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Optimization and evaluation of a novel real-time RT-PCR test for detection of parechovirus in cerebrospinal fluid

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ABSTRACT

Human parechovirus (HPeV) infections cause a broad array of clinical manifestations ranging from gastrointestinal or respiratory illness to central nervous system (CNS) diseases. Though nucleic acid amplification tests (NAATs) for detection of HPeVs have been described, a need exists for sensitive and specific NAATs with internal control (IC). This study describes optimization and evaluation of a novel, real-time reverse transcription PCR (RT-PCR) test for detection of HPeV from CSF using EliTech HPeV Research-Use-Only detection reagent, MS2 IC and quantified HPeV control. Four RT-PCR kits were compared to select an enzyme with optimal amplification efficiency. The optimal RT-PCR enzyme volume and the best approach to add MS2 to the easyMAG extraction platform were investigated. Following assay optimization, performance characteristics were determined. SuperScript was the most efficient one-step RT-PCR kit, with 0.5 μl/reaction of enzyme being most cost-effective. Adding MS2 to samples post-lysis was better than pre-lysis. The limit of detection of the new test was 570 copies/mL. Commercially-available HPeV 1–6 were detectable, and no cross-reactivity with other CNS pathogens was observed. This test was accurate and reproducible for detection of HPeV and IC. It demonstrated good performance characteristics and is a useful addition to a suite of molecular assays for detection of viral pathogens in CSF.

1. Introduction

Parechoviruses, members of the family *Picornaviridae*, have four species: A through D (http://www.picornaviridae.com/parechovirus/parechovirus.htm). Parechoviruses A, also known as human parechoviruses (HPeVs), currently consist of 19 different types (HPeV1-19) based on the genetic diversity in the VP1 gene (Esposito et al., 2014; McNeale et al., 2018; Olijve et al., 2017). HPeV1 is the most prevalent type in the United States and Europe (Selvaraju et al., 2013). Infection with HPeV1, 2 and 4–8 may be asymptomatic or cause diseases ranging from mild gastrointestinal or respiratory illness to serious diseases including myocarditis, encephalitis, meningitis and pneumonia (Khetsuriani et al., 2006a; Stanway et al., 2000). HPeV3 is recognized as an emerging etiological agent responsible for sepsis-like illness and severe central nervous system (CNS) diseases including encephalitis, meningitis and white matter abnormalities (Esposito et al., 2014; Gupta et al., 2010; Levorson et al., 2009; Selvarangan et al., 2011; Verboon-Maciolek et al., 2008; Wolthers et al., 2008).

Rapid and accurate detection of HPeVs from cerebrospinal fluid (CSF) specimens is essential for diagnosis of CNS diseases and for preventing inappropriate and costly treatments (Esposito et al., 2014; Lai et al., 2003; Leber et al., 2016; Rotbart, 1990; Tang et al., 1999; Verstrepen et al., 2001; Whitley and Gnann, 2002). Development of nucleic acid (NA) amplification tests (NAATs) for detection of HPeVs has evolved in recent years. A pan-HPeV real-time reverse transcription-PCR (RT-PCR) assay to detect HPeV1–6 was first described in 2008 (Nix et al., 2008). Subsequently, other laboratory-developed HPeV RT-PCR tests, including a HPeV3-specific RT-PCR, and droplet digital PCR (ddPCR) assays have been developed (Aizawa et al., 2016; Esposito et al., 2014; Selvaraju et al., 2013). Two commercial CNS panels which include detection reagents for HPeVs are available: the Meningitis Viral 2 ELITe MGB Panel, and the BioFire FilmArray Meningitis/Encephalitis Panel (Leber et al., 2016). These panels are costly and not suitable for all clinical laboratories.

The addition of a sensitive and specific method for detection of HPeVs to the current menu of laboratory-developed RT-PCR viral pathogen detection tests on CSF would be beneficial for appropriate patient care. EliTech HPeV Research-Use-Only (RUO) detection reagent

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with MS2 internal control (IC) recently became available commercially. This reagent contains primers and probes targeting HPeVs and MS2. This study describes the optimization and evaluation of a pan-HPeV RT-PCR test ("EliTech HPeV RT-PCR Test") based on the EliTech HPeV RUO detection reagent. Optimization involved selection of a one-step RT-PCR master mix, use of standardized control material, and use of IC. Performance characteristics studied included inclusivity of HPeV types detected, analytical sensitivity, analytical specificity, reproducibility and accuracy.

2. Materials and methods

2.1. Samples and HPeV run control

Specimen matrix consisted of pooled residual CSF found to have no detectable nucleic acids from common CNS viral pathogens or HPeV after testing with a panel of laboratory-developed PCR assays (for CMV, EBV, enteroviruses, HSV-1, HSV-2, JCV or VZV) and a previously published HPeV real-time RT-PCR test (primers AN345 and AN344, and probe AN257) (Nix et al., 2008). Analytical experiments were performed with a commercial preparation of HPeV-1 quantified by ddPCR (HPeV Run Control, 2.4×10^7 copies/mL, Exact Diagnostics, Fort Worth, TX).

2.2. Screening for optimal one-step RT-PCR master mix

Four commercial one-step RT-PCR master mixes were compared for reverse transcription and DNA polymerase efficiency using previously published HPeV real-time RT-PCR primers (AN345 and AN344) and probe (AN257) (Nix et al., 2008). SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (SSIII; Invitrogen, Carlsbad, CA), QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany), PrimeScript One Step RT-PCR Kit Ver. 2 (Takara Bio USA Inc., Mountain View, CA) and GoTaq Probe 1-Step RT-qPCR System (Promega, Madison, WI) were used according to the manufacturers' instructions. HPeV Run Control was diluted (10-fold) to concentrations of 2.4×10^4 - 2.4×10^1 copies/mL in negative CSF. Total nucleic acid extraction (500 µL input and 50 µL elution volume) was performed on the NucliSENS easyMAG automated extraction system with the generic protocol (bioMérieux Inc., Durham, NC). Eluate (5 µL) was added to 25 µl of reaction mix. Real-time RT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Thermal cycling conditions of different kits are listed in Table 1.

2.3. Optimization of the EliTech HPeV RT-PCR Test

The most efficient RT-PCR master mix was integrated with EliTech HPeV RUO detection reagent (purchased from EliTech Group, Paris, France) to establish the EliTech HPeV RT-PCR Test. To further optimize the EliTech HPeV RT-PCR Test, two methods of MS2 IC addition to samples during extraction were investigated: either before lysis or with silica beads post-lysis. Experiments were performed using 25 µl of MS2 stock solution (20,000 copies/µL; EliTech Group, Paris, France) added to HPeV Run Control (500 copies/mL HPeV RNA). Nine replicates for each IC addition method were assayed with the EliTech HPeV RT-PCR Test. Detection rate and threshold cycle (C_T) value for HPeV and MS2 were compared. Based on the results of this experiment, MS2 was added post-lysis at a dilution that produced C_T values between 30 and 33.

The impact of different SSIII enzyme volumes (0.25, 0.5 or 1 µL per reaction) on detection of HPeV and MS2 was determined (6 replicates for each volume). Two concentrations of HPeV Run Control were studied (500 and 1000 copies/mL).

Table 1
Thermal cycling conditions of four commercial one-step RT-PCR kits screened in this investigation.

One-step RT-PCR kits (manufacturer)	RT condition	PCR cycling conditions
SuperScript III (Invitrogen) PrimeScript (Takara) GoTaq (Promega)	50°C 30 min	95°C 2 min; 95°C 15 sec, 60°C 1 min, 45 cycles
QuantiTect (Qiagen)	50°C 30 min	95°C 15 min; 95°C 15 sec, 60°C 1 min, 45 cycles

RT, reverse transcription.

2.4. The optimized EliTech HPeV RT-PCR Test

After assay optimization, the EliTech HPeV RT-PCR Test was performed using the following protocol. CSF specimens were subject to total NA extraction using the NucliSENS easyMAG automated extraction system with the generic protocol (500 µL input and 50 µL elution volume). After sample lysis step, MS2 IC was subject to 1:10 dilution with PBS, and then 5 µl diluted MS2 was added to a lysed sample with 50 µl silica beads. After extraction, 5 µL eluate was added to 25 µl of reaction mix containing 12.5 µl SuperScript III Master Mix buffer (2x), 0.5 µl SuperScript III enzymes and 1.25 µl EliTech HPeV RUO detection reagent (20x). Real-time RT-PCR for HPeV and MS2 was performed on a 7500 real-time PCR system. Thermal cycling conditions were: 50°C 30 min; 95°C 2 min; 95°C 15 sec, 60°C 1 min, 45 cycles.

2.5. Performance characteristics of the EliTech HPeV RT-PCR Test

The optimized EliTech HPeV RT-PCR Test was evaluated for the following performance characteristics: (i) genotype inclusivity: HPeV1–6 NATtrol controls (ZeptoMetrix Corporation, Buffalo, NY) were diluted 1:100 in negative CSF and tested; (ii) the limit of detection (LOD): HPeV Run Control was serially diluted in negative CSF to 1000, 500, 250 and 125 copies/mL and tested (20 replicates per dilution). LOD was defined as the lowest HPeV concentration with a detection rate ≥ 95% (Forman et al., 2011; Yao et al., 2018); (iii) analytical specificity: cross-reactivity with a broad range of CNS pathogens (listed in Table 5) was assessed; (iv) reproducibility: three replicates of HPeV Run Control at 1000 copies/mL were tested over three days; (v) accuracy: a blinded panel of 10 HPeV-negative CSF specimens and 20 CSF specimens spiked with different concentrations of HPeV was tested. Contrived specimens were utilized because CSFs from patients with HPeV-associated CNS disease were not available.

2.6. Statistical analysis

Statistical differences were determined by t-test, analysis of variance or exact probabilities with SPSS 11.5 software where appropriate. The calculated LOD was determined by Probit regression. All P values were two-tailed and < 0.05 was considered statistically significant.

3. Results

3.1. Optimal one-step RT-PCR master mix

Various one-step and two-step RT-PCR kits can confer different reverse transcription, amplification, and detection efficiencies (Liu et al., 2016; Selvaraju et al., 2013; She et al., 2010; Wei et al., 2016). This study compared the sensitivity of four commercial one-step RT-PCR kits using a previously reported HPeV RT-PCR assay (Nix et al., 2008). SSIII, QuantiTect and PrimeScript showed a 100% detection rate for dilutions of run control from 2.4×10^4 to 2.4×10^2 copies/mL, whereas GoTaq only had a 66.7% (2/3) detection rate for 2.4×10^3 copies/mL and 0% (0/9) for 2.4×10^2 copies/mL (Supplemental Table S1). GoTaq was eliminated from further consideration due to its low sensitivity. Of the remaining three candidates, only SSIII was able to detect the most dilute run control at 2.4×10^1 copies/mL, with a 37.5% (3/8) positive

Table 2
Detection of HPeV and MS2 by the EliTech HPeV RT-PCR Test with MS2 internal control added either before or after sample lysis.

	Targets			
	HPeV[b]		MS2	
Addition of MS2 IC during NA extraction[a]	No. detected/ total no. (%)	Mean C_T ± SD	No. detected/ total no. (%)	Mean C_T ± SD
Pre-lysis (added to samples)	6/9 (66.7)	39.8 ± 0.8	9/9 (100)	39.0 ± 0.6
Post-lysis (added with silica beads)	8/9 (88.9)	38.9 ± 0.9	9/9 (100)	26.2 ± 0.2
P value	0.25	0.07	Not Applicable	< 0.001

NA, nucleic acid.
[a] 25 µL of MS2 stock solution (20,000 copies/µL) was added either before or after sample lysis.
[b] HPeV dilution concentration = 500 copies/mL.

detection rate (Supplemental Table S1). SSIII was selected as the best-performing one-step RT-PCR kit.

3.2. Impact of MS2 addition pre- and post-lysis on the EliTech HPeV RT-PCR Test

The effect of IC addition pre- and post-lysis on HPeV and MS2 amplification efficiency was determined. Although both methods resulted in a 100% detection rate for MS2, its mean C_T value was 39.0 ± 0.6 when MS2 was added to samples before lysis, but significantly ($P < 0.001$) decreased to 26.2 ± 0.2 when MS2 was added with silica beads post-lysis (Table 2). Interestingly, adding MS2 post-lysis resulted in a lower mean C_T value for HPeV (38.9 ± 0.9) and a higher detection rate (88.9%, 8/9) than adding it before sample lysis (HPeV mean C_T value: 39.8 ± 0.8; detection rate: 66.7%, 6/9), though neither difference was statistically significant ($P = 0.07$ and 0.25, respectively). In all subsequent experiments, MS2 was diluted 1:10 with PBS and added with silica beads post-lysis such that the MS2 C_T value fell between 30 and 33 (Tables 3–6).

3.3. Impact of SSIII enzyme volume on the EliTech HPeV RT-PCR Test

The volume of SSIII enzyme per reaction showing the best detection rates for HPeV, and the lowest C_T values for both HPeV and MS2 was determined. At 1000 copies/mL of HPeV, 0.5 and 1 µL of SSIII enzyme showed the same HPeV detection rate (100%), and similar HPeV mean C_T values (37.9 ± 0.8 and 37.4 ± 1.0, respectively) and MS2 mean C_T values (32.1 ± 0.1 and 32.0 ± 0.1, respectively) (Table 3). In contrast, 0.25 µL of SSIII enzyme was less sensitive as it resulted in a decreased HPeV detection rate (83.3%, 5/6), and significantly increased C_T values for both HPeV [41.2 ± 2.0 ($P = 0.004$)] and MS2 [33.1 ± 0.4 ($P = 0.001$)] compared to 0.5 µL. Similar results were observed with 500 copies/mL of HPeV (Table 3). With 0.25 µL of SSIII enzyme, two replicates of HPeV were not detected (detection rate: 66.7%, 4/6) and there was a significant increase in mean C_T values for

Table 4
LOD data of the EliTech HPeV RT-PCR Test.

HPeV dilution (copies/mL)	No. detected/ total no. (%)	Targets (Mean C_T ± SD)	
		HPeV	MS2
1000	20/20 (100)	37.7 ± 1.0	32.5 ± 0.1
500	18/20 (90)	38.2 ± 0.6	32.6 ± 0.1
250	12/20 (60)	38.9 ± 0.7	32.6 ± 0.1
125	7/20 (35)	39.8 ± 0.2	31.5 ± 0.3

LOD, limit of detection.

Table 5
Cross-reactivity of the EliTech HPeV RT-PCR Test with common CNS pathogens.

Spiked Organisms		C_T (HPeV)	C_T (MS2 IC)
Bacteria			
	Escherichia coli	Not detected	34.4
	Hemophilus influenzae	Not detected	31.1
	Streptococcus agalactiae	Not detected	30.8
	Streptococcus pneumoniae	Not detected	33.4
Viruses			
	CMV	Not detected	31.5
	coxsackievirus A21	Not detected	32.7
	coxsackievirus B5	Not detected	31.9
	EBV	Not detected	31.9
	echovirus 7	Not detected	31.4
	EV 70	Not detected	31.2
	HIV	Not detected	33.8
	HSV-1	Not detected	31.3
	HSV-2	Not detected	33.6
	JCV	Not detected	33.2
	VZV	Not detected	33.5
Yeast			
	Cryptococcus neoformans	Not detected	33.4

Table 3
Detection of HPeV and MS2 by the EliTech HPeV RT-PCR Test with different amount of SuperScript III enzyme.

		SuperScript III enzyme (µL/reaction)					
		0.25		0.5		1	
HPeV dilution	Indexes	HPeV	MS2	HPeV	MS2	HPeV	MS2
1000 copies/mL	No. detected/ total no. (%)	5/6 (83.3)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
	Mean C_T ± SD	41.2 ± 2.0[a]	33.1 ± 0.4[b]	37.9 ± 0.8[a]	32.1 ± 0.1[b]	37.4 ± 1.0	32.0 ± 0.1
500 copies/mL	No. detected/ total no. (%)	4/6 (66.7)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
	Mean C_T ± SD	41.0 ± 0.9[c]	33.8 ± 0.3[d]	39.5 ± 0.9[c]	32.7 ± 0.2[d]	39.1 ± 1.3	32.6 ± 0.2

[a] $P = 0.004$ between the two C_T values.
[b] $P = 0.001$ between the two C_T values.
[c] $P = 0.035$ between the two C_T values.
[d] $P < 0.001$ between the two C_T values.

Table 6
Reproducibility of the EliTech HPeV RT-PCR Test.

Day	No. detected/ total no. (%)[a]	Mean C_T ± SD (HPeV)	Mean C_T ± SD (MS2)
1	3/3 (100)	38.1 ± 0.9	32.3 ± 1.1
2	3/3 (100)	38.4 ± 1.1	32.4 ± 1.0
3	3/3 (100)	38.3 ± 0.3	32.3 ± 0.6

[a] HPeV concentration = 1000 copies/mL.

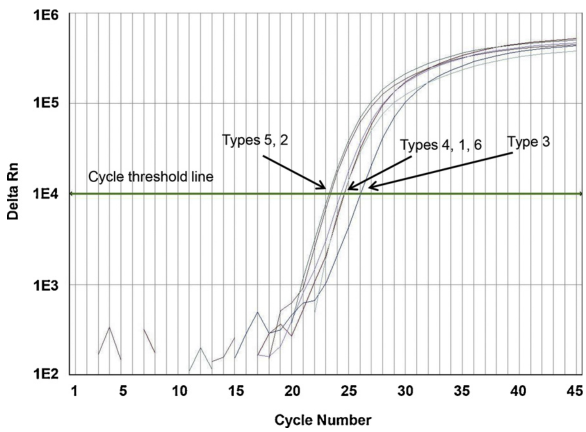

Fig. 1. Detection of HPeV 1–6 with EliTech RUO reagent in the EliTech HPeV RT-PCR Test. HPeV1–6 controls (ZeptoMetrix NATtrol) were diluted 1:100 with negative CSF and tested with the optimized assay conditions.

HPeV (41.0 ± 0.9; $P = 0.035$) and MS2 (33.8 ± 0.3; $P < 0.001$) compared with 0.5 µl (HPeV detection rate: 100%, 6/6; HPeV mean C_T: 39.5 ± 0.9; MS2 mean C_T: 32.7 ± 0.2). In all subsequent experiments, 0.5 µL/reaction of SSIII enzyme was used (Tables 4–6).

3.4. Performance characteristics of the EliTech HPeV RT-PCR Test

3.4.1. Genotype inclusivity

A panel of HPeV NATtrol controls (types 1–6) was tested by the EliTech HPeV RT-PCR Test. HPeV 1–6 showed sigmoidal amplification curves with C_T values ranging from 23 to 26, verifying detection of all HPeV types tested (Fig.1).

3.4.2. LOD

At HPeV concentrations of 1000, 500, 250 and 125 copies/mL, the detection rate was 100% (20/20), 90% (18/20), 60% (12/20) and 35% (7/20), respectively (Table 4), indicative of the LOD falling between 1000 and 500 copies/mL. Probit regression analysis determined the LOD to be 570 copies/mL (Fig. 2).

3.4.3. Analytical specificity

Cross-reactivity of the EliTech HPeV RT-PCR Test with common viral, bacterial, and fungal CNS pathogens was assessed (Table 5). All CNS pathogens were negative while MS2 IC was detected with expected C_T values.

3.4.4. Reproducibility

Stable and consistent HPeV and MS2 C_T values were obtained with three replicates of HPeV at 1000 copies/mL over three days (Table 6). The EliTech HPeV RT-PCR Test was highly reproducible.

3.4.5. Accuracy

A blinded panel of CSF specimens (10 negatives and 20 positives spiked with different concentrations of HPeV) was tested with the EliTech HPeV RT-PCR Test. HPeV RNA was detected in all positive specimens (Table 7; C_T values ranged from 35.1 to 41.4); no HPeV RNA was detected in negative CSF specimens. C_T values for MS2 were comparable in all reactions.

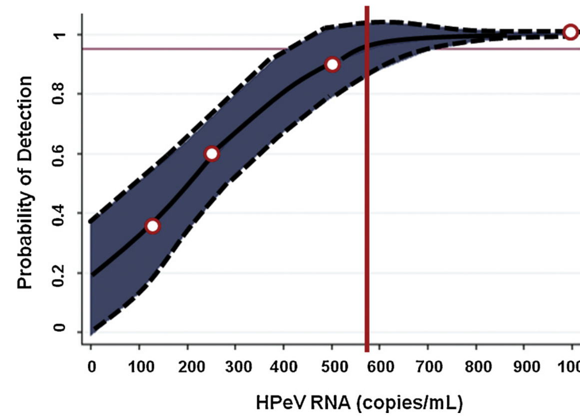

Fig. 2. Probit regression analysis determined the LOD of the EliTech HPeV RT-PCR Test to be 570 copies/mL. Probability of detection corresponds to percentage of replicates detected (n = 20 tested at each concentration). Open circles, LOD data (percentage of replicates detected). Arched solid line, probit regression line. Arched dashed lines, 95% confidence intervals of the regression line. Solid vertical line, predicted concentration of 95% detection limit.

Table 7
A blinded panel of 30 CSF specimens spiked without or with different concentrations of HPeV was tested by the EliTech HPeV RT-PCR Test.

Spike-in HPeV concentration (copies/mL)	No. detected/ total no. (%)	Mean C_T ± SD	
		HPeV	MS2
No spike-in	10/10 (100%)	Undetected	32.1 ± 0.8
1.0×10^3	11/11 (100%)	39.5 ± 1.0	31.9 ± 1.6
2.4×10^3	8/8 (100%)	38.2 ± 0.7	32.0 ± 1.8
2.4×10^4	1/1 (100%)	35.1	32.4
Total	30/30 (100%)		

4. Discussion

CNS diseases including meningitis, encephalitis or meningoencephalitis are usually acute infections with high morbidity and mortality. Laboratory diagnosis of these diseases remains challenging (Leber et al., 2016; Mace, 2010; Tang et al., 1999). HPeVs, especially HPeV3, have been increasingly recognized as significant etiologic agents of infection-related CNS diseases (Esposito et al., 2014; Gupta et al., 2010; Levorson et al., 2009; Selvarangan et al., 2011; Verboon-Maciolek et al., 2008; Wolthers et al., 2008). HPeV NAATs have been developed, including commercial multiplex CNS pathogen detection assays, e.g., BioFire FilmArray Meningitis/Encephalitis Panel (Leber et al., 2016), and Meningitis Viral 2 ELITe MGB Panel. However, reduced sensitivity due to reagent competition, and lack of flexibility to modify panel are major hurdles for the applications of the multiplex CNS pathogen detection panels in the clinical laboratory (Onyango et al., 2017). Laboratory-developed real-time HPeV tests have also been reported (Nix et al., 2008; Selvaraju et al., 2013). Although laboratory-developed tests (LDTs) for HPeV are sensitive and flexible, some have not incorporated IC, thus lacking means to detect PCR inhibition or monitor assay performance. Some LDTs report LODs in 50% cell culture infectious dose per mL ($CCID_{50}$/mL), making LOD comparison between assays difficult, as $CCID_{50}$/mL is semi-quantitative measure and does not reflect the true assay sensitivity. Consequently, there is an unmet demand for a sensitive, specific and accurate HPeV NAAT with IC.

This study describes the integration of SSIII and EliTech HPeV RUO detection reagent with MS2 to develop the EliTech HPeV RT-PCR Test, a novel HPeV RT-PCR with IC. Test performance was optimized by adding MS2 IC with silica beads post-lysis and adjusting the SSIII enzyme volume to be cost-effective with high amplification efficiency. Furthermore, a new HPeV run control quantified in copies/mL by ddPCR was used to define performance characteristics of the EliTech HPeV RT-PCR Test. This test demonstrated good performance characteristics, including analytical sensitivity (LOD 570 copies/mL), analytical specificity, reproducibility, accuracy, and the ability to detect the most common HPeV types (types 1–6).

SSIII was determined to be the most efficient one-step RT-PCR master mix among the four commercial kits compared in this study. This kit has been reported to be efficient and sensitive for enterovirus genotyping (She et al., 2010). Interestingly, SSIII was not the most sensitive one-step RT-PCR kit in a previous study by Selvaraju (Selvaraju et al., 2013). Two possible explanations for this discrepancy are that SSIII was compared to a different panel of one-step RT-PCR kit candidates, and the temperature for reverse transcription chosen in this study (50 °C) provided a higher cDNA synthesis efficiency than 55 °C that was used by Selvaraju et al. (Selvaraju et al., 2013). In fact, lowering RT temperature below 55 °C is recommended by the manufacturer of SSIII to enhance RT efficiency. The volume of SSIII enzyme played an important role in the HPeV and MS2 detection of the EliTech HPeV RT-PCR Test. The optimized volume of SSIII for RT-PCR was 0.5 µl per reaction, which was advantageous because it decreased the manufacturer's recommended enzyme volume with minimal effect on C_T values. Similarly, adding MS2 to sample post-lysis during NA extraction substantially improved detection of both MS2 and HPeV, which permitted dilution of the MS2 stock solution. These optimized parameters made the EliTech HPeV RT-PCR Test more efficient and cost-effective.

Limitations of this study include SSIII selection based on performance of one-step RT-PCR kits with published real-time primers and probe and not the EliTech HPeV RT-PCR Test, only HPeV types 1–6 tested for type inclusivity of the EliTech HPeV RT-PCR Test (types 7–19 were not available for testing), the small number of replicates tested for reproducibility, and the lack of CSFs from patients with HPeV-associated CNS disease to assess accuracy (though a spiking study into residual CSF was performed). HPeV infections have seasonality and peak in summer/fall seasons (Khetsuriani et al., 2006b; Olijve et al., 2017; Selvarangan et al., 2011). Also, the HPeV prevalence in Baltimore area population is unkown.

In summary, the EliTech HPeV RT-PCR Test is a novel qualitative HPeV RNA test with good performance characteristics. Its broad detection spectrum, and its high sensitivity and specificity make it a valuable addition for diagnosis of HPeV infection from CSF.

Declaration of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113690.

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