Journal of Clinical Virology 56 (2013) 65–68

Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Short communication

Virtual quantification of influenza A virus load by real-time RT-PCR

Antonio Piralla[a], Cristina Daleno[b], Elena Pariani[c], Piergiulio Conaldi[d], Susanna Esposito[b], Alessandro Zanetti[c], Fausto Baldanti[a,*]

[a] Molecular Virology Unit, Virology and Microbiology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[b] Department of Maternal and Pediatric Sciences, University of Milan, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy
[c] Department of Biomedical Sciences for Health, University of Milan, Milan, Italy
[d] Institute of Microbiology and Virology, Mediterranean Institute for Transplantation and Advance Specialised Therapies, University of Pittsburgh Medical Center-Italy, Palermo, Italy

ARTICLE INFO

Article history:
Received 12 July 2012
Received in revised form
18 September 2012
Accepted 24 September 2012

Keywords:
Influenza quantification
Real-time RT-PCR
Cycle threshold
Virtual quantification tool (VQT)

ABSTRACT

Background: The pan-influenza A real-time RT-PCR detection assay developed by the Centers for Disease Control and Prevention (CDC) during the 2009 pandemic is widely utilized. A quantitative version of the assay may be useful to monitor influenza A infection and response to treatment.
Objectives: To prove in principle the possibility that a virtual quantification tool (VQT) would allow conversion of CDC real-time RT-PCR cycle threshold (Ct) values in virus RNA copy number.
Study design: A plasmid carrying the CDC real-time RT-PCR target region of the influenza A Matrix (M) gene was generated. In a multicenter study, a set of 5 ten-fold dilutions (equivalent to 1×10^2 to 1×10^6 copies/reaction) were prepared and distributed to the 4 participating virology laboratories and then amplified to generate a virtual quantification standard curve. Clinical samples ($n = 120$) were quantified in parallel by interpolation with locally generated standard curves and using the VQT.
Results: A total of 40 standard curves were obtained by the participating centers (ten from each center). The intra- and inter-laboratory variability showed a coefficient of variation (CV) $\leq$5%. Influenza A virus quantification in 120 respiratory samples showed a significant correlation between interpolation with locally generated standard curves and the VQT ($R^2 = 0.9655$). Bland Altman analysis showed that the majority (no. 111, 92.5%) of clinical samples had <0.5 $\log_{10}$ variation.
Conclusions: VQT proofs the concept that qualitative results from real-time RT-PCR assays can be converted into quantitative determination of virus load in clinical samples without running standard curves in parallel.

1. Background

The emergence of a new influenza A/H1N1 variant in early 2009, prompted the release by the Centers for Disease Control and Prevention (CDC) of a real-time assay for detecting and typing influenza A virus.[1] The CDC assay is currently utilized worldwide for diagnosis of influenza A infection. The use of this assay in a quantitative format would require the generation of quantification standards to be run in parallel with clinical samples to interpolate results with a standard curve. However, quantification of serial amounts of plasmid DNA or in vitro transcribed RNA carrying the real-time RT-PCR target sequence is cumbersome, time consuming and lacks standardization among different laboratories.

2. Objectives

The objective of the present study was to provide proof of concept that a virtual quantification tool (VQT) would allow conversion of cycle threshold (Ct) values in influenza A RNA copy number in clinical samples.

3. Study design

The influenza A virus M gene region conserved among all influenza A strains targeted by the real-time RT-PCR assay developed by the CDC was used to produce the standard.[1] An amplicon of the A/Brisbane/59/2007-like(H1N1) virus strain was cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA). A set of 5 ten-fold dilutions equivalent to 1×10^2 to 1×10^6 copies/reaction was prepared and distributed to participating laboratories and then

Abbreviations: C_T, cycle threshold; CV, coefficient of variation; PCR, polymerase chain reaction; RT, retrotranscription; LGS, Clocally generated standard curve; VQT, virtual quantification tool.

* Corresponding author at: Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo, Via Taramelli 5, 27100 Pavia, Italy. Tel.: +39 0382 502420; fax: +39 0382 502599.
E-mail address: f.baldanti@smatteo.pv.it (F. Baldanti).

1386-6532/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jcv.2012.09.011

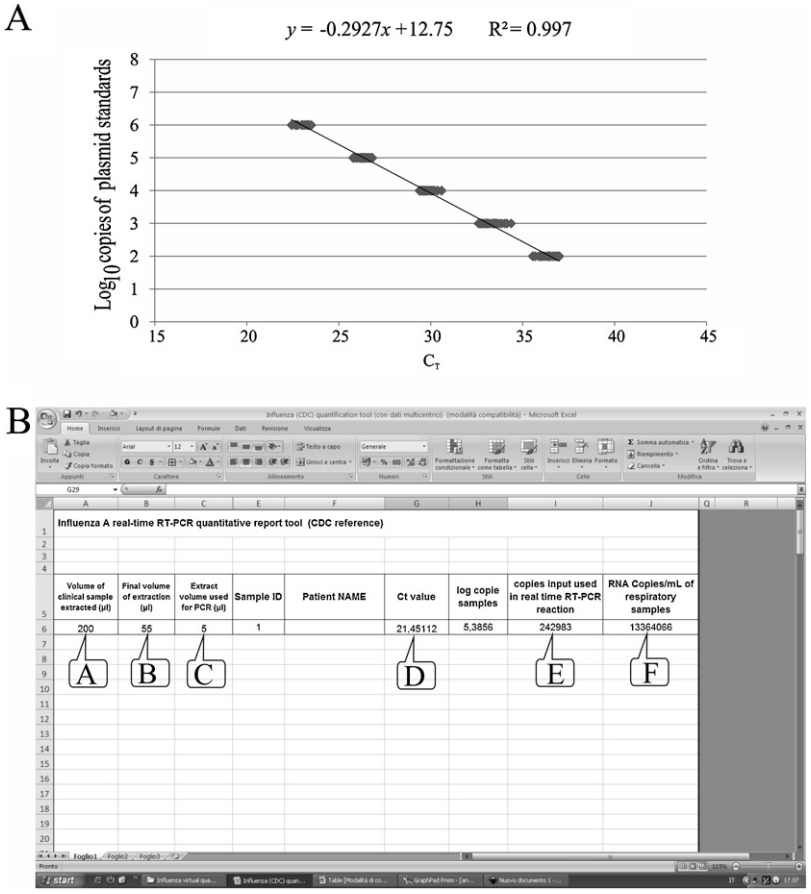

Fig. 1. Calibration curves ($n = 40$), generated by four participant centers were used to create the virtual calibration standard (A). The virtual quantification tool user interface (B). The set of parameters required for adjusting the quantification: the volume of clinical sample extracted (μl); extraction eluate volume (μl); the input extract volume in real-time RT-PCR (μl); the Ct obtained for each clinical sample.

were amplified to generate a standard curve for quantification of viral load in test samples.

The real-time RT-PCR amplification was performed in a 7300 Real-Time PCR System (Applied Biosystem, Foster City, CA) with AgPath-ID™ One-Step RT-PCR kit (Ambion, Applied Biosystem, Foster City, CA). The reaction was performed in a total of 25 μl reaction volume containing 5 μl of plasmid or nucleic acid extract, 12.5 μl of 2× RT-PCR buffer, 400 nM of each forward and reverse primer,[1] 120 nM labeled probe,[1] 1 μl 25× RT PCR enzyme mix and H_2O was added to achieve the final 25 μl volume. The set of 5 ten-fold dilutions of plasmid (from 1×10^2 to 1×10^6 copies/reaction) were run in 4 different virology centers (one in Pavia, two in Milan and one in Palermo) in order to generate calibration curves to be included in the virtual quantification tool (VQT).

In order to evaluate the performance of VQT, 30 influenza A(H1N1)pdm09 positive samples were collected in each center and quantified by locally generated standard curves and in parallel by the VQT. Overall, 120 samples were analyzed from as many patients (median age 24 years; range 6 months to 80 years) with respiratory syndromes. Among the 120 collected samples, 82 (68.3%) nasal swabs and 30 (25.0%) nasopharyngeal swabs were from patients with mild or moderate infections, while 8 (6.7%) bronchoalveolar lavage were from patients with severe infections. Fifty-six out of 120 (46.6%) were inpatients and 64/120 (54.4%) were outpatients.

The geometric mean was calculated for all standard dilutions. The intra-laboratory assay and inter-laboratory assay variability were expressed as coefficient of variation (CV). Correlation between quantification obtained with locally generated standard curves and VQT was expressed with the Spearman coefficient. Bland–Altman analysis was performed and the percentage of samples showing <0.5 $\log_{10}$ difference was calculated.

4. Results

A total of 40 standard curves were obtained from the four participating centers (10 from each center). The mean Ct values at each ten-fold dilution of forty independent runs were considered for the standard curve. Fig. 1A shows the linear curve obtained by plotting the Ct values (x axis) and the $\log_{10}$ values of the copy numbers (y axis). The resulting quantitative equation was: $y = -0.2927x + 12.75$ ($R^2 = 0.997$), where x and y represent the Ct value of real-time RT-PCR and the logarithmic ($\log_{10}$) value of the viral copy numbers, respectively. This equation was also used to calculate the viral RNA copy number in each clinical sample. The standard curve equation was inserted into an excel spreadsheet to obtain a quantification tool (Influenza A CDC real-time RT-PCR virtual quantification tool).

An overview of the VQT is shown in Fig. 1B. Four parameters (A–D in Fig. 1B) were required to obtain the quantification output: box A, the volume (μl) of extracted sample (e.g. 200 μl), box B, the extraction eluate volume (μl) (e.g. 55 μl), box C, the input extract volume (μl) in the real-time RT-PCR (e.g. 5 μl), and in box D the Ct obtained for each clinical sample. Hence, the value of influenza A RNA copy input in the real-time RT-PCR assay is reported (RNA copies input = $10^{-0.2927*Ct+12.75}$) is displayed in box E. Finally, the output information is reposted in box F, where the value of influenza A RNA copies/ml sample is reported (box E value*multiplication factor for ml of sample).