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RESEARCH ARTICLE

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A concordance study of the Altona RealStar Varicella-Zoster virus real-time quantitative PCR and in-house conventional qualitative PCR

Simon Grandjean Lapierre MD, MSc[1,2] | Karel Boissinot[3] | Christian Renaud[2,3]

[1] Centre Hospitalier de l'Université de Montréal, Montreal, Quebec, Canada

[2] University of Montreal, Montreal, Quebec, Canada

[3] Centre Hospitalier Universitaire Sainte-Justine, Montreal, Quebec, Canada

Correspondence
Simon Grandjean Lapierre, MD, MSc, Centre Hospitalier de l'Université de Montréal, 850 rue St-Denis, Montreal, QC H2X0A9, Canada.
Email: simon.grandjean.lapierre@umontreal.ca

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This study compared the Altona RealStar™ VZV Kit 1.0 real time quantitative VZV PCR with in-house qualitative conventional VZV PCR on cerebrospinal fluid, mucocutaneous, and other uncommon clinical specimens. Overall, positive and negative agreement percentages were respectively 97.9% (95%CI: 93.8-99.6), 100.0% (95%CI: 93.1-100.0), and 96.3% (95%CI: 89.4-99.2) while Cohen's kappa statistic value was 0.96 (95%CI: 0.91-1.00). RealStar™ VZV quantitative PCR assay reported average quantitative viral loads of 4.4×10^5 and 1.1×10^7 copies/mL in cerebrospinal fluid and cutaneous specimens, respectively ($P < 0.01$). RealStar™ VZV PCR assay showed excellent agreement with in house conventional assay for various clinical specimens.

KEYWORDS
nervous system, skin, Varicella-Zoster virus

1 | INTRODUCTION

Varicella Zoster Virus (VZV) or *Human herpes virus 3* belongs to the *Herpesviridae* family and the *Varicellovirus* genus. It is a common vaccine preventable pathogen[1] that is responsible for cutaneous, cerebral, ocular, and disseminated infections.[2,3] Rapid and accurate laboratory diagnosis of VZV infections in a large variety of specimens is essential for optimal clinical management. Effective antiviral treatment and prophylaxis are available and early therapy can improve patients' outcome. VZV meningoencephalitis and myelitis are mainly caused by viral reactivation in the central nervous system parenchymal tissue. Therefore, DNA concentrations in cerebrospinal fluid (CSF) specimens are lower than in vesicle specimens, which are the primary viral replication site in cases of mucocutaneous disease. Quantification of VZV DNA in specimens such as CSF[4,5] and aqueous humour[6] has been shown to have a prognostic value for cases of meningoencephalitis and retinal necrosis, respectively.

Although *Herpesviridae* viral culture used to be considered the gold standard, nucleic acid amplification tests (NAAT) based on Polymerase Chain Reaction (PCR) techniques, including conventional gel electrophoresis, have been shown to be more sensitive.[7] To improve standardization, reduce workload, and shorten turn-around time many clinical microbiology laboratories implement real-time quantitative PCR assays. Several commercial and laboratory designed VZV PCR assays have been evaluated in clinical settings. So far, only the Lyra™ direct HSV 1+2/VZV multiplex real-time PCR assay (Quidel, San Diego, CA) has received FDA approval for testing on cutaneous specimens.[8–10] The RealStar™ VZV PCR assay (Altona Diagnostics, Hamburg, Germany) is a real-time quantitative PCR assay commercially available for research use only. A previous study has evaluated its performance against a consensus of multiple assays including viral culture and direct immunofluorescence assay.[11] An *Alphaherpesvirinae* multiplex PCR assay commercialized by the same manufacturer has also been used as a comparator assay for the evaluation of new Herpes simplex PCR assays[12] and as a diagnostic tool for the evaluation of aseptic meningitis.[13] This is the first comparative evaluation study of the RealStar™ VZV PCR assay performed with a broader variety of specimen.

The primary objective of this study was to evaluate the concordance between RealStar™ VZV real-time quantitative PCR assay and in-house conventional qualitative PCR assay. The secondary

Abbreviations: CSF, cerebrospinal fluid; PCR, polymerase chain reaction; VZV, Varicella-Zoster virus.

objective was to evaluate the ability of the RealStar™ VZV PCR assay to differentiate between expected high and low positive specimens such as mucocutaneous and CSF', respectively.

2 | MATERIALS AND METHODS

2.1 | Study specimens

This study was performed on clinical specimens submitted for routine VZV PCR testing at the clinical microbiology laboratory in Sainte-Justine University Hospital Center, Montreal, Canada from January to June 2015. These specimens were prospectively selected and included in the study after standard of care in-house conventional PCR testing and result reporting to the clinician. To ensure sufficient study power and reduce selection bias, positive and negative specimens for each specimen types were included. A total of 145 specimens including 62 CSF (43%), 61 mucocutaneous (43%), and 22 other specimen types (14%) (11 serums, four respiratory secretions, three corneal scraping, two aqueous humour, one tissue biopsy, and one College of American Pathologist external control) were included. RealStar™ VZV PCR assay was subsequently performed on all specimens.

2.2 | DNA extraction and specimen conservation

Paramagnetic particles based DNA extraction and purification were performed using Maxwell™ 16 Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI). Two-hundred microliter of clinical sample was used in the extraction and nucleic acid was eluted in 80 µL. Both assays were performed with the same nucleic acids eluate, stored at −80°C. As this study was mostly performed retrospectively using these frozen extracted nucleic acids, no extraction control could be used.

2.3 | Conventional qualitative PCR assay

The study center's laboratory routinely performs conventional qualitative VZV PCR assay using the following published protocol.[14] PCR primers targeting the UL21 gene are; forward 5′-AAG TTT CAG CCA ACG TGC CAA TAA A-3′; reverse 5′-AGA CGC GCT TAA CGG AAG TAA CG-3′. PCR reaction is performed with 5 uL of eluate from nucleic acids. Amplification is performed on the ABI 7500 thermal cycler (ThermoFisher Scientific, Inc., Mississauga, ON, Canada) and the 647-bp generated product was detected by standard agarose gel electrophoresis (Figure 1). Internal control is spiked directly to extracted specimen. External proficiency testing and positive and negative quality controls on each PCR reaction are routinely performed as recommended by the College of American Pathologists. Amplification, gel electrophoresis and assay interpretation has a turn-around time of 105 min.

2.4 | Altona RealStar™ VZV Kit 1.0 real-time quantitative PCR

RealStar™ VZV PCR assay was performed according to manufacturer's recommendations with the following reaction mix:

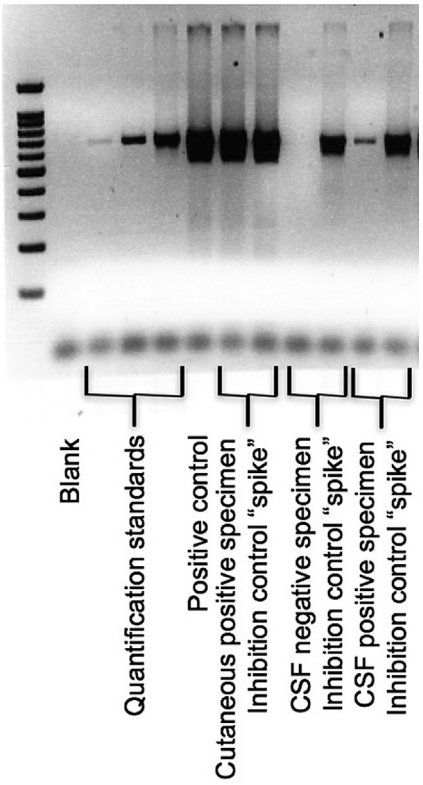

FIGURE 1 Conventional qualitative VZV PCR gel electrophoresis. Positive cutaneous specimen (left), negative CSF specimen (right), and appropriate controls

RealStar™ mastermix (20 µL/reaction), internal control (1 µL/reaction), and eluate from nucleic acid extraction (10 µL/reaction). Amplification was performed on the ABI 7500 thermal cycler (ThermoFisher Scientific, Inc., Mississauga, ON, Canada) as per the RealStar™ VZV protocol: 10 min at 95°C, followed by 45 cycles consisting of 15 s at 95°C, and 1 min at 58°C for a total combined amplification and detection time of 66 min. Probes specific for VZV DNA and for the internal control were labeled with FAM and JOE fluorophores, respectively. Provided quantification standards ranging from 1×10^1 to 1×10^4 copies/µL are used both as positive controls and for standard curve generation. Analytical sensitivity is reported by the manufacturer as 0.1 copies/µL (95%CI: 0.05-0.3 copies/µL). The qualitative aspect of the PCR defines a sample positive if the FAM (VZV) and JOE (Positive control) channels are positive, negative if the FAM channel is negative and the JOE channel is positive and undetermined and retest needed if both channels are negative. However, there is no defined parameter to identify very low copy numbers. While establishing the performance of the test, very low copy specimens (<1 copy/µL) were retested. For this study, a total of three PCR runs and one retest run were performed. Generated standard curves all met the manufacturer's validity criteria with the following average parameters; slope −3.15 (validity range: −3.0 to −3.74), R^2 0.999 (validity range: >0.98), *y*-intercept of 36.03, efficiency of 107.9% (validity range: 85 to 115%) (see Figure 2).

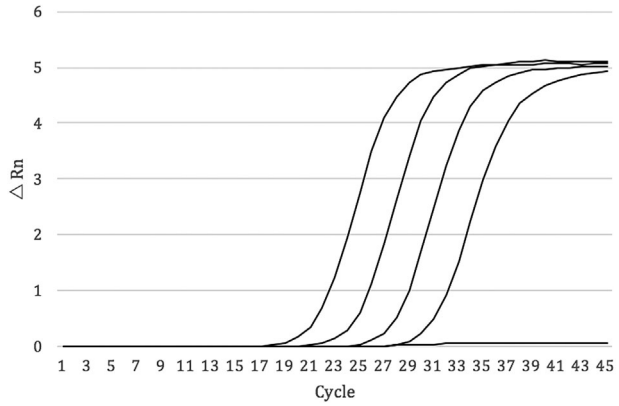

FIGURE 2 Altona RealStar™ Kit 1.0 commercial quantitative VZV PCR standard curves and positive controls. From left to right, amplification of 1.00E+04, 1.00E+03, 1.00E+02, and 1.00E +01 copies/µL calibration controls

2.5 | Statistical analysis

Conventional and quantitative real-time PCR techniques have intrinsic characteristics influencing assay's performance. Establishing a gold standard for new highly sensitive nucleic acid amplification tests is a challenge. Therefore, concordance analysis between both assays using overall rates of agreement (ORA), positive and negative percent agreements (PPA, NPA), and Cohen's kappa statistic value were performed. As kappa values are calculated to ascertain agreement between assays that is in excess of that due to chance, they are more robust than agreement percentages. By definition, Kappa values above 0.75 indicate excellent agreement, values between 0.40 and 0.75 indicate fair to good agreement, and values below 0.40 represent poor agreement beyond chance.[15] Conventional PCR assay was considered as the reference method for the PPA and NPA calculations. The modified Wald method was used to calculate 95% confidence intervals (CI) around the proportions of the binomial distribution. Unpaired *t*-test statistical test was used to compare mean DNA quantification between different specimen types. All statistical analyses were performed with GraphPad Prism™ statistics software version 6.0 (GraphPad Software, Inc., LaJolla, CA).

3 | RESULTS

For 142 specimens out of 145, RealStar™ VZV PCR assay, and the in-house assay yielded the same result. Three specimens were found to be positive on RealStar™ VZV PCR assay and negative on conventional PCR (see Table 1). Repeated testing was performed with both assays on these three discordant samples confirming conventional and real-time PCR results. Two discordant CSF specimens had relatively low viral load with RealStar™ VZV PCR assay (discordant one initial and repeat values: 3920 copies/mL and 9620 copies/mL; discordant two initial and repeat values: 14 copies/mL and 272 copies/mL). One discordant endobronchial aspiration specimen had low viral load (initial and repeat values: 133 copies/mL and 80 copies/mL) with RealStar™ VZV PCR assay.

3.1 | Both assays concordance analysis

Overall rates of agreement, PPA and NPA were, respectively, 97.9% (95%CI: 93.8-99.6), 100.0% (95%CI: 93.1-100.0), and 96.3% (95%CI: 89.4-99.2). Cohen's kappa statistic value was 0.96 (95%CI: 0.91-1.00) indicating excellent agreement beyond chance between both assays.

3.2 | Quantification analysis

Positive specimens on RealStar™ VZV PCR assay resulted in an average Ct of 23.98 (SD 6.18) and quantitative viral loads ranging from 1.40×10^1 to 6.67×10^8 copies/mL. Mean and median quantitative DNA values were 4.43×10^6 and 0.13×10^6 copies/mL in CSF, and 1.09×10^8 and 0.27×10^8 copies/mL in mucocutaneous specimens, respectively ($P < 0.01$).

4 | DISCUSSION

This study evaluated concordance between RealStar™ VZV quantitative PCR assay and in-house conventional qualitative PCR assay in clinical specimens of various nature. Sensitivity, specificity, positive and negative predictive values could not be calculated in the absence of an established gold standard assay. Concordance analysis were performed according to best statistical practices. Both assays performed similarly with only few discordant results. Real-time quantitative PCR assays typically target and amplify significantly smaller DNA fragments than the 647-bp fragment amplified by the conventional PCR comparator assay. Intrinsic analytic sensitivity of the RealStar™ VZV PCR assay could therefore be superior but false-positive results cannot be excluded.

This study was not designed to establish correlations between clinical severity of disease and quantification of viral DNA in clinical samples. Other studies have shown this correlation to exist in certain clinical entities (4,6). Samples containing very low DNA concentrations (below 0.1 copies/µL) can result in variable quantification. In this study,

TABLE 1 Conventional and RealStar™ commercial VZV PCR results

	Conventional+/RealStar™+	Conventional−/RealStar™+	Conventional+/RealStar™−	Conventional−/RealStar™−
CSF	24	2	0	36
Mucocutaneous	24	0	0	37
Other	15	1	0	6
Total	63	3	0	79

these specimens were negative with the conventional qualitative assay. The capacity of the RealStar™ VZV PCR assay to differentiate between known low and high positive clinical samples such as CSF and mucocutaneous lesions was evaluated. Difference between mean quantitative values the two specimen types was found to be statistically significant ($P < 0.01$).

Implementing real-time quantitative PCR assays has many advantages over conventional electrophoresis based qualitative PCR assays. It allows DNA quantification, reduces cross contamination risks, and decreases turn-around time. This is particularly important for a high throughput assay such as VZV PCR. The implementation of real-time PCR assay can facilitate laboratory testing and optimise patient management. In this study, post extraction total turnaround time was 66 min for the RealStar™ VZV PCR assay compared to 105 min for the in-house conventional PCR assay.

5 | CONCLUSION

This is the first study presenting concordance data between the RealStar™ VZV PCR assay and comparative conventional qualitative PCR assay in various clinical specimens. RealStar™ VZV PCR assay has excellent concordance with conventional PCR assay in CSF, mucocutaneous and other specimen types. RealStar™ VZV PCR assay is also quantitatively discriminant between low and high positive specimens and has shorter post extraction turnaround time than conventional PCR assay. More exhaustive studies including detailed clinical correlations or multiple molecular assays as imperfect composite gold standard comparator are needed to further evaluate the clinical performance of the RealStar™ VZV PCR assay.

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CONFLICTS OF INTEREST

The authors declare having no financial or other conflict of interest that might be construed to influence the contents of the manuscript, including the results, or interpretation of publication. Reagent kits for the RealStar™ VZV Kit 1.0 real-time quantitative PCR assay were provided free of charge by Altona Diagnostics (Hamburg, Germany). Altona Diagnostics was not implicated in study design, specimens testing, results collection, or manuscript writing and submission.

ORCID

Simon Grandjean Lapierre http://orcid.org/0000-0003-3646-1573

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