

Are three generations of quantitative molecular methods sufficient in medical virology?

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SUMMARY

In the last two decades, development of quantitative molecular methods has characterized the evolution of clinical virology more than any other methodological advancement. Using these methods, a great deal of studies has addressed efficiently *in vivo* the role of viral load, viral replication activity, and viral transcriptional profiles as correlates of disease outcome and progression, and has highlighted the physio-pathology of important virus diseases of humans. Furthermore, these studies have contributed to a better understanding of virus-host interactions and have sharply revolutionized the research strategies in basic and medical virology. In addition and importantly from a medical point of view, quantitative methods have provided a rationale for the therapeutic intervention and therapy monitoring in medically important viral diseases. Despite the advances in technology and the development of three generations of molecular methods within the last two decades (competitive PCR, real-time PCR, and digital PCR), great challenges still remain for viral testing related not only to standardization, accuracy, and precision, but also to selection of the best molecular targets for clinical use and to the identification of thresholds for risk stratification and therapeutic decisions. Future research directions, novel methods and technical improvements could be important to address these challenges.

KEY WORDS: Quantitative methods, Competitive PCR, Real-time PCR, Digital PCR, Viral load.

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THE QUANTITATIVE REVOLUTION IN MEDICAL VIROLOGY

From the first development of a molecular method for the absolute quantitation of viral nucleic acids directly *in vivo* (Menzo *et al.*, 1992), the pathogenic potential of human viruses and the virus-host interactions have largely been addressed using the quantitative approaches (Clementi *et al.*, 1993; Clementi *et al.*, 1996; Clementi *et al.*, 2000). Before this advancement, clinical virologists were limited to the use of labor-intensive culture techniques (only applicable to viruses capable of growing

in cell cultures), of antigen assays characterized by low sensitivity, and, finally, of improbable applications to clinical diagnosis of electron microscopy (Gentile and Gelderblom; 2014); these techniques are imprecise from a quantitative point of view, because reproducibility of these assays are depending on numerous variables.

Starting from the early '90, a great deal of studies has demonstrated within a short time that the degree of virus activity documented by quantitation of virus load in blood or tissues is a crucial correlate of disease outcome in most acute and persistent infections, including human immunodeficiency virus type 1 (HIV-1) (Bagnarelli *et al.*, 1991; Bagnarelli *et al.*, 1992; Michael *et al.*, 1992; Gupta *et al.*, 1993; Bagnarelli *et al.*, 1994; Bagnarelli *et al.*, 1996), hepatitis C virus (HCV) (Manzin *et al.*, 1995; Manzin *et al.*, 1996; Manzin *et al.*, 1997; Fanning *et al.*, 1999), Epstein-Barr virus (EBV) (Stevens *et*

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al., 1999), and human cytomegalovirus (hCMV) (Spector *et al.*, 1998; Gerna *et al.*, 1998) infections. The opportunity to directly quantify virus nucleic acids from clinical samples has allowed natural history and pathogenicity studies of viral diseases to be carried out efficiently in a relatively short time. These studies have strongly contributed to a better understanding of virus-host interactions, have revolutionized research strategies in basic and medical virology, and importantly from a medical point of view, have provided a rationale for the specific antiviral therapeutic intervention and therapy monitoring. Over the time, these researches have clarified that systemic viral activity, consisting in a sum of dynamic processes, is influenced by viral and host factors. The major virus-related variables include the degree of virus expression and the host-cell range (Bonhoefer *et al.*, 1997), while the host factors include the specific immune response and polymorphism of genes coding for cell receptors of viruses, as documented for HIV-1 (Feng *et al.*, 1996; Balotta *et al.*, 1997). In this context, the introduction of new antiviral agents into the clinical use has generated the need for sensitive and reliable parameters to evaluate the efficacy of these drugs and to monitor them. Overall, absolute quantitation of viral nucleic acid species directly in clinical samples has emerged as a crucial tool in medical virology. However, despite the advances in technology, great challenges still remain for viral testing related not only to standardization, accuracy, and precision, but also to the identification of the best molecular targets for clinical use.

THREE GENERATIONS OF QUANTITATIVE METHODS THROUGH THE YEARS

The basis of the first generation of viral load assays is represented by competitive PCR (cPCR) (Clementi *et al.*, 1993). The general concept of cPCR is the coamplification of target and calibrator (competitive) templates with similar length and the same primer binding sequences; the amount of competitive template is known and, after amplification of sequences with identical thermodynamics and amplification efficiencies, the products from both templates are

distinguishable from each other. As mentioned before, this approach has undoubtedly represented a first concrete aid for both a more accurate experimental approach to many biological fields and a less empiric medical management of viral diseases.

The second generation of quantitative assays is represented by real-time amplification (real-time PCR). This methodology has progressively been accepted during the past decade for molecular quantification of pathogens *in vivo* (Mackay *et al.*, 2002). In real-time PCR, the fluorescent signal generated during the reaction and measured during the exponential phase of amplification is proportional to the concentration of the target DNA or RNA. The advantages of real-time PCR that have contributed to its wide use include:

- a) real time monitoring of the quantitative analysis;
- b) reduced possibility of contamination by eliminating the post-reaction processes;
- c) improved multiplexing. Additional advantages in this assay are that;
- d) the reaction components are not limiting, as in conventional PCR.

At present, real-time PCR is the most commonly used quantitative method in virology. However, for amplification with real-time PCR, a standard curve with known concentrations of the target sequence is necessary to transform the output values of real-time PCR (threshold cycles) into concentration values (target molecules/volume). Unfortunately, most of the methods based on real-time PCR have a high degree of result variability when compared to each other (Hayden *et al.*, 2008; Hoffman *et al.*, 2008; Hirsch *et al.*, 2013). The reasons for this variability are of multiple origin, and several factors have been shown to play a role (24), including the co-extraction of substances that influence the amplification efficiency and, more importantly, the lack of universally available calibrators (Holden *et al.*, 2011). Although the World Health Organization (WHO) has recently made available quantitative standards (biologic standards) for EBV, CMV, HIV-1, HBV and HCV, the subsequent generation of secondary standards by commercial entities has introduced another potential source of variability.

The third generation of quantitative PCR meth-

ods is represented by digital PCR (dPCR), a method originated from the studies of target quantification by limiting dilutions (Vogelstein and Kinzler, 1999) and now also applied to viral diagnostics (Sedlak and Jerome, 2013). In the dPCR procedure, the reaction mixture containing primers and probes and purified nucleic acid sample is divided into hundreds to millions of partitions (partitions are chambers or droplets, depending of the digital platform used).

The number of partitions affects the accuracy of quantification. A fraction of the droplets is occupied by a single to a few copies of the target nucleic acid, while a fraction of compartments remains target free. After amplification, positive and negative compartments are counted and the absolute concentration of target molecules is derived using Poisson's distribution without the need of any standard (Dube *et al.*, 2008). Under these conditions, the number of positive and negative reactions is counted after end-point PCR amplification, and the final result is independent of variations in the PCR amplification efficiency and less prone to intra- and inter-laboratory variations (Sedlak *et al.*, 2014). An additional advantage of dPCR, is that low frequency targets do not compete during amplification with those present in high frequency, thus allowing the detection of rare viral mutants in a background of wild type sequences (Pekin *et al.*, 2011).

Validation of dPCR for viral diagnostics is still in its very early stage (Kiss *et al.*, 2008; White *et al.*, 2012; Henrich *et al.*, 2012), but, the initial studies have highlighted the potential clinical utility of dPCR for rapid, sensitive, and accurate quantification of viral load in patient samples. As indicated before, a second potential application of dPCR in viral diagnostics is the detection of rare point mutants in a background of wild-type sequences (Yung *et al.*, 2009; Wang *et al.*, 2010; Pekin *et al.*, 2011). Although the studies on this aspect have principally been focused on oncology diagnostic applications, the principles can be translated to virology, where detection of low abundance mutant sequences, such as those mediating antiviral resistance, can significantly impact treatment outcome. Otherwise, despite the potential of dPCR, possible limitations to utilizing this technology in

clinical virology have to be considered. In fact, some (but not all) of the commercial dPCR platforms have a relatively small number of partitions that can only be scaled up using multiple and expensive microfluidics chips; moreover, although dPCR should theoretically be more sensitive and more precise at low virus levels than real-time PCR, practical aspects concerning limits on sample volume and master mix compatibility on the dPCR platform should be considered. Finally, the digital platforms also add another layer of complexity to any assay, potentially slowing the laboratory workflow. In conclusion, a process of adaptation to the virus diagnostics is necessary for dPCR for a wide use of this methodology in routine laboratories.

FUTURE NEEDS AND RESEARCH DIRECTIONS

Extensive research within the last two decades to develop molecular methods for the absolute quantitation of viral nucleic acid species does not seem sufficient to the purpose, and further technical development appears to be necessary. As an example, one should consider that, despite hundreds molecular studies in medical virology and long-term monitoring of therapies, viral load testing thresholds for the risk stratification and therapeutic decision have not yet defined, with only few exceptions. In fact, the precise identification of these thresholds requires reduction of intra- and inter-laboratory variability, in order to perform reliable clinical studies in many centres.

The variability generated by nucleic acid extraction methods, selection of target sequences, methods used for the detection of amplified viral nucleic acids, and use of different calibrators can strongly influence the results of real-time PCR. As indicated before, dPCR reduces variability, increases sensitivity, and it has a great potential for practical use in diagnostic virology, allowing simple multiplexing (Brunetto *et al.*, 2014). Multiplexing assays can be central in medical virology in many applications where comparative analysis of multiple targets can be performed within the same experimental run. For all these principal reasons, dPCR provides a real opportunity for medical virologists; how-

ever, wide clinical validation is necessary in the near future to optimize the new methodology in the field. Additionally, the extensive use of dPCR in virus diagnostics would require a complete automation, that is currently lacking for the different platforms. Finally, the running costs of dPCR are presently higher than those of real-time PCR. However, it is reasonable to hypothesize that an extensive use of the new methodology can be coupled to a lower cost in the near future.

In conclusion, the answer to the question of the title is therefore no. Although advanced methods have been developed and important data have been obtained in medical virology and in the monitoring of therapies after three generations of molecular methods, further research in the field is presently necessary.

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