w mililitrze, początkowo istotne, po przeliczeniu na IU/mL uległy zmianie na nieznamienne statystycznie.

Wnioski: Wykazano pozytywny wpływ standaryzacji na ocenę liczby kopii wirusowego DNA, wyrażające się mniejszymi rozbieżnościami między średnimi wartościami liczby kopii DNA EBV mierzonymi przy użyciu różnych metod. Różnice oznaczeń ilościowych były mniejsze w przypadku próbek zawierających większą liczbę kopii DNA EBV (> 3,5 $\log_{10}$), niezależnie od jednostek, w których wyrażano kopijność DNA. Wyniki wykazały przewagę niektórych komercyjnych testów diagnostycznych nad metodą opracowaną samodzielnie.

Keywords: EBV, HHV-4, real-time PCR standardization, EBV WHO/NIBSC Standard
Słowa kluczowe: EBV, HHV-4, standaryzacja real-time PCR, wzorzec EBV WHO/NIBSC

Introduction

The real-time PCR for diagnosis of Epstein-Barr virus (EBV, Human Gammaherpesvirus 4, family *Herpesviridae*) infections has been introduced into laboratories at the end of the millennium [1]. Clinical value of the quantitative detection of EBV DNA load (EDL) in blood is now widely accepted, being especially valuable in diagnosis and monitoring of infections in hematopoietic stem cell transplant recipients (HSCT) [2, 3, 4]. The most important information includes identification of viremia and dynamics of EDL (initial and in response to treatment). Recently there was a number of valuable information published concerning the practical performance of qPCR methods for EBV DNA detection and quantitation [5, 6, 7]. However, the measurement of EDL may be burdened with significant lack of repeatability, even if analyses utilize single blood compartment. Differences in both intra– and interlaboratory comparisons exist, and the most important sources of variability include method of viral nucleic acids isolation, target of the EBV gene, type of qPCR probe(s) chemistry, variant of quantitative calibrators and units in which EDL is presented [8]. In interlaboratory assessment of qPCR results, accumulation of above factors may result in differences of measured EDLs reaching 3-4 $\log_{10}$ copies/mL in sample-to-sample comparisons [9, 10].

Introduced in 2011, the 1st International WHO Standard for Epstein-Barr Virus for Nucleic Acid Amplification Techniques, is addressed to calibrate secondary reference material and in consequence, to reduce between-run variation. Up to date, this is sole certified reference material for application in quantitative real-time PCR for EBV detection.

There were several publications presenting results of investigations concerning practical use of the 1st EBV WHO Standard, focused on commutability of the results between assays and interlaboratory performance in EDL quantitation with the use of laboratory reference material [10-13]. An extended report from collaborative group responsible for the Standard establishment also has been published [14]. The aim of the present study was to assess the influence of 1st International WHO Standard on the EDL results in clinical serum samples, obtained with use of two automated methods for nucleic acids isolation, three quantitative real-time PCR tests for EBV detection and two qPCR platforms.

Materials and Methods

Test samples. Material comprised of 90 serum samples obtained from HSCT recipients diagnosed for EBV infection in tertiary clinical hospital. Samples were collected and tested as a part of the routine diagnostic procedure, utilizing LightCycler MagNAPure for nucleic acids isolation and qPCR with use of LightCycler EBV Quantitative Kit in LightCycler 2.0 apparatus (equipment and test produced by Roche Diagnostics®, Switzerland). This method was used as a reference in the present study, and quantitative results of EDL of 80 consecutive samples tested positive with this method were used as a reference dataset. Ten randomly chosen serum samples, in which EBV DNA was not detected in routine testing, was used as a negative control group. Directly after collection, sera were aliquoted to obtain set of three 250 mL-volume samples and stored at -20°C (beside the part used for routine testing). Finally, all tested sera were de-identified and new sample numbers were attributed randomly. Samples were used for *de-novo* DNA isolation and EDL measure with qPCR, as described below.

WHO Standard. 1st International WHO EBV Standard, (National Institute for Biological Standards and Control, United Kingdom, ver. 3.0), referred further as "WHO Standard", was used for recalculation of quantitative results obtained with compared methods. Standard was prepared according to manufacturer's instruction; briefly: first, lyophilized standard containing whole EBV virions was resuspended in 1 ml of ultrapure water to achieve the virus titer warranted by producer ($5x10^6$ IU/ml), and obtained stock was stored frozen at -20°C. Prior to nucleic acids isolation, WHO Standard stock was diluted in the Standard Negative Serum (Bio-Rad, USA), to obtain series of aliquots containing 5000, 50 000 and 500 000 IU/mL, according to a protocol described by Semenova et

Table I. Characteristics of real-time PCR methods compared in this study.

	Type of qPCR probe/ reporter	EBV genome region targetted with primers and probe	Volume of DNA template used for analysis	Total volume of reaction mixture	Limit of EBV DNA detection (95% LOD)	Internal control included	Linearity range covered by calibrators	Number of calibrators	Quality assessment`
LightCycler EBV Quant Kit Roche®	Scorpion Technology® FAM	LMP2	10 µl	20 µl	229 copies/ml	Yes	$6x10^2$ – $6.5x10^8$	4	IVD*
EBV R-gene Quantification Kit bioMérieux®	Taqman® FAM	BXLF1	10 µl	25 µl	182 copies/ml	Yes	$2.5x10^3$ – $2.5x10^6$	4	IVD*
"*In house*" real-time PCR EBV	Taqman® FAM	BALF5	10 µl	20 µl	320 copies/ml	No	$5x10^2$ – $5x10^5$	4	RUO**

*) for *in-vitro* diagnostics, compliant with 98/79/EC European Union Directive
**) for research use only

al. [12]. This protocol was also used for calculation of conversion factor, used for recalculate of EDL values from copies per milliliter (c/mL) to international units per milliliter (IU/mL).

Nucleic acids isolation. Isolation of nucleic acids (NA) was performed with two automated methods: NucliSENS EasyMAG® (bioMérieux, France, further reffered to as "EasyMag") and LightCycler MagNA Pure® (Roche Diagnostics, Switzerland, further reffered to as "MagnaPure"). Isolation of NA in EasyMag system was performed according to manufacturer's instruction with the protocol "Specific B", and for MagnaPure system – Total Nucleic Acid Kit – Plasma, Serum, Blood (Roche Diagnostics) was used, with respective protocol. In both systems, nucleic acids were isolated from 200 ml of serum samples or from 200 µl of WHO Standard aliquots repetitions, suspended in a final volume of 50 mL of elution buffer, and stored at -20°C. As study involved retrospective examination of clinical samples, all experiments were performed within 25 days, what should minimize impact of storage time on quantitative results, and each serum sample was frozen and thawed once [15]. For qPCR, samples were divided into 8 groups for analyses of operator and day-to-day impact, what included two operators and four consecutive days.

Real-time PCR. Three qPCR methods were used for the EBV DNA detection: two commercially available, and one developed in the Department of Medical Microbiology, Medical University of Warsaw, Poland (further referred to as *In-house*) [16]. Commercial tests used in present study were: LightCycler EBV DNA Quant Kit (Roche Diagnostics®, Switzerland, further referred to as "EBV QK") and R-gene EBV (Argene®, France, further referred to as "R-gene EBV"). Preparation of reaction components, samples and standards in commercial tests were performed strictly as indicated in manufacturers' instructions. Design of the *In-house* method, and its validation for serum samples, were published previously [16]. Basic characteristics of qPCR methods compared in this study are presented in Table I.

Amplification, detection and quantitation of EBV DNA were conducted with use of two qPCR platforms (both manufactured by Roche Diagnostics®): LightCycler 2.0 (further referred to as "LC2.0", a platform certified for *in vitro* diagnostics) and LightCycler 96 (further referred to as "LC96", a platform for research use).

For the purpose of this study, cross-analysis of six following combinations of NA isolation, amplification and detection was performed: EasyMAG /LC96/EBV R-gene, MagnaPure /LC96/EBV R-gene, MagnaPure /LC2.0/EBV QK, MagnaPure /LC96/*In-house*, EasyMAG /LC96/*In-house* and EasyMAG /LC2.0/*In-house*.

Statistical analysis. All WHO Standard and EDL values were transformed to their decimal logarithms prior to statistical analysis. Negative results were excluded from quantita-

Table II. Impact of WHO EBV Standard on EBV DNA viral load in serum samples (n=80).

Method	WHO EBV Standard-derived conversion factor	Number of samples tested positive	EDL, range	Average EDL (SD)*	95% C.I.** of average EDL
EasyMAG LC 96 R-gene EBV	0.998	61	0.778-5.557	2.846[a] (1.008)	0.258
			0.777– 5.545	2.840[b] (1.006)	0.258
MagnaPure LC 96 R-gene EBV	1.023	60	1.255– 6.004	3.107[a] (0.893)	0.231
			1.284– 6.143	3.178[b] (0.914)	0.236
MagnaPure LC 2.0 EBV QK	1.001	72	1.421– 6.699	3.241[a] (0.746)	0.175
			1.453– 6.707	3.244[b] (0.747)	0.176
MagnaPure LC 96 *In-house*	1.057	59	1.204– 6.642	2.678[a] (1.141)	0.297
			1.273– 7.021	2.831[b] (1.207)	0.314
EasyMAG LC 96 *In-house*	1.052	66	0.845– 6.719	2.934[a] (1.181)	0.290
			0.889– 7.072	3.088[b] (1.242)	0.305
EasyMAG LC 2.0 *In-house*	1.138	65	0.301– 6.303	2.838[a] (1.219)	0.302
			0.343 –7.172	3.229[b] (1.387)	0.344

a) EDL before conversion, expressed as log copies/ml
b) EDL after conversion, expressed as log IU/ml
*) Standard Deviation
**) Confidence Interval

tive analyses. Comparison of mean EDLs was performed for both non-standardized and standardized values with the use of t-test, and 95% confidence intervals were calculated. F-test was used to test for equality of variances. Differences in methods capacity of EBV DNA detection in particular samples were analyzed with use of chi-square test. *P* value for significance was set at 0.05.

Results

None of six evaluated combinations of DNA isolation, EBV amplification/detection method and qPCR platform was able to detect EBV DNA in all 80 serum samples, identified previously as positive. Method utilizing MagnaPure, *In-house* qPCR and LC96 detected EBV DNA in the smallest number of samples (59 of 80), and the combination of MagnaPure, LC2.0 and EBV QK was the most effective method (72 positive samples); chi-square *P*=0.014. Statistically significant differences, assessed with chi-square test, were also observed between MagnaPure/LC2.0/EBV QK and R-gene/LC96, both in combination with EasyMag (60 positive samples, *P*=0.022) or MagnaPure (61 positive samples, *P*=0.034). None of 10 samples, determined previously as negative, produced a positive result in any of compared methods.

For each method, conversion factor obtained with the WHO Standard was calculated (Table II). Analysis of operator and day-to-day impact did not reveal a significant influence on measured EDLs (data not shown).

Before recalculation with the WHO Standard, the average EDLs ranged from 2.678 $\log_{10}$ c/mL (MagnaPure/LC96/*In-house*) to 3.241 $\log_{10}$ c/mL (MagnaPure/LC2.0/EBV QK), thus the highest difference between EDL averages was 0.563 $\log_{10}$ c/mL, while the lowest difference was observed between EasyMAG/LC96/R-gene EBV and EasyMAG/LC2.0/*In-house* (0.008 $\log_{10}$ c/mL). Using t-test, we identified statistically significant differences in average EDLs between MagnaPure/LC2.0/EBV QK and three other combinations of DNA isolation system, qPCR platform and assay, namely: MagnaPure/LC96/*In-house* (VL difference 0.563 $\log_{10}$ c/mL, *P*=0.001); EasyMAG/LC2.0/*In-house* (0.403 $\log_{10}$ c/mL, *P*=0.020); and EasyMAG/LC96/R-gene EBV (0.395, *P*=0.011). Comparison of two other combinations, MagnaPure/LC96/R-gene EBV vs. MagnaPure/LC96/*In-house,* also revealed significant difference (EDL difference 0.429 $\log_{10}$ c/mL, *P*=0.024). In remaining comparisons differences in average EDLs ranged from 0.008 $\log_{10}$ c/mL to 0.307 $\log_{10}$ c/mL and were not statistically significant (Fig. 1a).

After recalculation of above results with respective conversion factors, in two out of four cases, significant differences in average EDLs were normalized to a level recognized as statistically non-significant (MagnaPure/LC2.0/EBV QK vs. EasyMAG/LC2.0/*In-house,* and MagnaPure/LC96/R-gene EBV vs. MagnaPure/LC96/*In-house;* Fig. 1b). Application of conversion factors for comparison between MagnaPure/LC2.0/EBV QK and MagnaPure/LC96/*In-house* decreased difference in average EDLs from 0.563 $\log_{10}$ c/mL to 0.414 $\log_{10}$ IU/mL, but resulting difference was still significant (*P*=0.018). On the other hand, for comparison between EasyMAG/LC96/R-gene EBV and MagnaPure/LC2.0/EBV QK, conversion led to increase of the difference between average EDLs (from 0.395 $\log_{10}$ c/mL to 0.405 $\log_{10}$ IU/mL, respective *P* values 0.011 and 0.009). For the remaining eleven comparisons, decrease in differences between average EDLs was observed in seven of them, and increase in four of them, nevertheless this increase have not led to appearance of significant differences (Table 2). Analysis of influence of conversion with the WHO Standard on dispersion measures with use of F-test for equality of variances did not show significant differences.

For illustration of the distribution of EDLs assessed with examined methods, sample-to-sample comparisons of EDLs were performed, both before and after conversion with the WHO Standard (Fig. 2). It has revealed that the highest differences were observed for samples with average EDLs lower than 3.0 $\log_{10}$ IU/mL (similarly for c/mL).

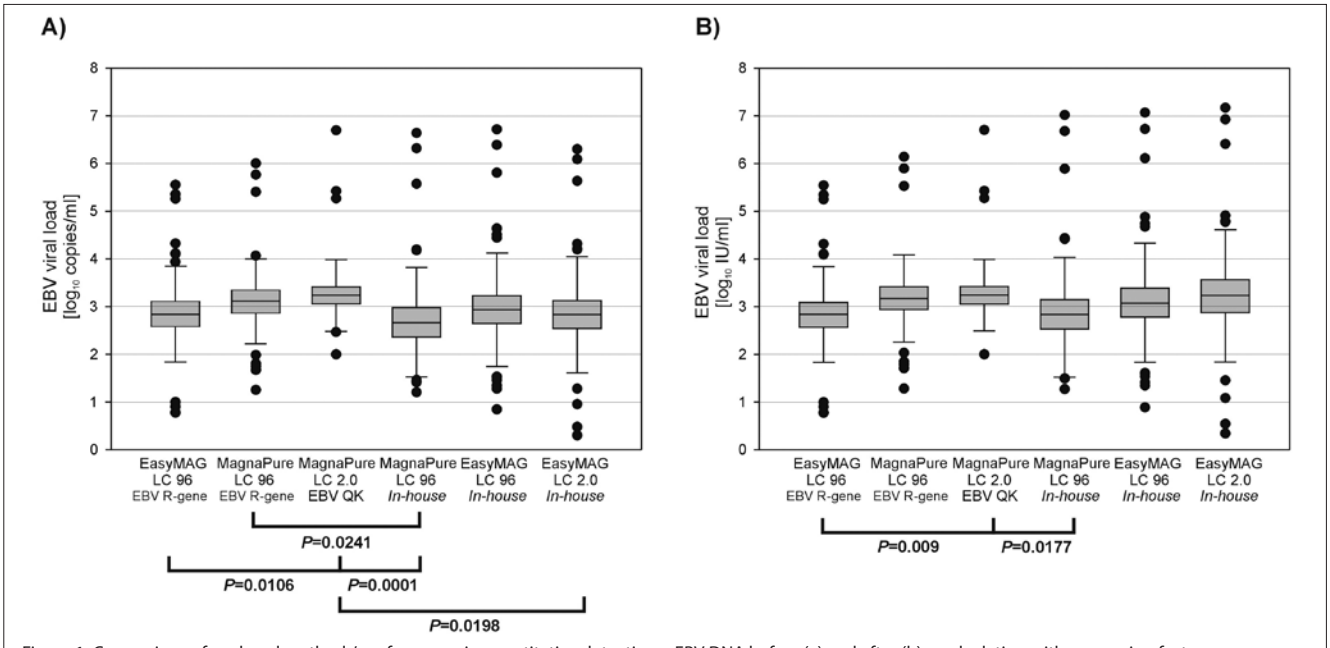

Figure 1. Comparison of analyzed methods' performance in quantitative detection o EBV DNA before (a) and after (b) recalculation with conversion factors.

Discussion

The monitoring of EBV DNA in peripheral blood has become a routine test for care of *inter alia* transplant recipients at high risk of post-transplant lymphoproliferative disorders. A viral load measurement using quantitative real-time PCR is significant for the diagnosis and management of EBV infection; moreover, it's critical for preemptive intervention in order to prevent the development of disease and for controlling the response to therapy [4, 14, 17]. With increasing number of the newly introduced commercially developed tests for EBV DNA detection in clinical settings and establishment of EBV WHO standard, the quality of EBV diagnostics in people at risk greatly improved.

Concerning qualitative analysis, none of six compared methods was able to detect EBV DNA in all 80 serum samples, identified previously as positive. It included also re-testing with the reference method for this study (MagnaPure/LC2.0/EBV QK). There were significant differences in method's sensitivities (73,8% – 90%), but most of discrepant results represented samples with an average values of EDL below 2.5 $\log_{10}$ c/mL (Fig. 2), which is close to the declared limits of detection of the methods. On the other hand, 36 out of 80 tested samples were identified as a negative by at least one method and as a positive by the other; moreover, in 11 of them the highest measured EDL was high enough to consider it clinically significant (3.0 – 3.5 $\log_{10}$ c/mL, Fig. 2). This problem is apparent and it was present from the very beginning of qPCR technique, and in our opinion, it represents one of the most important issue in everyday diagnostic practice.

Methods analyzed in the present study revealed good performance in the quantitation of EBV DNA, as for 15 analyzed combinations of assays, significant differences of average EDLs were observed only in four of them. Conversion of the results to IU/mL decreased two of these differences to a non-significant level, what indicates overall positive impact of WHO EBV Standard. Still, in the remaining two panels, impact of standardization was too low to reduce an average EDL differences. Of the possible reasons, the character of samples population used in this study may be important. Out of 80 samples tested, 29 (36.3%) had an average EDL below 182 copies/mL, which was the detection limit of the most sensitive method used in this study (Argene® EBV). Intrinsic characteristic of qualitative real-time PCR is the high variability in DNA measurements close to the method's limit of detection (regardless of units used), what in this particular analysis might have a negative impact on the "susceptibility" of the results to be normalized with the WHO EBV Standard. This problem was also underlined in the work of Semenova et. al [12], and in our opinion it shouldn't be treated as the argument against expression of the results in IU/mL, as the clinically significant values of EDL are almost always higher than 1000 copies/mL, and exact quantitation of low-copy samples is of lower clinical importance. However, it should be mentioned that analyzed set of samples have not been intentionally chosen or adjusted in any way in terms of EBV DNA copy number, but included 80 EBV DNA-positive sera collected consecutively during routine EBV testing, thus is probably representative for this particular group of HSCT recipients.

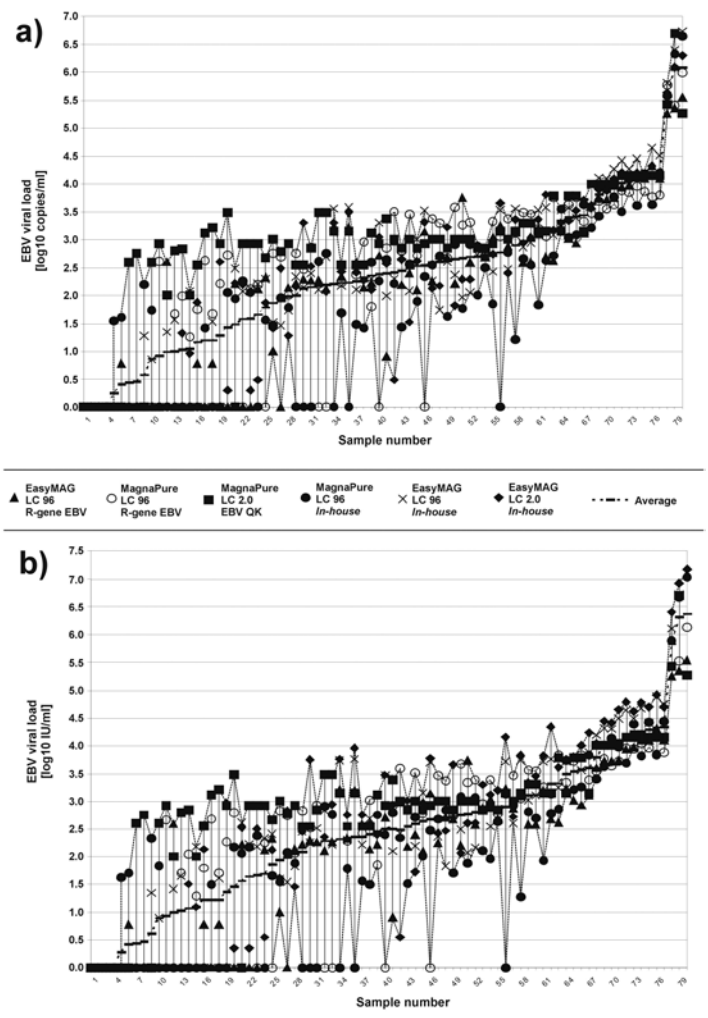

Figure 2. Graphic representation of all quantitative measurements of EBV DNA used in this study, (a) shows measurements expressed in copies/ml, and (b) represents EBV DNA expressed in IU/ml, after conversion with WHO EBV Standard.

There is another issue, which in our opinion is worth to be mentioned: the WHO EBV Standard represents an example of almost ideal test sample, while the quality of clinical samples varies, despite proper treatment of blood samples and obtained sera, what may be a reason for some incoherency between measured EDLs and value of obtained conversion factor.

Use of the WHO EBV Standard-derived conversion factor should reduce steady bias, which represents, in fact, difference in an average EDL values, but from theoretical point of view it shouldn't have a significant impact on statistical dispersion of EDL values. In the study of Semenova et al. [12], the standard deviation (SD) of analyzed samples ranged from 0.41 to 0.55 when the results were expressed in $\log_{10}$ c/mL, and SD decreased, and ranged from 0.17 to 0.32 after recalculation with conversion factor. In our research we have not observed such a trend, as SD of samples populations analyzed in this study was similar before and after conversion (Table 2). In consequence, we cannot confirm the observations presented in the same study, saying that dispersion of EDL results was significantly different depending on the

mode of expression (in log c/mL or log IU/mL). However, we agree that the impact on variability was lower for samples with a EDL higher than 3.5 log, regardless the unit used [12].

In conclusion, we agree that the wide introduction of the WHO EBV Standard into laboratory practice will eventually lead to the standardization of EDL results, what should improve management, especially of patients at high risk of PTLDs, and may simplify comparison of results received from other transplant centers [11-13, 18]. However, there are many conditions which must be fulfilled to achieve this goal. One of the most important problems to overcome is a difference of EDL values resulting from the various methods of isolation, amplification and detection of EBV DNA [14, 18, 19]. To decrease the impact of these sources of results variations, IVD/CE labelled (certified for *in vitro* diagnostics, according to UE regulations), fully automated test systems should thus be employed [12].

Acknowledgements

We thank bioMérieux® company for their support. We also thank IFMSA Students, Andreia Patricia Machado Fino and Ramona Andrea Bologa for their help.

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Otrzymano: 26.01.2018
Akceptacja do druku: 26.06.2018

Conflict of interest

This study was partially funded by the bioMérieux®, Poland, what included Argene® qPCR tests for EBV DNA detection and an access to EasyMAG Nuclisens apparatus for the purpose of this study. No other conflicts of interests are declared.