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Development of a new diagnostic tool for the detection of Human Parechovirus in RT PCR

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INTRODUCTION

Human Parechoviruses (HPeV) have an estimated prevalence of 95% of the world population. They are responsible for infections that, although mostly asymptomatic, may still lead to severe diseases (e.g. encephalitis, meningitis).
The diagnosis is usually based on viral culture, potentially followed by a sero-neutralization. This technique is very sensitive for most HPeV but is long and tedious.
The bioMérieux Parechovirus r-gene® Ref# 71-020 significantly improves the diagnosis of HPeV allowing early detection and improved sensitivity with all Human Parechovirus serotypes.
The internal control (IC) provided allows to check the whole process of extraction and amplification, thus preventing a false negative result due to presence of inhibitor or extraction issue.

MATERIAL AND METHODS

Extraction step:
Nucleic acids were extracted from Cerebrospinal Fluid (CSF) by using NucliSENS® easyMAG® system with Specific B protocol.

200µl of sample were extracted after addition of 10µL of IC1 then eluted in 50µL.
A negative extraction and amplification control (W0) with IC1 was added from the lysis step to check contamination and/or inhibition during the whole process of extraction and amplification.

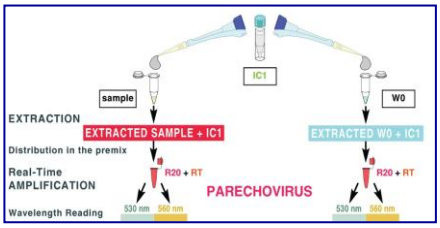


Figure 1: Principle for Parechovirus Detection

Amplification step:
0.15µL of diluted (1/10) reverse transcriptase (RT) was added to 15µL of Parechovirus and IC1 amplification premix. Then 10µL of eluted samples, W0_IC1 and a Positive Control (PC20) were added.
Parechoviruses and IC1 were detected at 530nm & 560nm respectively on Bio-Rad Dx Real Time System (Dx RTS) or Applied Biosystems 7500Fast.

Analytical performance of the Parechovirus r-gene® assay were established :
- Performance on External Quality Assessment Panel (EQA Panel)
- Analytical sensitivity
- Precision
- Analytical specificity

✓EQA Panel:
This Panel was obtained from Quality Control for Molecular Diagnostics (QCMD), an independent International organisation. The 2012 EQA PeVRNA panel consisted of 8 samples containing various concentrations and serotypes of Human Parechovirus, 1 sample containing Human Enterovirus and 1 negative sample. Each vial of lyophilized sample was reconstituted with 1.0 mL of molecular grade water then 200µL were extracted following the protocol described above. The amplification was done on Applied Biosystems 7500Fast.

✓Analytical sensitivity:
The HPeV1 and HPeV2 serotypes were obtained from ATCC (VR-52 & VR-53 respectively). The titers of the pathogens panel were established by the Reed-Muench method for the viruses to determine the $TCID_{50}$/mL. The Human Parechoviruses were prepared by spiking the viruses into CSF, previously characterized Parechovirus negative. A wide dilution range of the stock solution was first performed to determine six dilutions tested in 15 replicates from extraction to amplification (on Dx RTS), for a total of 90 results allowing the Limits of Detection 95% ($LoD_{95\%}$) by Probit Analysis (Minitab 16 statistical software), for HPeV1 and HPeV2.

✓Precision:
The intra-experimental reproducibility (or repeatability) & inter-experimental studies of the Parechovirus r-gene® kit were carried out on titrated samples of HPeV1 (ATCC VR-52) and HPeV2 (ATCC VR-53) diluted in CSF negative for Parechovirus.
1. Intra-assay: 10 RT-PCRs assays from 1 eluted sample were tested on the same experiment.
2. Inter-assay: 10 eluted samples were tested on 10 successive RT-PCRs assays.

✓Analytical specificity:
A panel of 22 viruses (including HPeV1 et HPeV2) representing pathogens commonly present in the CSF or belonging to Enterovirus Genus was constituted to evaluate the analytical specificity. Virus strains were obtained from ATCC or internal collections. The titers of the pathogens panel were established by the Reed-Muench method for the viruses to determine the $TCID_{50}$/mL. Each virus was individually spiked, at a relevant concentration, into CSF specimen. Amplification was done on Applied Biosystems 7500Fast.

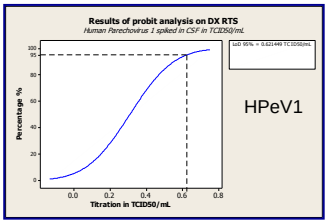
RESULTS

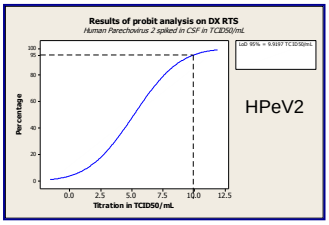
Parechovirus RNA EQA 2012 QCMD Panel:
«Panel members are designated 'core proficiency samples' on the basis of scientific information, clinical relevance and clinical experience (...). Laboratories are expected to correctly analyse and report the core proficiency samples in order to show acceptable proficiency.» QCMD-2012-general-announcement.
Consequently the other samples are considered as challenging due to very low concentrations, they are clearly detection limits.

PeVRNA 2012 EQA Panel QCMD Results						Parechovirus r-gene® REF# 71-020 Results Dx RTS BioRad
Panel Code	Sample Content	Sample Type	Sample Status	Dilution factor	Expected Result	CT (cycles)
HPeV 2012-01	Parechovirus 1		Detected	1.0×10^{-6}	Positive	36.36
HPeV 2012-02	Parechovirus 3		Infrequently detected	1.0×10^{-6}	Positive	39.62
HPeV 2012-03	Parechovirus 1	Core	Detected	1.0×10^{-5}	Positive	32.75
HPeV 2012-04	Parechovirus 3	Core	Frequently detected	1.0×10^{-4}	Positive	36.53
HPeV 2012-05	Parechovirus 4	Core	Frequently detected	1.0×10^{-4}	Positive	36.59
HPeV 2012-06	Coxsackievirus A21	Core	Negative	1.0×10^{-3}	Negative	-
HPeV 2012-07	Parechovirus 2	Core	Frequently detected	1.0×10^{-5}	Positive	35.94
HPeV 2012-08	Negative	Core	Negative	N.A	Negative	-
HPeV 2012-09	Parechovirus 5	Core	Frequently detected	1.0×10^{-4}	Positive	33.84
HPeV 2012-10	Parechovirus 3		Infrequently detected	1.0×10^{-7}	Positive	-

- The 5 "Core" Parechovirus positive samples from Panel HPeV 2012 are detected.
- The 2 "Core" negative samples are undetected as expected.
- No cross reaction is observed with Enterovirus (Coxsackievirus A21).
- 2/3 "No Core" samples (challenging samples) are detected.

Analytical sensitivity:
The following curves show the analysis of the probability of detecting HPeV1 (ATCC VR-52) and HPeV2 (ATCC VR-53).





The limits of detection at 95% in CSF are respectively 0.62 $TCID_{50}$/mL for HPeV1 and 9.92 $TCID_{50}$/mL for HPeV using NucliSENS® easyMAG® and Dx RTS.

Precision:
The following tables give the mean CTs obtained for each dilution. The standard deviation and coefficients of variation were determined.

Intra variability assay

	[LoD95%]	Mean CT	Standard deviation	Coefficient of variation (%)
HPeV1	1 000	28.06	0.14	0.51
	100	30.99	0.29	0.95
	10	34.54	0.32	0.92
	0.01	-	-	-

	[LoD95%]	Mean CT	Standard deviation	Coefficient of variation (%)
HPeV 2	1 000	27.26	0.23	0.85
	100	30.95	0.20	0.66
	10	33.00	0.38	1.16
	0.01	39.12	0.55	1.42

The coefficient of variation was ranged between 0.51% and 0.95% for HPeV1 and between 0.66% and 1.42% for HPeV2.

Inter variability assay

	[LoD95%]	Mean CT	Standard deviation	Coefficient of variation (%)
HPeV1	1 000	27.36	0.23	0.85
	100	30.46	0.49	1.61
	10	34.01	0.71	2.08
	0.01	39.35	0.46	1.17

	[LoD95%]	Mean CT	Standard deviation	Coefficient of variation (%)
HPeV 2	1 000	27.12	0.39	1.44
	100	30.47	0.41	1.36
	10	33.87	0.98	2.90
	0.01	38.47	0.44	1.15

The coefficient of variation was ranged between 0.85% and 2.08% for HPeV1 and between 1.15% and 2.90% for HPeV2.

Analytical specificity:
The recognition specificity of the primers and probes selected for the detection of Human Parechovirus serotypes was determined after the sequence analysis (viral, bacterial and human) present in the banks. It was checked experimentally by real-time PCR on the following pathogens:
HSV1, HSV2, VZV, CMV, EBV, HHV6, HHV7, HHV8, BKV, JCV, HRV14, HRV87, HRV1B, Echovirus 9, Echovirus 25, Echovirus 30, Coxsackievirus B2, Coxsackievirus B4, Coxsackievirus A9, Poliovirus S3, HPeV1 and HPeV2.
HPeV1 and HPeV2 were amplified. No amplification was observed with other pathogens tested.

CONCLUSIONS

Analytical performance studies of the Parechovirus r-gene® real time PCR assay demonstrated robustness and reliable detection of Human Parechoviruses from CSF specimens. The high quality associated with its compatibility with the major extraction and real time PCR platforms allows an immediate integration of Parechovirus r-gene® in most routine diagnostic labs.