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Development and evaluation of a four-tube real time multiplex PCR assay covering fourteen respiratory viruses, and comparison to its corresponding single target counterparts

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Background: Multiplex real time PCR is increasingly used to diagnose respiratory viruses and has shown to be superior to traditional methods, like culture and antigen detection. However, comprehensive data on sensitivity, specificity and performance of the multiplex PCR compared to the single target PCR’s is limited for most published respiratory multiplex real time PCR assays.

Objectives: Development and extensive analysis of an internally controlled multiplex real time rt-PCR for detection of respiratory viruses.

Study design: The assay was validated in comparison to single-target PCR’s using plasmid targets and prospectively collected nasopharyngeal aspirates.

Results: Using plasmid targets the multiplex format was found to be at least as sensitive and specific as the single-target PCR and no competition was observed when different targets were present at different amounts in one tube. Clinical validation showed high concordance for all viruses tested except for samples with low levels of enterovirus.

Conclusion: This multiplex showed excellent specificities for all 14 respiratory viruses and sensitivity was high except for clinical samples with low levels of enterovirus.

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1. Background

Demonstrating the presence of a viral pathogen in patients with respiratory symptoms has long been challenging. Traditional methods like viral culture and antigen detection have moderate sensitivity and specificity. Moreover, the relative long turnaround time for some of these techniques makes them less useful for diagnosis of acute clinical problems. With the development of real time (RT) reverse transcriptase (rt) PCR, direct detection of amplified nucleic acids became possible, minimizing cross-contamination and reducing hands on time. In addition, RT rtPCR provides the possibility of easy quantitation of the amplified target. This progress has led to the development of a large number of rtPCR assays for the detection of individual viral pathogens. The introduction of multiplex RT rtPCR assay has increased the efficiency of routine molecular diagnostics of respiratory viruses and has been shown to be cost effective.1–3

Past studies have extensively proven superior sensitivity of (real time) multiplex PCR over traditional methods.4–6 However comprehensive analysis of analytical sensitivity and specificity of a multiplex PCR as compared to single-target assays has not been described for respiratory viruses. Puppe et al.7 compared a multiplex assay with corresponding single-target PCR assays and showed a mean loss of 1.3 log for the multiplex assay. However their PCR was qualitative and did not use a real time format. Gunson et al.8 reported similar median crossing-point (Cp) values when comparing duplex versus triplex real time assays.

2. Objective

In the study described here, a systematic analysis of multiplex RT rtPCR was performed. Sensitivity and specificity of the multiplex assay were compared to its single-target counterparts. This was done in serial dilutions of plasmids, samples with mixed plasmid input and in prospectively gathered nasopharyngeal aspirates (NPA).
Table 1

| Virus                        | Sequence 5’→3’ | Target gene | Labels 5’/3’ |
|------------------------------|---------------|-------------|--------------|
| Influenza A                  |               |             |              |
| F                            | GACAAAGAACCGAACTCCTGTACAYTTCAGTG | M           |              |
| R                            | AAGCCTCCTAGCGTCGAGTCC |              |              |
| P                            | TTTACCGGCTACCGGAGCAGTGG |              |              |
| Influenza B                  |               |             |              |
| F                            | TGGCTGTTTGGACACAACAAT |              |              |
| R                            | TTTTCCCACCACGACAA | M           |              |
| P                            | AGAAATGAGAAAGCGCAACGACACT |              |              |
| Enterovirus                  |               |             |              |
| F                            | GCCCGCTGAACTGCGGTAAAT |              |              |
| R                            | GGGATTTGGCTACATGACAGG |              |              |
| P                            | GCGGAAACGACTATTGGTTGG |              |              |
| Adenovirus                   |               |             |              |
| F                            | CAGAGGCGCTCGGCTGCGCTTASG |              |              |
| R                            | GGAGGACCGTGGGTTAGGRT | Hexon       |              |
| P                            | CAGGCTGCTGAGTTGGGCGCG |              |              |
| RSV                          |               |             |              |
| F                            | ATCAACGTTATACATACCAGACT |              |              |
| P1                           | TGGATCCTAAAACAGATGGAACGCCTCC |              |              |
| P2                           | TTATGACATCAAAACGACATAAGCATCAG |              |              |
| Rhinovirus                   |               |             |              |
| F                            | GCGACCAAGCCACCCGC |              |              |
| P1                           | TAGAGCAGGATGATAG+G+CT |              |              |
| P2                           | TAGTTGTGTCGA+A+G+GCT |              |              |
| P3                           | CAGACCTTTTGTTTCC |              |              |
| Coronavirus                  |               |             |              |
| F1                           | GGGTYGTTGAGATATTTAGC |              |              |
| R1                           | KRITTCGCTGAGCATACCA |              |              |
| R2                           | CARYTTKTCATCAAAAGTAGGACAGCA |              |              |
| P1                           | ATGTTGACAAAYCTGCTACTGGTTTGG |              |              |
| P2                           | CAGAARTATTTGATGTAATGTATGAGACA |              |              |
| Metapneumovirus              |               |             |              |
| R                            | CCGATCAGGATYGGACATG |              |              |
| P                            | TGTTTGCGAGTTTTCAGACATGC |              |              |
| Parechovirus                 |               |             |              |
| F                            | GCTACCTCTGCGGTCATCTTC |              |              |
| R                            | AAAACTGATGTGTGWCCCC |              |              |
| Parainfluenzivirus 1         |               |             |              |
| F                            | CATCTTACCTTTACGTACATCG | HN         |              |
| R                            | AGGTGTGAGTGATAGTACATCG |              |              |
| Parainfluenzivirus 2         |               |             |              |
| F                            | CTGAGCTATGAGATATC |              | CYAN500/DB   |
| R                            | TGATGAGCGATCCTGGAAT |              |              |
| Parainfluenzivirus 3         |               |             |              |
| F                            | ACTTATCACATCTGACAGC |              |              |
| R                            | GATCAGCTTACATCTGACAGC |              |              |
| Parainfluenzivirus 4         |               |             |              |
| F                            | GATCAGCTTACATCTGACAGC |              |              |
| R                            | AGGGACACGGCGGTCTTCCATC |              |              |
| HBoV                         |               |             |              |
| R                            | CACATTCTTCTTCGGCTAAGCAGC |              |              |
| P                            | ATGTTCGGACGGCGTTAATACCTCACC |              |              |
| IC (EAV)                     |               |             |              |
| F                            | CATCTTCTTCTTCTCTTATAG |              |              |
| R                            | AGCCCGACCTTCTACATG |              |              |
| P                            | TGGCCGAGATAATGTTGTCTGACAGC |              |              |

* denotes a locked nucleic acid (LNA) nucleotide.

3. Study design

3.1. Preparation of controls

DNA targets controls were constructed by elongation of oligonucleotide linkers followed by amplification with specific primers, as described before. The PCR products were ligated into PCRII-TOPO vector (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. The sequences of all plasmids obtained were confirmed by sequencing using the BigDye sequencing kit (ABI, Nieuwerkerk a/d Ijssel, The Netherlands).

Equine arteritis virus (EAV) was used as internal control (IC) and was measured during multiplex PCR in respiratory viral package (RVP) number one.

3.2. Primers and probes

Specific primers and probes for all targets (Table 1) were designed using published sequences from Gene Bank. Primers and 6FAM and HEX labeled probes were obtained from Biolegio (Nijmegen, the Netherlands). CYAN500, LCRED610, and LCRED670 labeled probes from TIB Molbiol (Berlin, Germany) and MGB probes from ABI (6FAM or VIC labeled).

3.3. Nucleic acid extraction and real-time multiplex RT rtPCR

Extraction of nucleic acids (NA) from 200 μl of NPA was performed by MagNA Pure LC extraction using the total nucleic acid extraction kit (Roche Diagnostics, Penzberg, Germany). cDNA synthesis was performed as described earlier.11 All PCR (multiplex and single-target) reactions were performed on a Roche LC480 (Roche Diagnostics, Penzberg, Germany) using exactly the same conditions. Reactions contained 10 μl of 2× Probes Master (Roche Diagnostics, Penzberg, Germany), 900 nM of primer (each) 200 nM of probe (each), and 5 μl of a cDNA in total volume of 20 μl. Cycling conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles each consisting of 15 s at 95 °C and 1 min at 60 °C.
Data were analyzed using the LC480 software. Color compensation and calculation of Cq-values was done using LC480 software. The crossing point (Cq) value, which is comparable to a cycle threshold (CT) value, was used as an approximation of the amount of virus present. The Cq-value reflects the cycle at which a positive PCR signal is detected (the lower the Cq-value the more target DNA or RNA is present in the sample). The LC480 software obtains a Cq-value using the second derivative maximum of the amplification curve, which is more precise than cycle thresholds or crossing point algorithms with normalized, proportional, arithmetic or no background adjustment. However, small differences between Cq value of mono and multiplex PCR seem inevitable, since PCR reaction mixture between single and multiplex intrinsically different and to some degree will influence outcome of amplification curves used to determine Cq value.

A sample was considered positive if the results showed Cq-values ≤ 40. Samples were considered negative if Cq-values were above 40 and the Cq-value for the corresponding IC was <32.8 (i.e. two times the standard deviation) above the mean IC for the negative samples.

3.4. PCR design

We have recently described an internally controlled multiplex RT rtPCR for the detection of influenza virus A (InfA) and B (InfB), enterovirus (EV), and adenovirus (AdV) which will be referred to as respiratory virus package 1 (RVP1). In the current study we have broadened our diagnostic repertoire by designing three additional RVP’s: RVP2 for the detection of respiratory syncytial virus (RSV), rhinovirus (RV) and human-metapneumovirus (hMPV); RVP3 for the detection of parainfluenzavirus 1–4 (PIV1–4) and human-parechovirus (hPeV); RVP4 for the detection of human-coronavirus (hCoV: HKU1, NL63, 229E and OC43), and human-bocavirus (hBoV).

3.5. Assessment of sensitivity and specificity

Sensitivity and specificity of the multiplex assay was analyzed using the single target PCR’s as the gold standard.

This was done in four ways. One; dynamic ranges of the multiplex and single-target PCR’s were assessed by testing serial dilutions of plasmids. Two; testing of mixed plasmid solutions containing different viral DNA targets. Each target was tested by spiking one target in an end dilution of 10^6 copies/reaction (i.e. 10^7 copies/ml) in a background of 10^5 copies/reaction (i.e. 10^6 copies/ml) of all other targets for respectively RVP1, RVP2, RVP3 and RVP4. Samples were analyzed by multiplex PCR and single-target PCR and Cq-values were determined and compared. Three; testing of prospectively gathered clinical samples. A panel of 133 NPA’s was taken from cohort of children who were admitted for a suspected respiratory tract infection to the Academical Medical Centre of Amsterdam, during the winter season of 2007–08. The study was approved by the local ethical committee and participants were included after written informed consent by one of the parents. A basic population description is presented in Table 2. Four; for viruses species that were detected in less than 4 NPA samples, QCMD (Quality Control for Molecular Diagnostics, Glasgow Scotland) control panels were run (Table 3). These samples consisted of cultured virus in various concentrations.

Assessment of specificity was done by analyzing potential “crosstalk” (i.e. the fluorescence of a single target influencing detection of one of the others targets, generating a false positive signal) and potential false positivity due to unspecified primer–primer combinations (some multiplex packages contained up to 10 primers) in prospectively gathered NPA.

### Table 2

| Patients characteristics. |
|---------------------------|
| Clinical characteristics from children (n = 133) admitted to the Academical Medical Centre for an acute respiratory tract infection during the winter of 2007–08. |
| Age in months; median (inter quartile range) | 12.1 (4.8–31.5) |
| Sex | ♀ 82 (62%), ♂ 51 (38%) |
| Reason of admittance: |
| Upper respiratory tract infection + imminent dehydration | 15 (11%) |
| Wheezing illness | 30 (23%) |
| Pneumonia | 12 (9%) |
| Impeding respiratory failure | 17 (13%) |
| Dyspnoea not further classified | 13 (10%) |
| Fever without specific respiratory symptoms | 24 (18%) |
| Other (not classifiable) | 22 (16%) |

4. Results

4.1. Analyses of dynamic ranges and mixed input of plasmids

Dynamic ranges were determined to be linear between 500 and 10^6 copies/PCR of target plasmid for both multiplex and single target assay’s. The lower limit of detection for the multiplex assay was between 40 and 50 copies/reaction for every target, which was similar to the individual single-target PCR’s (data not shown).

When analyzing mixed plasmid input, median Cq-values in the multiplex PCR were never higher than the single-target assay’s, and in most cases the median Cq-value of multiplex analyses was even statistically lower than its single-target counterpart (Fig. 1). Variation was slightly higher for the multiplex analysis, with a coefficient of variation (CV) range for the multiplex assay between 0.4% (hCoV) and 3.3% (PIV3), compared to the CV range for single-target assay’s between 0.3% (hPeV) and 1.0% (hBoV).

4.2. Analyses of clinical samples and quality control panels

Overall, good concordance was observed between the multiplex PCR in comparison with the single-target PCR assays for clinical samples (Table 3 and Fig. 2). Of the 133 tested samples 70% (93/133) gave a positive result in one or more of the single-target assays (52% [70/133] mono-infections, 16% [22/133] double and <1% [1/133] triple infections). In 68% (90/133) the multiplex assay gave one or more positive results (50% [67/133] mono-infections, 17% [23/133] double-infections, no triple-infections). All discrepancies are described in Table 4. Opposed to plasmid analysis, the multiplex assay failed to detect EV in clinical samples with low EV loads, since Cq-values > 33 in the single-target PCR were not
Fig. 2. Analysis of sensitivity and specificity using prospective gathered nasopharyngeal samples (grey dots) and QCMD samples (white dots). Crossing point (Cp) values of single target assays (i.e. singleplex) are plotted against Cp values of multiplex analysis, for every individual virus species. Dots with a Cp > 40 represent discrepant PCR results (i.e. sole detection by one of the assays).
Table 3
Clinical evaluation.

(A) Analysis of prospectively collected nasopharyngeal aspirates (n = 133) from children admitted to the Academic Medical Centre for an acute respiratory tract infection during the winter of 2007–08

|                  | Single target PCR | Multiplex PCR |     |
|------------------|-------------------|---------------|-----|
| InflA            | 9/133             | 9/133         | (p = 1.00*) |
| InflB            | 3/133             | 3/133         | (p = 1.00*) |
| EV               | 7/133             | 1/133         | (p = 0.01*) |
| ADV              | 15/133            | 14/133        | (p = 0.86*) |
| RSV              | 36/133            | 36/133        | (p = 1.00*) |
| RSV              | 23/133            | 22/133        | (p = 0.89*) |
| hMPPV            | 7/133             | 8/133         | (p = 0.80*) |
| PIV-1            | 2/133             | 2/133         | (p = 1.00*) |
| PIV-2            | 1/133             | 1/133         | (p = 1.00*) |
| PIV-3            | 1/133             | 2/133         | (p = 1.00*) |
| PIV-4            | 1/133             | 1/133         | (p = 1.00*) |
| hBoV             | 4/133             | 3/133         | (p = 0.70*) |
| hCoV             | 3/133             | 4/133         | (p = 0.70*) |
| hNoV             | 8/133             | 7/133         | (p = 0.80*) |
| Total            |                   |               |     |
| Excluding EV     | 113/133           | 112/133       | (p = 0.96*) |
| Including EV     | 120/133           | 113/133       | (p = 0.73*) |

(B) Quality control panel (2007) for PIV 1, 2, 3, 4, InflB, hPeV and hCoV (n = 10)

|                  | Single target PCR | Multiplex PCR | QCMD |     |
|------------------|-------------------|---------------|------|-----|
| PIV-1            | 4/10              | 4/10          | 4/10 | (p = 1.00*) |
| PIV-2            | 1/10              | 1/10          | 1/10 | (p = 1.00*) |
| PIV-3            | 1/10              | 1/10          | 1/10 | (p = 1.00*) |
| PIV-4            | 3/10              | 3/10          | 3/10 | (p = 1.00*) |
| InflB            | 1/14              | 1/14          | 1/14 | (p = 1.00*) |
| hCoV             | 5/14              | 5/14          | 5/14 | (p = 1.00*) |

* Chi square
* Fisher exact test.

detected in the multiplex PCR. Moreover low viral loads of hBoV (CP-values > 36) seemed to be detected alternately by single-target PCR or by multiplex assay.

Median CP-values in clinical samples of the single-target assay was 29.7 (Inter Quartile Range [IQR]; 27.5–33.3) which did not differ statistically from the median CP-value of the multiplex assay 28.8 (IQR 26.9–32.0) (p = 0.2). Species specific analyses did also not show statistical differences for median CP-values. However, somewhat lower CP-values for the multiplex assay were observed for InflB, RSV and RV in samples with high CP-values (Fig. 2).

Analyses of quality controls was performed to test viral targets that were less prevalent (i.e. PIV 1, 2, 3, 4, InflB and hCoV) in the clinical samples. Results are summarized in Table 3 and Fig. 2. Complete concordance was observed between the multiplex assay and the individual single-target assays for quality controls.

5. Discussion

Real time multiplex PCR is a technique that has been shown to be fast and cost effective for the diagnosis of respiratory viral infections. Most papers on respiratory multiplex PCR’s use traditional methods like virus culture, antigen tests or direct immune fluorescence as comparison, hence questions can be raised about their diagnostic accuracy compared to sensitive single-target assays. We report

Table 4
Discrepancies.

| Virus | Single target CP value | Multiplex CP value | Remarks |
|-------|------------------------|--------------------|---------|
| hBoV  | 39.1                   | neg                | Co-infection with RV (Cp 27.76) |
| hBoV  | 38.4                   | neg                | Co-infection with ADV (Cp 36.04) |
| hBoV  | 37.35                  | neg                | No remarks |
| hBoV  | 36.48                  | neg                | No remarks |
| hBoV  | 37.25                  | neg                | Co-infection with RSV (Cp 28.83) |
| hBoV  | 37.1                   | neg                | Co-infection with RV (Cp 28.83) |
| hBoV  | 36.94                  | neg                | Co-infection with CoV (Cp 34.11) |
| EV    | 39.11                  | neg                | Co-infection with RV (Cp 26.33) & ADV (Cp 37.83) |
| EV    | 39.06                  | neg                | Co-infection with ADV (Cp 34.17) |
| EV    | 38.47                  | neg                | Co-infection with RV (Cp 27.13) |
| EV    | 36.25                  | neg                | Co-infection with RV (Cp 27.04) |
| EV    | 33.92                  | neg                | Co-infection with RV (Cp 26.27) & ADV (Cp 33.23) |
| ADV   | 37.83                  | neg                | Co-infection with RV (Cp 26.55) & EV (Cp 39.11) |
| hMPPV | neg                    | 35.94              | Co-infection with InflA (Cp 23.65) |
| PIV 3 | neg                    | 35.14              | No remarks |
| hPeV  | 33.32                  | neg                | No remarks |
| CoV   | neg                    | 38.11              | Co-infection with ADV (Cp 28.38) |
| RSV   | 38.45                  | neg                | No remarks |
the development and the extensive validation of an internally con-
trolled four-tube RT rPCR for the detection of 14 different viruses
associated with respiratory infections.

5.1. Sensitivity

Combining primers and probes in a single reaction can influence
the sensitivity of the assay as primer–primer interactions may lead
to a lower availability of specific primers. In addition, the presence
of multiple targets in one reaction may result in competition for
enzymes and nucleotides. Analysis of dynamic ranges and mixed
input of plasmids, revealed no loss of sensitivity. In fact, for most
viruses Cp-values were slightly, but significantly, lower in the mul-
tiple analyses. Most likely, this difference is due to the algorithm
used by the LC480 software to calculate crossing point. The LC480
software calculates the second derivatives of the entire amplifi-
cation curve and determines where this value is at its maximum.
This value is the Cp-value and represents the cycle at which the
increase of fluorescence is highest (i.e. the start logarithmic phase
of the PCR). Using Cp values by the second derivative maximum of
the amplification curve is known to be more precise than cycle
thresholds values or other crossing point algorithms.\textsuperscript{13} We
have observed that in our multiplex reactions the total fluorescence is
slightly lower and the slope of the amplification curve is less steep
as compared to the corresponding single-target PCR’s (which is
most likely due to inevitable minute differences in PCR reaction
mixtures of singleplex versus multiplex PCR’s). This difference could
lead to slightly lower Cp-values for the multiplex reactions, but does
not represent a difference in absolute sensitivity.

In prospectively collected clinical samples the multiplex PCR
showed concordant results to the single-target PCR’s. For InFA,
hCoV, PIV1, PIV2, PIV3, PIV 4, hMPV, AdV and hPeV Cp-values were
nearly identical for multiplex and single-target assays. However for
certain viruses (InFb, PIV 1, 2, 3 & 4, hPeV, and hCoV) the number of
clinical samples was relatively small and extensive comparisons
could be merely performed in the mixed input of plasmids anal-
ysis, but not by clinical samples. For RSV, RV and InFb multiplex
showed slightly lower Cp-values than the single target PCR, particu-
larly when single-target Cp-values were \textgtr 30. This is probably due
to a comparable phenomenon as described for slightly lower mul-
tiplex Cp-values in the plasmid evaluation. Discrepancies in clinical
samples were mostly seen for hBoV and EV. Samples with a very
low hBoV load (Cp-value \textgtr 36) were variable positive in either mul-
tiplex or single-target assay. This seems to represent a stochastic
process around the detection limit for both multiplex and single-
target assays. Most of the EV positive respiratory samples, with
Cp-values higher than 33 in single-target assays were negative in
the multiplex assay, suggesting reduced sensitivity of EV detection
of our multiplex assay. Additional analysis of dilution series of 4 dif-
f erent EV subtypes (representing group A, B, C and D EV’s) showed
concordant results for EV Cp-values in the multiplex and the sin-
gleplex assay (data not shown). However the fluorescence plateau
of the EV multiplex assay was notably lower compared to its sin-
gleplex equivalent. It might be possible that in samples with low
abundance of multiple viral targets this lower fluorescence could
lead to false-negativity. In this respect, it is noteworthy that all
clinical EV samples that were “missed” by the multiplex PCR were
samples positive two or three respiratory viruses (Table 4).

5.2. Specificity

Another explanation the missing EV in the multiplex PCR could
be due to possible cross reactivity between EV PCR and RV. The
EV primers and probes we used were previously developed in
our department and described by Beld et al.\textsuperscript{19} EV specificity was
assessed in this publication and did not show any cross-reactivity
with a panel of culture typed rhinoviruses. Since then, we have also
tested the EV PCR against a panel of RV type C virus isolates (N = 9)
which showed absent of cross reactivity against these viruses as
well. Morever extensive in silico analysis (data not shown) did not
show cross reactivity between our EV primers and RV sequences.
However, as RV comprises over a hundred subtypes we cannot
completely rule out cross reactivity with less common HRV strains.
In literature cross-reactivity of molecular test between rhinovirus
and enterovirus is not uncommon\textsuperscript{13} and some authors even sug-
gest that complete specificity for Rhinovirus and Enterovirus is
impossible.\textsuperscript{14} Yet the ongoing discovery of new respiratory EV’s
implies that separate analyses of EV in respiratory samples is of
clinical importance\textsuperscript{15} and optimization RV and EV pcr specificity is
an important topic for future study.

In addition when addressing specificity issues in multiplex PCR:
false positive results due to fluorescence cross-talk from one detec-
tion channel into other detection channels one was not seen in
analysis with mixed plasmid input. Furthermore false positivity
in the multiplex by other causes, like non-specific primer–target
interactions, was studied by analyzing prospective clinical sam-
ple (including viral negative samples) of which the results were
not known before testing. Some discrepancies were seen in which
positive multiplex results were not confirmed by single-target
testing. Most of these samples were hBoV positive samples with
Cp-values > 36 and are probably due to a stochastic process at the
detection limit, as mentioned earlier.

In summary, we present here an extensively evaluated multiplex
PCR assay for the detection of 14 major causative agents of
respiratory tract disease. Good concordance was observed between
multiplex and single target analysis, except for clinical samples
with low level of enterovirus. The assay is internally controlled,
does not need post-PCR manipulations and can be used in routine
diagnostic laboratories, providing a fast and proper validated way
for the detection of respiratory viruses.

Conflicts of interest

None.

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