Proficiency Study for Quantitative Detection of Herpes Simplex Virus 1 and 2 in Respiratory Samples by Q-PCR

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ABSTRACT

A multicenter proficiency study was organized for the detection of Herpes Simplex Virus (HSV) DNA in tracheal and bronchial aspirates by quantitative real time PCR (Q-PCR).

Analysis of a first batch of clinical samples resulted in some discrepancies in the HSV DNA quantities between the labs. Training focused on contamination-risk and follow-up with more batches of clinical samples, resulted in concordant data. All centers reported very comparable results for the analysis of HSV DNA in tracheal and bronchial aspirates by Q-PCR : 7 samples were negative and 10 samples were found positive with minimal intra- and inter-laboratory variation (less than 0.25 $\log_{10}$ copies HSV genomes/mL).
After the proficiency study, the observational study was initiated with monthly monitoring of the assay performance. The reproducibility remained high: 3 negative samples and 9 positive samples were analyzed with minimal inter-laboratory variation (less than 0.4 $\log_{10}$ copies HSV genomes/mL).

Reliable conclusions from a multicenter data set can only be guaranteed by
- assay standardization
- training on assay performance
- repeated monitoring

INTRODUCTION

Herpes Simplex Virus infection in the upper respiratory tract of intubated patients has been associated with an increased morbidity compared to HSV-negative patients. To evaluate the frequency and the clinical relevance of HSV infection in the lower respiratory tract of mechanically ventilated patients, an observational study was recently started in 4 intensive care units in Belgium. To guarantee correct conclusions from a multicenter data set, an interlaboratory proficiency study was established for the detection of HSV DNA in tracheal and bronchial aspirates by quantitative real time PCR.

METHODS (1)

Protocol HSV DNA detection :

1st DNA extraction of respiratory samples using QiaAmp DNA mini kit (Qiagen)
Clinical samples (tracheal and bronchial aspirates) and validated protocol were provided by the core lab

2nd Q-PCR on DNA extracts to quantify HSV DNA using Rotorgene 3000 or 6000 (Corbett)
Real time Q-PCR reagents (PCR mix, primers, TaqMan probe) and 4 HSV-plasmid solutions (10^3, 10^5, 10^7 and 10^9 copies/mL) for standard curve and internal control were provided by the core lab along with a validated protocol

Protocol proficiency study :

For each participating laboratory, the proficiency testing consisted of 2 evaluation steps:

1st Standard curve analysis
Quality assessment of the PCR efficiency by performing 5 runs of a standard curve with each HSV-plasmid concentration (10^3, 10^5, 10^7 and 10^9 copies/mL) tested in duplicate. Results were reported in Ct values.

2nd Clinical samples analysis
Quality assessment of the DNA extraction and PCR efficiency by analyzing twice several batches of aspirates containing varying amounts of HSV. A standard curve with 3 HSV-plasmid concentrations (10^3, 10^5 and 10^7 copies/mL) was used for quantification. Results were reported in copies HSV genomes/mL.

METHODS (2)

The tests of the proficiency study had to meet well defined criteria :
- ° negative PCR control and negative DNA extraction control served as control on possible contamination
- ° internal PCR control per sample : Ct < 30 to eliminate sample-associated inhibition of PCR reaction
- ° standard curve : R^2 > 0,99 and amplification efficiency between 90% and 110%
- ° < 2 Ct variation between replicates of standard points
- ° < 0.5 $\log_{10}$ variation on copies HSV genomes/mL sample

RESULTS (1)

1. Standard curve analysis All labs obtained results meeting the criteria :

° Negative PCR control : negative
° Standard curve : R^2 > 0,99 and amplification efficiency between 90% and 110%
° Within-run variability per lab : up to 1 Ct variation between duplicates (except for 10^3 copies/mL, 3 labs had differences up to 1.7 Ct's)
° Between-run variability per lab : up to 1 Ct variation between the means of 5 runs (except for 10^3 copies/mL, 1 lab had differences up to 2 Ct's)
° Total variability per lab : up to 1 Ct variation between 10 replicates over 5 runs (except for 10^3 copies/mL, 2 labs had differences up to 2 Ct's)
up to 0,3 sd on 10 replicates over 5 runs (except for 10^3 copies/mL: sd between 0,4 and 1,0) (see Table 1)

copies/mL	Ct Lab1	sd	%CV	Ct Lab2	sd	%CV	Ct Lab3	sd	%CV	Ct Lab4	sd	%CV
10^3	31,4	0,5	1,6	31,1	1,0	3,2	31,5	0,6	1,9	32,2	0,4	1,3
10^5	24,6	0,3	1,2	24,2	0,3	1,2	24,3	0,2	0,7	25,5	0,2	0,8
10^7	17,9	0,2	0,9	17,5	0,3	1,6	17,7	0,1	0,4	18,8	0,2	1,1
10^9	11,3	0,1	1,1	10,5	0,2	2,2	11,3	0,1	0,7	12,3	0,1	0,7

Table 1 : Total variability per lab : mean Ct ± sd on 10 replicates spread over 5 runs (sd = standard deviation)

° Between-lab variability up to 0,7 sd on 40 replicates over 20 runs and 4 labs (see Table 2)

copies/mL	mean Ct	sd	%CV
10^3	31,6	0,7	2,2
10^5	24,7	0,6	2,4
10^7	18,0	0,6	3,3
10^9	11,4	0,7	6,4

Table 2 : Between- lab variability : mean Ct ± sd of 40 replicates spread over 20 runs and 4 labs (sd = standard deviation)

=> conclusion : all labs presented comparable PCR performances

2. Clinical samples analysis All labs obtained results meeting the criteria :

° Negative PCR control and negative DNA extraction control : negative
° Internal PCR control per sample : Ct < 30
° Standard curve : R2 > 0,99 , amplification efficiency between 90% and 110% , Ct's within postulated regions: 30-33 for 10^3 copies/mL, 23-26 for 10^5 copies/mL, 16-19 for 10^7 copies/mL and 12-15 for delta Ct [10^3 - 10^7] copies/mL
° Within-lab and between-lab variability :

2.1 *first batch aspirates* : - concordant results for 6 positive samples
- discrepancies for 5 samples with low viral load (see Table 3)
=> contamination probably caused by handling of viscous samples during DNA extraction

RESULTS (2)

sample n°	copies/mL Lab1		copies/mL Lab2		copies/mL Lab3		copies/mL Lab4		consensus after re-analysis	
	run 1	run 2	run 1	run 2	run 1	run 2	run 1	run 2		
1	ND	ND	ND	24	ND	<1000	670	ND	ND	ND
2	ND	ND	2577	876	ND	ND	ND	ND	ND	ND
3	ND	ND	348	467	ND	ND	3145	ND	ND	ND
4	ND	ND	2379	686	ND	ND	245	ND	ND	ND
5	ND	ND	101	112	ND	ND	923	ND	ND	ND

Table 3 : Within- and between-lab variability for 5 'discrepant' samples (ND = not detected)

2.2 *training* : - special attention on contamination-risk
- introducing a negative extraction control
repeat result => within and between labs 100 % concordant results (see 'consensus' in Table 3)

2.3 *follow-up batches aspirates* : 100% concordant results
- 7 negative samples
- 10 positive samples (range 4.9 10^3 to 1.4 10^9 copies/mL) with minimal within-and between-lab variation : less than 0.25 $\log_{10}$ copies HSV genomes/mL (see Table 4)

sample n°	copies/mL Lab1	copies/mL Lab2	copies/mL Lab3	copies/mL Lab4	copies/mL mean	acceptable interval (± 0,25 $\log_{10}$) lowest limit	highest limit
1	1.1 10^4	4,8 10^3	8,5 10^3	1.3 10^4	8,6 10^3	4,5 10^3	1.4 10^4
2	7.1 10^8	4.5 10^8	5.3 10^8	3.8 10^8	5.2 10^8	2.8 10^8	8.9 10^8
3	1.2 10^5	1.4 10^5	7.5 10^4	1.5 10^5	1.2 10^5	7.1 10^4	2.2 10^5
4	1.6 10^5	1.6 10^5	9.6 10^4	9.9 10^4	1.3 10^5	7.1 10^4	2.2 10^5
5	1,3 10^9	1,1 10^9	1,6 10^9	1,6 10^9	1,4 10^9	0,7 10^9	2,2 10^9
6	1.2 10^5	5.8 10^4	9.6 10^4	1.7 10^5	1.1 10^5	5.6 10^4	1.8 10^5
7	4,6 10^3	7,1 10^3	2,8 10^3	5,1 10^3	4,9 10^3	2,8 10^3	8,9 10^3
8	7.8 10^7	1.2 10^8	1.3 10^8	6.9 10^7	9.8 10^7	5.6 10^7	1.8 10^8
9	4,8 10^6	3,6 10^6	3,4 10^6	2,7 10^6	3,6 10^6	2,2 10^6	7,1 10^6
10	6,8 10^3	1.3 10^4	4,8 10^3	4,7 10^3	7,3 10^3	4,5 10^3	1.4 10^4

Table 4 : Between-lab variability (based on ± 0,25 $\log_{10}$) for 10 positive samples : mean copies HSV genomes/mL of 2 extractions spread over 4 labs

=> conclusion : all labs presented excellent DNA extraction and PCR performances

3. Monthly monitoring during 6 months after the proficiency study : 100% concordant results
- 3 negative samples
- 9 positive samples with minimal inter-laboratory variation (less than 0.4 $\log_{10}$ copies HSV genomes/mL)

=> conclusion : all labs presented continuous excellent DNA extraction and PCR performances

CONCLUSIONS

=> Discrepant results for first aspirates: negative samples were reported low positive.
=> Training stressed on contamination-risk : elimination of false positives
=> Highly reproducible results: mean ± 0.25 log10 copies/mL
=> Repeated monitoring can ensure continuous high quality performance.

Assay standardization + training ⇒ highly reproducible Q-PCR assay in a multicenter setting