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Letter to the Editor

Unreliable Real-Time PCR Analysis of Human Endogenous Retrovirus-W (HERV-W) RNA Expression and DNA Copy Number in Multiple Sclerosis

EDITOR: The potential role of human endogenous retroviruses in the pathogenesis of multiple sclerosis (MS) has been the subject of many studies since the discovery of MSRV,[1] founder member of the HERV-W family.[2] Two such studies from Prof. Power's group in Canada,[3,4] recently published in *AIDS Research and Human Retroviruses*, give us serious cause for concern. The findings of both studies were based on the use of real-time polymerase chain reaction (PCR) assays for the quantification of HERV-W RNA and DNA levels in brain, blood, and cerebrospinal fluid samples from patients and controls. We consider that technical flaws in the real-time PCRs employed in these studies are of such a severe and fundamental nature that the assays are unable to generate accurate or reliable data and that the conclusions of the papers are therefore unlikely to be valid.

One of the critical parameters used to assess the quality of real-time PCR assays is the slope of the regression line derived from 10-fold dilutions of calibration standards. Ideally, in a perfect PCR with 100% efficiency, the slope should be −3.32 ($2^{3.322} = 10$), but for practical purposes slopes within a range from −3.10 to −3.59 are generally regarded as acceptable.[5] This represents an acceptable efficiency range of 90–110%. In view of this, we were disturbed to see slope values of −1.365 for syncytin-1 DNA and −2.276 for GAPDH DNA presented in the legend to Fig. 1, and −1.857 for syncytin-1 RNA in the legend to Fig. 2 of the paper by Antony *et al*.[3] (NB: Syncytin-1 is a member of the HERV-W family and GAPDH is a reference gene.) Applying the equation, $E = 10^{(-1/\text{slope})} - 1$ to the syncytin-1 DNA slope value of −1.365 gives an apparent PCR efficiency (E) of 4.4, i.e., 440%, which is entirely implausible since *Taq* polymerase cannot produce more than a doubling of the number of DNA molecules with each PCR cycle. An efficiency of 440% would imply a 5-fold increase in DNA with each PCR cycle, which is impossible on theoretical grounds. Implausibly high apparent PCR efficiencies are a well-recognized problem, especially with SYBR Green I quantitative real-time assays, and may be due to the generation of primer dimers or spurious amplification products yielding excessive fluorescent signal, systematic pipetting errors, or to dilution of reverse transcriptase polymerase chain reaction (RT-PCR) inhibitors with increasing template dilution.[6] A quantitative PCR assay with such an extremely aberrant regression line slope value would not be expected to be capable of yielding accurate or reliable data suitable for publication.

Evidence of unreliable quantification resulting from the use of such defective PCR assays is seen in Fig. 1d of the same paper.[3] Figure 1d shows GAPDH DNA copy numbers in brain-derived DNA extracted from multiple sclerosis patients and controls. Since GAPDH is a "single copy" gene we would expect a fixed number of GAPDH DNA copies per μg of extracted DNA ($\sim 3 \times 10^5$ copies/μg DNA). However, Fig. 1d does not show a fixed number of copies but an extraordinarily wide spread of copy numbers that extends over a 100-fold range. Even if we take into account the presence of a number of GAPDH pseudogenes that might be detected by the PCR assay, the problem of extremely high variability remains because the number of pseudogenes would not be expected to vary significantly between different individuals.

The more recent paper by Antony *et al*.[4] uses some of the same PCR assays as those discussed above. The grossly aberrant slope value of −1.365 for the ERVWE1 (syncytin-1) PCR is the same in both papers (Table 2 of reference 4). Further evidence of the unreliability of the data generated by these assays is provided by Table 3, which presents the coefficients of variation (CV%) of the PCR assays.[4] Table 3 indicates, for example, that the overall intraassay variation for ERVWE1 (non-MS brain) is 10.15% based on raw Ct values. The figure of 10.15% is not worrying per se until we consider that this is the figure derived from raw Ct values that are logarithmic in nature and therefore grossly underestimate the true degree of variation that would be evident if the Ct values were converted into linear values (i.e., copies/ml) prior to calculating the CV%. Schmittgen *et al*.,[7] referring to this matter, state that the "Presentation of statistical data calculated from the raw Ct values falsely represents the error and should be avoided." A 10.15% raw Ct figure for ERVWE1 would give an unacceptably large CV% (>100%) upon conversion to linear values. Reliable and accurate quantification cannot be achieved by assays with such high coefficients of variation.

In addition to these major failings, we note that neither publication assesses RNA integrity and purity, which are critical parameters for evaluating the validity of RNA quantification by real-time PCR.[8] Furthermore, correct usage of the $2^{-\Delta\Delta Ct}$ method,[9] applied in both papers, requires evidence that PCR efficiencies for targets used for relative quantification are equal. The authors' data clearly show that they are not equal, hence this method is inappropriate.

Given these fundamental problems with the performance of the quantitative real-time PCR employed in these papers, we very much doubt the reliability of any of the conclusions based upon the assays. Our primary concern is that the literature in this important area of multiple sclerosis studies must not be inadvertently confounded by misleading data based on unreliable quantification. We believe that the publication of these studies represents an unfortunate failure of the peer review system and that the potential for future such failures could be significantly reduced by universal adoption of guidelines[10] similar to those in place for DNA microarray analysis.[11]

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