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Pitfalls in interpretation of CT-values of RT-PCR in children with acute respiratory tract infections

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ABSTRACT

Background: The relation between viral load and disease severity in childhood acute respiratory tract infections (ARI) is not fully understood.
Objectives: To assess the clinical relevance of the relation between viral load, determined by cycle threshold (CT) value of real-time reverse transcription-polymerase chain reaction assays and disease severity in children with single- and multiple viral ARI.
Study design: 582 children with ARI were prospectively followed and tested for 15 viruses. Correlations were calculated between CT values and clinical parameters.
Results: In single viral ARI, statistically significant correlations were found between viral loads of Respiratory Syncytial Virus (RSV) and hospitalization and between viral loads of Human Coronavirus (HCoV) and a disease severity score. In multiple-viral ARI, statistically significant correlations between viral load and clinical parameters were found. In RSV-Rhinovirus (RV) multiple infections, a low viral load of RV was correlated with a high length of hospital stay and a high duration of extra oxygen use. The mean CT value for RV, HCoV and Parainfluenza virus was significantly lower in single- versus multiple infections.
Conclusion: Although correlations between CT values and clinical parameters in patients with single and multiple viral infection were found, the clinical importance of these findings is limited because individual differences in host-, viral and laboratory factors complicate the interpretation of statistically significant findings. In multiple infections, viral load cannot be used to differentiate between disease causing virus and innocent bystanders.

1. Background

Acute respiratory infections (ARI) frequently occur in young children. Most infections have a viral cause and are usually mild. However, some patients need to be hospitalized because of a more severe course of disease. Factors determining disease severity are not fully understood.

Patient factors related to disease severity, like prematurity or congenital heart disease have been well known for many years [1]. Other factors, such as the role of the immune system are studied recently. CD4 and CD8T cells, as well as NK cell numbers were found to be reduced in the peripheral blood of severe cases of RSV [2]. Genetic factors also play an important role. Recently, a relation between gene expression profiles and disease severity in children with respiratory syncytial virus (RSV) infection was published,

Abbreviations: ARI, acute respiratory tract infection; CT, cycle threshold value; RT-PCR, real-time reverse transcription-polymerase chain reaction test; LOS, length of hospital stay; RSV, respiratory syncytial virus; RV, rhinovirus; HCoV, human coronavirus; HMPV, human metapneumovirus; FLU, influenza virus; HAdV, human adenovirus; HBoV, human bocavirus; PIV, parainfluenza virus.

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Table 1
Viral characteristics.

Virus	Genetic material	Incubation period[a]	Period of viral shedding[a]	Asymptomatic infection[a]
RSV A, B	RNA	2–8 days	3–8 days young infants and immunosuppressed people: up to 28 days	– young infants: not common – reinfections occur frequently
RV	RNA	1 day	7–14 days	common
HCoV 229E, OC43, NL63	RNA	2–5 days	unknown	common
hMPV	RNA	3–5 days	7–14 days up to weeks to months in immunocompromised hosts	common recurrent infection; usually mild or asymptomatic
FLU A, B	RNA	1–4 days	3–7 days	not common
HAdV	DNA	2–14 days	unknown, persistent and intermittent up to months is common	common
HBoV	DNA	Unknown	– up to 75 days	common
PIV 1, 2, 3, 4	RNA	2–4 days	7–21 days	unknown

[a] According to the American Academy of Pediatrics, Red Book(25) and Fields, Virology(26).

showing that expression of specific genes provides a promising biomarker of disease severity for RSV [3,4].

Viral factors may also influence disease severity. Viruses such as RSV are known for their great burden of disease [5]. Thus far, it is not known if the amount of virus in an individual patient is a determinant of disease severity. The amount of virus per milliliter body fluid is called viral load and this parameter is inversely correlated with cycle threshold (CT) value, defined as the number of cycles that are needed to yield a positive fluorescent amplification signal in a real-time reverse transcription-polymerase chain reaction test (RT-PCR). The lower the CT value, the higher the viral load.

The literature on the relation between disease severity and viral load is conflicting. Some investigators suggest a positive relationship [6–15], although others cannot confirm these results [16–20]. Multiple viruses are detected rather frequently in children with respiratory infections and in these cases it is almost impossible to determine the causative pathogen and the relation between viral load and disease severity [21–23].

2. Objectives

Since the relation between viral load and disease severity may be virus dependent, we studied this correlation for 15 different viruses. First, we studied infections in which only one virus was detected. With this information we subsequently studied cases in which multiple viruses were detected.

3. Study design

This study is part of the Evaluation of Viral Diagnostics on Respiratory Infections in Children-trial (EVIDENCE-trial), a multicenter controlled clinical trial, designed to evaluate the clinical impact of rapid result communication using RT-PCR as a diagnostic method in pediatric patients with ARI. The study protocol with in- and exclusion criteria is described elsewhere [24]. In summary, nasal wash specimen samples were obtained at first presentation at the emergency room or outpatient clinic from previously healthy children with ARI during two winter seasons between 2007 and 2009 in two Dutch hospitals. Clinical data were prospectively collected using standardized forms. Informed consent was obtained from all parents and the study protocol was approved by the national Medical Ethics Committee (CCMO # ML13839.098.06).

3.1. RT-PCR method

Duplex RT-PCR assays were performed with all nasal wash specimen samples. The RT-PCR method and its validation procedure have been described previously [24]. In summary, nucleic acid extraction was performed after addition of an internal control (RNA viruses: phocine distemper virus; DNA viruses: artificial plasmid) with an Xtractor gene nucleic acid extraction robot (Qiagen, Hilden, Germany) with an Invisorb virus RNA HTS 96 kit (Invitek, Berlin, Germany) and a Corbett DX DNA extraction kit (Qiagen). The input volume was 200 μl and output was set to 100 μl. For RNA viruses, a random-primed reverse transcription reaction was performed with 57 μl of RNA (MultiScribe RT [Applied Biosystems, Carlsbad, CA]). RT-PCR was performed with an in-house assay adapted from the Erasmus Medical Center (courtesy of Dr M. Schutten, Erasmus Medical Center, Rotterdam, The Netherlands) on an ABI 7500 thermocycler (Applied Biosystems). The PCR reaction was terminated at 45 cycles. Each reaction tube was internally controlled.

3.2. Pathogens

All samples were tested for RSV with a rapid bedside test and supplementary RT-PCR for 15 viruses. Viral subtypes were clustered into virus groups. The characteristics of these virus groups are summarized in Table 1.

3.3. Disease severity score (DSS)

The DSS used in this study is a modification of the severity score used by Gern et al [25,26] (Supplementary Table 1). A score of 0–7 represents mild disease, 8–18 moderate disease and 19–27 severe disease. The score system was used at initial presentation of children with respiratory symptoms at the emergency department or outpatient clinic.

3.4. Statistics

SPSS for Windows, version 21.0 (SPSS inc., IBM Company, Chicago, Illinois) was used for statistical analysis. Mann-Whitney *U* test was used to calculate the relation between CT value and hospitalization (Table 2) and to compare median CT values of viruses in single- and multiple infections (Table 3). Spearman correlation test was used to calculate correlations between CT value and continuous variables with an abnormal distribution, like length of hospital

Table 2
Association between CT-value and the clinical parameters in single- and multiple viral ARI.

		CT value Median (IQR)	CT value, median Hospitalization			Length of hospital stay		Length of oxygen use		DSS at initial presentation	
	N/admitted		yes	no	P[1]	rho	p[2]	rho	p[2]	Rho	p[2]
Single infection	337/255										
RSV	202/163	23.90 (5.6)	23.17	24.57	0.040	−0.036	0.652	0.018	0.824	−0.029	0.678
RV	32/23	31.09 (7.82)	29.85	34.83	0.126	0.298	0.167	0.131	0.551	−0.045	0.809
HCoV	24/10	27.40 (9.42)	24.19	27.88	0.598	0.333	0.347	−0.276	0.440	−0.548	0.006
HMPV	22/15	28.17 (7.65)	28.31	26.77	0.916	−0.223	0.425	−0.210	0.452	−0.106	0.640
FLU	22/16	28.16 (9.1)	25.54	34.77	0.065	0.456	0.076	0.550	0.027	0.289	0.192
HAdV	15/11	28.93 (12.8)	33.37	25.45	0.240	0.413	0.207	0.542	0.085	0.403	0.137
HBoV	11/10	30.57 (17.2)	26.92	39.50	0.114	−0.187	0.604	0.180	0.618	−0.120	0.726
PIV	9/7	35.12 (7.9)	30.79	37.40	0.242	0.559	0.192	0.582	0.170	−0.042	0.914
Multiple infection											
RSV in (RSV +Any other)	91/69	24.43 (7.39)	24.4	25.3	0.584	−0.176	0.149	−0.028	0.818	−0.184	0.080
RSV in (RSV + HCoV)	22/19	23.5 (4.2)	23.5	21.8	0.356	−0.526	0.021	−0.156	0.522	−0.041	0.856
RSV in (RSV + RV)	18/12	25.1 (7.7)	24.7	26.1	0.682	0.270	0.396	0.071	0.826	−0.089	0.725
RSV in (RSV + HAdV)	14/9	23.6 (8.4)	24.2	22.9	0.898	−0.303	0.429	−0.306	0.423	−0.478	0.084
RV in (RV + Any other)	43/27	35.73 (6.18)	36.1	34.9	0.721	0.401	0.052	0.552	0.005	0.122	0.460
RV in (RSV + RV)	18/12	36.4 (3.2)	37.4[b]	35.7[a]	0.099	0.781	0.008	0.771	0.009	0.508	0.053
RV in (RV + any other, except RSV)	17/10	34.7 (8.4)	34.3	34.9	0.380	−0.106	0.771	0.089	0.807	−0.140	0.592
HCoV in (RSV + HCoV)	22/19	32.6 (11.2)	34.0[a]	29.4	0.307	0.004	0.987	0.200	0.425	0.049	0.833

Abbreviation: DSS: disease severity score.
[1] Mann-Whitney *U* tests; [2] Spearman correlation.
[a] CT value data from 1 patient is missing.
[b] CT value data from 2 patients are missing.

Table 3
Comparison of CT values of a specific virus in a single- or in a multiple infection.

Mono infection	N	CT value Median (IQR)	Multiple infection	N	CT value of first mentioned virus in the 4th column Median (IQR)	P value[1]
RSV	202	23.90 (5.6)	RSV + any other virus	92	24.43 (7.39)	0.127
			RSV + HCoV	23	23.1 (4.2)	0.812
			RSV + RV	18	25.1 (7.7)	0.126
			RSV + HAdV	14	23.6 (8.4)	0.704
RV	32	31.09 (7.82)	RV + any other virus	43	35.73 (6.18)	0.009
HCoV	24	27.40 (9.42)	HCoV + any other virus	47	31.60 (9.81)	0.015
HMPV	24	28.17 (7.65)	HMPV + any other virus	20	26.78 (13.71)	0.821
FLU	23	28.16 (9.1)	FLU + any other virus	13	25.37 (7.46)	0.749
HAdV	15	28.93 (12.8)	HAdV + any other virus	32	32.33 (9.89)	0.460
HBoV	11	30.57 (17.2)	HBoV + any other virus	14	32.05 (10.7)	0.120
PIV	9	35.12 (7.9)	PIV + any other virus	20	41.82 (5.51)	0.019

[1] Mann-Whitney *U* test.

stay (LOS), length of oxygen use and DSS, both in single and in multiple infections (Table 2). In multiple infections, analyses were made in group sizes with a minimum of 14 children. A p-value of <0.05 was considered as significant. The interpretation of the Spearman's correlation coefficient (rho) is independent of the statistical significance of the test. The rho indicates the "strongness" of the correlation found between two continuous variables that are tested. It is generally accepted that a rho of 0–0.19 means a very weak, between 0.2–0.39 a weak, between 0.4–0.59 a moderate, between 0.6–0.79 a strong and between 0.8–1 a very strong correlation [27].

4. Results

The detection rate using RT-PCR in all ARI episodes was 81.8%. Single viral infections were detected in 342/582 (58.8%) patients. Multiple viral infections were detected in 134/582 (23%) patients of which 110/582 (18.9%) were dual-infections, 22/582 (3.8%) were triple infections and 2/582 (0.3%) were quadruple infections. Within the group of dual infections, RSV was found most frequently. CT values were available in 471 out of 476 patients (98.9%). CT values ranged from 9.13 to 44.62.

In single infections, there was a small, but statistically significant difference between median CT values of RSV for hospitalized versus non-hospitalized children (23.17 versus 24.57, p 0.04) (Table 2). For Human Coronavirus (HCoV), there was a moderate correlation between CT value and disease severity (rho −0.548, p 0.006). For influenza virus (FLU), there was a moderate correlation between CT value and length of oxygen use (rho 0.55, p 0.027).

In RSV-HCoV multiple infections, there was a moderate correlation between CT values of RSV and LOS (rho −0.526, p 0.021) (Table 2). This correlation was not found for single HCoV in the same children. In RSV-Rhinovirus (RV) multiple infections, there was a strong correlation between CT values of RV and LOS (rho 0.781, p 0.008) and between CT values of RV and length of oxygen use (rho 0.771, p 0.009). In RV multiple infections in which RSV was excluded (RV + any other, except RSV), no significant correlations were found between CT values of RV and clinical parameters. In RV-multiple infections (RV + any other virus, including RSV) there was a moderate correlation between the CT values of RV and length of oxygen use (rho 0.552, p 0.005). These correlations were not found for RSV in the same children.

For RV, HCoV and for parainfluenza virus (PIV), the median CT values in single infections were significantly lower than for multiple infections (Table 3). For RSV, no significant differences of

median CT values were found between single and multiple infections.

5. Discussion

In this study we explored whether CT value is a useful marker for disease severity in children with ARI. Pitfalls in the interpretation of viral load are to be found in five domains: the host, the virus, the manner in which samples are collected, the laboratory techniques and the statistical methods. Our study illustrates the difficulties to interpret CT values in children with ARI in all these domains.

Host factors will be influenced by underlying disease, or genetic factors which predispose to a more severe disease course [1–4]. It is unknown whether the amount of virus in an individual plays an additional role in this context. We therefore suggest that CT values of different individuals do not reflect disease severity at the same level.

Viral factors may also influence the interpretation of CT values. The period of viral shedding differs between viruses (Table 1). For example, RSV and Influenza virus can only be detected during a short timeframe and a positive test usually reflects active infection. Other viruses can be detected for a long time even when clinical symptoms are not present anymore. In HBoV infections a high rate of co-infection occurs [28] and prolonged HBoV shedding is common [29,30]. This is important in the interpretation of the test results with regard to the time of sampling: early or late in the disease period. Most RT-PCR studies are cross-sectional, based on one single nasal washing specimen sample in a period of clinical symptoms, while ideally, a rise and fall in CT value would be necessary to correlate CT value with disease course. The cross-sectional design of our study is a limitation with regard to this. Furthermore, respiratory pathogens may also be detected in asymptomatic children, probably reflecting the natural virus colonization or asymptomatic infection in an individual in a certain period [31,32].

Laboratory factors may also influence the outcome of viral load. Nasopharyngeal aspirates, washes, swabs and brushes are usually considered suitable for RT-PCR analysis [33]. However, the sample size itself is difficult to quantify and some methods are more invasive than others. The quality and volume of nasal wash fluid and other samples (concentration of epithelial cells and/or leucocytes), may vary considerably. Most of the studies have not corrected for the amount of human DNA, epithelial cells or leucocyte counts in nasal wash specimen samples. This is also a limitation of our study. In an individual (adult) patient in our laboratory we found a 1000 fold difference in human DNA concentration between two broncho-alveolar lavage samples, taken by the same pulmonologist, potentially reflecting a similar difference in viral sample size (unpublished observation). Differences in the RT-PCR methods itself may influence sensitivity of the test too. The sensitivity of multiplex RT-PCRs is generally lower than that of single target PCRs [33]. The efficiency of the first step of the RT-PCR procedure, nucleic acid extraction, may vary between samples with different composition and nucleic acid concentrations. The efficiency of both steps may vary considerably between different target sequences. A given RNA or DNA concentration may result into different CT values depending on the target sequence, on the reverse-transcription and polymerase enzymes, and on the reaction conditions used. Furthermore, RNA viruses have a linear amplification step during the reverse transcription before the (exponential) PCR reaction. Therefore, the CT-value as such cannot be used to determine which virus is most important in multiple viral infections. In addition, interpretation of differences in viral load between RNA and DNA is complicated (Table 1). Finally, cut-off values of CT in RT-PCR are also important. Karpinnen et al. found significant differences in the amount of virus between cases and controls, suggesting that cut-off levels for CT have to be defined in order to provide adequate interpretation of positive test results [34]. The cut off value for a positive RT-PCR test result of 45 in our study is probably too high.

The statistical method is important for a proper interpretation of the relation between viral load and disease severity. CT values and continuous clinical parameters with an abnormal distribution like LOS, length of oxygen use and DSS at initial presentation should be correlated with each other using Spearman correlation. We suggest that only statistically significant correlations with a rho > 0.6 are relevant for clinical decision making. Studies on this topic, correlating CT values with disease outcome frequently lack information on Spearman's coefficient and should therefore be evaluated with much caution. We did not find a correlation between the CT value of RSV single infections and disease severity at initial presentation (rho −0.029, p 0.678, n = 202), as was also reported by Houben et al. in children during their first respiratory tract infection in life (rho −0.68, p 0.02, n = 11) [11]. For HCoV, the correlation between CT values and DSS was statistically significant. However, the Spearman's rho (−0.548) indicates only a moderate correlation. We therefore do not consider this finding as relevant for clinical practice. The significant correlation between CT value and length of oxygen use in children with infection by influenza also had a moderate rho (rho 0.550, p 0.027), indicating little clinical importance. Besides these difficulties in the interpretation of rho values for continuous variables, statistically significant differences in dichotomous variables are sometimes not of clinical importance. We showed a statistically significant difference between median CT values of RSV single infection of hospitalized versus non-hospitalized children (23.17 versus 24.57, p = 0.040). However, although this relation is statistically significant, the median CT values are close together and their interquartile ranges are overlapping (5.64 versus 5.14, data not shown in table). This does not help the clinician to discriminate between sick and non-sick children.

The correlation between CT values and clinical parameters is even more difficult to interpret in multiple infections. A strong correlation was found between CT value of RV and LOS (rho 0.781, p 0.008) and length of oxygen use (rho 0.771, p 0.009) in RSV-RV multiple infections. This correlation was not found in RV single infections, suggesting that a low viral load of RV in RSV-RV multiple infections is correlated with a more severe disease course. In multiple infections including RV (RV + any other virus), the correlation between CT value of RV and oxygen use was moderate (rho 0.552, p 0.005) and for LOS this relation was not significant. In multiple infections in which RV is included, but RSV is excluded (RV + any other virus except RSV), no relations between CT values of RV and clinical parameters were found. A recent study suggests that the presence of RSV reduces the probability of RV infection, but if a co-infection occurs, both viruses cause clinical symptoms [35]. Unfortunately, CT values were not reported in this study. Our study adds that in case of these RSV-RV multiple infections, a more severe disease course may be correlated with a low viral load of RV. As our results are based upon data from only 12 hospitalized children, this observation should be confirmed in further studies.

The viral load in single infections with RV, HCoV and PIV was significantly higher than for the same viruses in multiple infections (Table 3). This may suggest that RV, HCoV and PIV are bystanders in multiple infections. Thus far, there are no studies with appropriate sample sizes that show that finding multiple viruses in a patient is accidental, season-related, or the result of true synergism between viruses [31].

6. Conclusions

In children with RSV-RV multiple infections, a more severe disease course may be correlated with a low viral load of RV. Fur-

thermore, the viral load in single infections with RV, HCoV and PIV was significantly higher than for the same viruses in multiple infections, suggesting that these viruses are bystanders in multiple infections. These observations need to be confirmed in studies with a larger cohort in future.

However, for daily clinical practice at this moment, we conclude that viral load is not a helpful determinant to assess disease severity in children with ARI. Even when the relation between viral load of a single virus and clinical outcome is statistically significant, the Spearman's rho is often weak and therefore findings are unlikely to be of clinical importance. In multiple infections, viral load does not help the clinician to differentiate between disease causing virus and innocent bystanders in a single patient, as viral loads of different viruses may not be compared with each other, because technical specifications of RT-PCR assays differ between viruses.

Conflicts of interest

The final manuscript was reviewed and approved by all of the co-authors. We declare that we have no financial or personal conflicts of interest for the publication of this article.

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Appendix A. Supplementary data

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