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RESEARCH ARTICLE

Unexpected Factors That Influence Viral Detection and Viral Load Using Real-Time PCR

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ABSTRACT
This communication takes note of unexpected factors that can influence the results of RT-PCR in quantitation of copy numbers such as in determination of viral loads and in viral identification. We show that the presence of serum separator gel in authorized collection tubes for hepatitis C (HCV) viral load determinations causes underestimation of viral loads by blocking viral diffusion into plasma and that the presence of more than one targeted virus in a multiplex RT-PCR viral assay, while not affecting analytical specificity, results in raising of the minimal detectable viral titer and therefore a decreased analytical sensitivity. Our results suggest the possibility that HCV samples should be placed in cell lysis buffer for full viral load determination and that multiplex assays should be carefully validated and modified if necessary to minimize loss of sensitivity.

Keywords: RT-PCR, viral load, viral titers, serum separator gel, respiratory viruses

1. Introduction

A rapid laboratory diagnosis is a powerful decision-making tool for patient management and disease control, especially for infections that spread rapidly[1-2]. Molecular diagnostic techniques, such as real-time polymerase chain reaction (PCR), have developed into highly sensitive and reproducible means of detection and quantitation of both human genetic materials and microbial agents in clinical samples. These methods have become the standards for definitive diagnosis of viral-induced diseases such as hepatitis C (HCV)[3-4], human immunodeficiency virus (HIV)[5], and respiratory diseases[6-7], including COVID-19 (SARS-CoV-2), influenza (Flu), respiratory syncytial virus (RSV), and adenovirus among others.

In the course of performance of routine real-time reverse transcription PCR, we have found that certain unexpected factors formerly not considered can result in mis-determination of accurate viral loads and loss of analytical sensitivity. These are the type of sample tubes used for detection of viral load and the presence of more than one virus in a sample that can cause loss of analytical sensitivity.

Tube Type. A wide variety of sample tubes are required for the multitude of analytes whose presence and/or whose concentrations are determined. Use of incorrect tubes will almost invariably lead to erroneous results. However, even where more than one tube type can be used for an assay, results can vary as has been found for example, for serum glucose levels in serum separator (speckled top) tubes, gray top tops with fluoride ion and green top tubes with anti-coagulant. As we discuss below, in RT-PCR analysis of viral loads, the assumption has been made that the viral particles are present in plasma and are not present intracellularly. Therefore collection tubes that contain anti-coagulant such as lavender top tubes and tubes with anticoagulant that contain plasma separator are both acceptable for quantitative analysis of viral load. our findings as presented in this report suggest that serum separator may block viral diffusion from cells into the plasma layer resulting in underestimation of the viral load.

Multiplex Assays Affect Analytical Sensitivity. Especially as the result of the recent SARS-2 (Covid-19) pandemic, rapid RT-PCR methods have been developed to detect this virus. Since the onset of the pandemic, it has been found that Covid-19 viral infections are difficult to distinguish from other respiratory viruses. This finding has resulted in the advent of multiplex testing for Covid-19 and other major respiratory viruses. Thus highly specific primers for each virus are present in the same tube so that the presence of any of these viruses in a sample can be detected simultaneously. As we describe below, while these multiplex assays are effective and reliable, the presence of more than one virus in a sample has been found to cause a decrease in the analytical sensitivity of the multiplex assay by significant factors. We describe this effect in multiplex assays for samples containing Covid-19, influenza A and B, and respiratory syncytial virus (RSV) and suggest possible causes of this observation with potential remedies.

2. Methods

Sample Preparation and Viral Load Determinationof HCV. The methods used in these studies have been described in detail in ref. 8.

Nucleic Acids Extraction and Multiplex RT-qPCR Analysis of Flu/SARS-CoV-2/RSV

All viral samples consisted of nasal swabs from healthy donors to which viral suspensions (from American Type Culture Collection, Manassas, VA, and/or ZeptoMetrix, Buffalo, NY) at different titers were added. Nucleic acids were extracted from 200 microliters of nasal swab using the MagMAX ™ Viral/Pathogen II Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, catalog #A48383) on the KingFisher Flex platform (Thermo Fisher Scientific, Waltham, MA) per the manufacturers' protocol. To detect the pathogen RNAs, 2.5 microliters of purified nucleic acid were loaded to the PCR well alone with 1.25 microliters of 4X Luna Probe One Step RT-PCR mix (New England Biolabs, Massachusetts, USA, catalog #M3019B) and Flu-SARS-CoV-2-RSV Primer-Probe-Water mix (Mirimus Inc., Brooklyn, NY). Each sample was tested in triplicate and the PCR was performed under the following cycling conditions: 25 ◦C for 2 min, followed by 50 ◦C for 15 min (reverse transcription) and 95 ◦C for 3 min (initial denaturation), and 45 cycles of 95 for 15 s (denaturation) and 55 ◦C for 30 s (annealing/extension). A sample was determined positive if the cycle threshold (Ct) value was equal or under 35.

3. Results

Effects of Different Approved Tube Types on Quatitative PCR for Viral Load.

Several years ago, we reported that the two types of acceptable blood tubes for determination of hepatitis C viral loads (VL), i.e., lavender top tubes (LTT) and plasma processing tubes (PPT),

rendered significantly different viral load values[8]as determined RT- PCR using the m2000 System (Abbott Molecular Inc). This was unexpected given the fact that both tubes contain the same anti-coagulant in identical concentration and differed only in the presence of a gel in the PTT that separates plasma from cells. This finding led to a study in which 202 patient samples were analyzed for HCV viral load in each tube type; 103 were found to be negative in both tube types. Of the remaining 99, 73 showed significant discrepancies in viral load. For these 73 samples for which quantitative results were obtained for both tube types, VLs were statistically higher in LTTs (means 1,817,821.8 in LTTs and 1,083,669.1 in PPTs, p=0.006, alpha=0.05). Further, chi square analysis of paired values for the two tube types showed significantly higher VL values for LTTs compared with those for PPTs. Similar findings occurred for low VLs in the additional 26 paired tubes.

Serum Separator Gel Inhibits Viral Detection.

To determine the cause of this discrepancy, we first tested whether the gel present in the PTTs might sequester virus by transferring the plasma in LTTs to fresh PTTs and then re-performing PCR. For all tubes tested, the VLs were the same ruling out viral sequestration by the gel. This study was followed by another one in which we replaced assayed plasma in PTTs with fresh plasma that was devoid of virus. We then re-centrifuged these tubes and performed RT-PCR on the virus-free plasma. The viral titers in these tubes were significantly high indicating that viral particles were being released from the cell fraction. In the absence of gel, this release appears to be rapid and allows for viral diffusion into the plasma layer while the presence of the gel retards this diffusion. These studies indicate that HCV enters cells and that perhaps VL determinations for HCV should be performed on whole blood in lysis buffer that would result in release of intracellular virus.

In this regard, it is of interest to note that HCV entry into cells (mainly hepatocytes) requires binding of its E2 protein to the CD-81 receptor; binding and viral transport across the cell membrane is thought to be aided by low density lipoprotein (LDL)[8]. Of the hematopoietic cells in whole blood only B lymphocytes have been found to express CD-81 on their membranes[9]. Thus, this cell fraction may be the one responsible for viral sequestration.

Effects of the Presence of More Than One Virus on Viral Amplification.

Multiplex Platform.

At the start of the recent pandemic, our laboratory was called upon to devise and perform rapid testing for COVID-19) in saliva and nasal swab samples. We have developed a rapid RNA extraction method, and we have designed primers for SARS-CoV-2 ORF-10 and N1-coding genes that have unique sequences identified using in silico (BLAST) searches. We have been able to detect SARS-CoV-2 at 3 copy/µL. In view of increased requests for assays for other respiratory viruses, we have expanded our testing to include these respiratory viruses and have adapted our PCR methodology to multiplex analysis. As we did with COVID-19 testing, we developed primer sequences that are unique to each of three other respiratory viruses: influenza A (Flu A), influenza B (Flu B) and RSV.

In these studies, all PCR reactions contain primer/probes for all four target viruses (SARS-CoV-2, Flu A, Flu B, and RSV). We first determined the Limit of Detection (LoD) (defined as the minimum target concentration which can be detected by its specific primer/probes) for each virus alone using a dilution series of contrived samples that was generated by spiking quantitated viruses into the pooled negative specimen (Figure 1A). Three samples at each concentration were generated and subjected to nucleic acid extraction followed by real time PCR, with each sample tested in triplicate PCR wells. The LoD is referred to the lowest concentration where each target (SARS-CoV-2, Flu A, Flu B, or RSV) showed positive (cycling time or Ct $\leq$ 35) in all 3 replicates. Additionally, potential cross-reactivity testing was performed against a range of the commonly found respiratory pathogens. The pathogens (from Zeptometrix, Buffalo, NY) were subjected to the same experimental protocol. All specimens were negative for all four targeted viruses (i.e., no cross-reactivity and good specificity) in all samples tested.

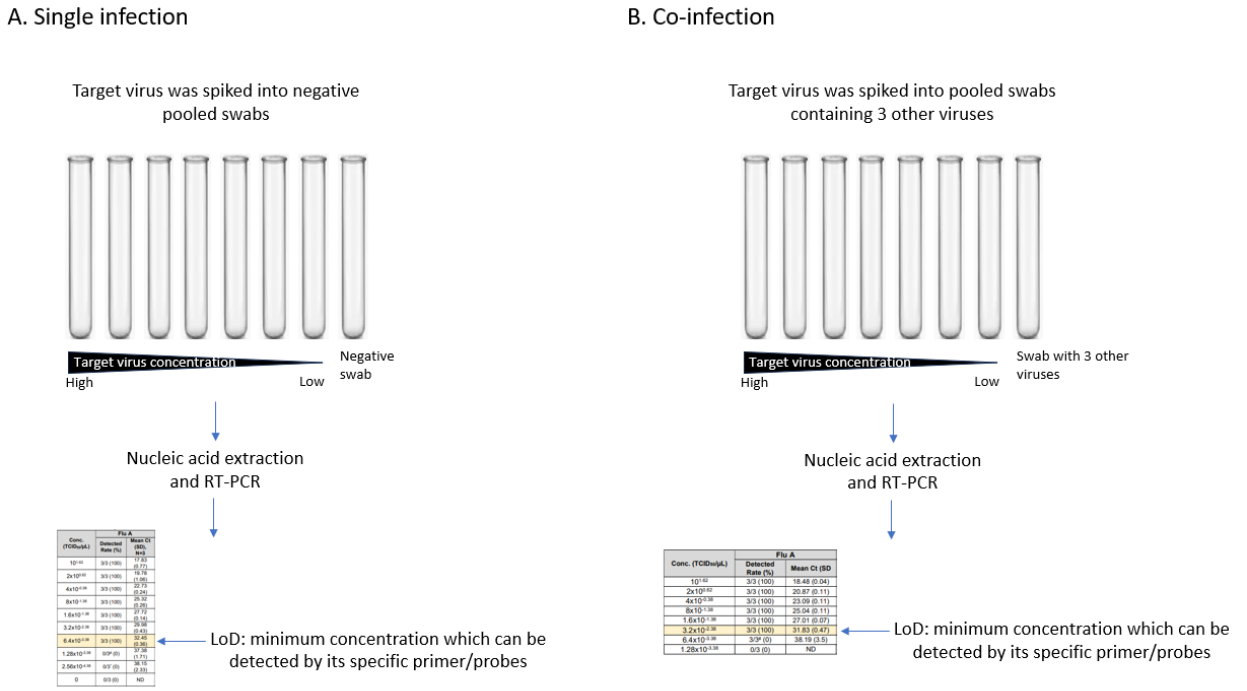

Figure 1. Schematic diagram of single infection (A) and coinfection (B). In A, the upper figure shows that the assayed virus was added to tubes, each containing a virus-negative pooled swab, at different titers (indicated as "low" to "high"). The lower figure shows the lowest detectable titer value highlighted in yellow. In B, the same experiment was repeated except that the tubes contained pooled swab samples that were spiked with three respiratory viruses other than the virus that was being assayed. The viral titer of each of the three viruses was the same in all assay tubes. The LoD in A was one fifth of the LoD in B indicating a decreased analytical sensitivity.

Multi-Virus-Associated Decrease in Method Sensitivity.

We further determined the LoD for each virus in the presence of fixed high titers of the other three viruses (co-infection scenario; Figure 1B). To determine the LoD in the scenario of co-infection, the target virus was serially diluted in the specimens containing high-titer concentration of the other target in the assay. The background viruses were first spiked into the pooled specimen, and a serial dilution of the experimental target was performed using the aforementioned pooled specimen in the presence of the background pathogens. The samples were then extracted and tested with the multiplex assay. This finding is summarized in Figure 2. In this figure -Log LoD is plotted for each of the four respiratory viruses tested in nasal swab samples against paired samples in which the virus is present alone (dark bars) and in the presence of all three other viruses at titers representing their individual LoD (open bars). Higher values therefore indicate increased sensitivity (detection of lower titers of virus).

As can be seen for each virus, there is a large increase in the LoD in the presence of three other viruses (listed as “mixes,” e.g., Mix 1 is Flu A, Flu B and RSV present in tubes assayed for SARS-CoV-2). The increases are factors of 5 for three viruses as shown on the connectors between dark and open bars. Interestingly the increase in LoD is highest for RSV, a factor of 25. Thus, while there is no interference in viral identification in our RT-PCR assays, there is a decreased analytical sensitivity induced by the presence of more than one target virus.

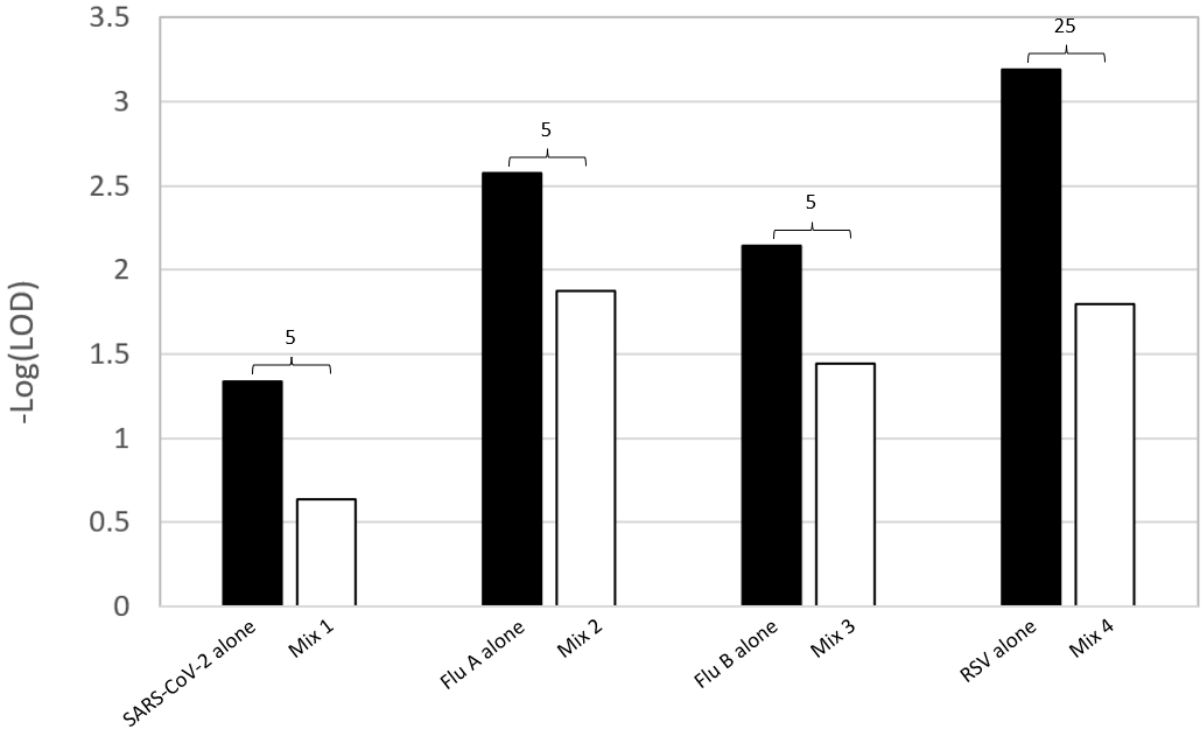

Figure 2. Effects of presence of viruses on the LoD values for amplification of specific viral sequences. In these experiments, two tubes were prepared. In each tube, unique primers for amplification of DNA sequences of each of the four respiratory viruses (SARS-CoV-2, Flu A, Flu B, and RSV) were present. In the single infection scenario, a sample containing one of the four respiratory viruses was added to the tube. In the co-infection scenario, each sample contained different titers of the assayed virus in the presence of constant titers of the three other viruses (see Figure 1). Both tubes were then subjected to analysis for the lowest detectable titer of each virus measured as the LoD. The negative logarithm of the LoD_{50} values for each virus alone (dark bar graph) and in the presence of the other viruses (white bar graphs) are plotted on the Y-axis in that order. Viral identification and presence of the other viruses, shown as "mix," are displayed on the X-axis. Mix 1 is for SARS-CoV-2 in the presence of Flu A, Flu B and RSV; mix 2 is for Flu A in the presence of SARS-CoV-2, Flu B and RSV; mix 3 is for Flu B in the presence of SARS-CoV-2, Flu A and RSV; mix 4 is for RSV in the presence of SARS-CoV-2, Flu A, and Flu B. The factors for decrease in analytical sensitivity for amplification of each virus alone and in the presence of the other three viruses are shown on the connecting lines at the top of the bar graph pairs for each virus.

4. Discussion

Multiplex PCRs to detect more than one target sequence in a single reaction have improved the diagnostic capacity and the cost of the test. However, optimization of multiplex PCRs can be complicated. The effect on increasing the LoD occurs in all coinfection scenarios suggesting that the presence of more than one targeted virus in a patient sample analyzed in a multiplex assay may result in a decreased analytical sensitivity. This effect appears to be non-specific since the viral composition is different in each case. One of the causes could be the competition of reaction components. In one of the multiplex PCR studies for the dystrophin gene (nine targets), the authors showed a 4-5 times greater Taq DNA polymerase concentration (with an appropriate increase in $MgCl_2$ concentration) than that required in a singleplex PCR was necessary to achieve optimal nucleic acid amplification[10]. In the presence of high amounts of the other target pathogen DNAs, the desired target DNA, if at lower concentration, can be outcompeted by the amplification of other targets leading to decreases in the efficiency of the amplification of the desired targets and thus the sensitivity of the reaction[11-12]. Optimization of the PCR components such as PCR buffer constituents, dNTPs, and enzyme concentrations in multiplex PCRs that are designed for simultaneous amplification of multiple targets may prove beneficial. The finding of the decrease in the analytical sensitivity when multiple target pathogens are present simultaneously suggests that thorough evaluation and validation of new multiplex PCR procedures is essential.

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