Diagnostic accuracy of digital RNA quantification versus real-time PCR for the detection of respiratory syncytial virus in nasopharyngeal aspirates from children with acute respiratory infection


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ABSTRACT

Background: Virus-specific molecular assays such as real-time polymerase chain reaction (RT-PCR) are regularly used as the gold standard to diagnose viral respiratory tract infections, but simultaneous detection of multiple different pathogens is often challenging. A multiplex digital method of RNA quantification, nCounter (NanoString Technologies), can overcome this disadvantage and identify, in a single reaction, the presence of different respiratory viruses.

Objectives: To evaluate the accuracy of nCounter to identify and quantify RSV-A and RSV-B in nasopharyngeal aspirates (NPA) of children (6–23-months-old) with acute respiratory infection.

Study design: NPA was collected at enrolment in a prospective cross-sectional study conducted in Salvador, Brazil. A quantitative RT-PCR with a subgroup-specific primer and probeset for RSV-A and RSV-B was performed in parallel with a customized nCounter probeset containing viral targets in NPA.

Results: Of 559 NPA tested, RSV was detected by RT-PCR in 139 (24.9%), by nCounter in 122 (21.8%) and by any method in 158 (28.3%) cases. Compared to the gold standard of qRT-PCR, sensitivity of nCounter was 74.3% (95%CI:63.3%–82.9% RSV-A) and 77.6% (95%CI:66.3%–85.9% RSV-B); specificity was 98.4% (95%CI:96.8%–99.2% RSV-A) and 97.8% (95%CI:96.0%–98.8% RSV-B); positive predictive value was 87.3% (95%CI:76.9%–93.4% RSV-A) and 82.5% (95%CI:71.4%–90.0% RSV-B) and negative predictive value was 96.1% (95%CI:91.1%–97.5% RSV-A), and 96.9% (95%CI:95.1%–98.2% RSV-B). Accuracy was 95.2% (95%CI:93.1%–96.9%) for RSV-A and 95.3% (95%CI:93.3%–96.9%) for RSV-B, while both methods significantly correlated for RSV-A (r = 0.44, p = 8 × 10⁻⁵) and RSV-B (r = 0.73, p = 3 × 10⁻¹²) quantification.

Conclusions: nCounter is highly accurate in detecting RSV-A/B in NPA. Robustness and high-throughput multiplexing indicate its use in large-scale epidemiological studies.
1. Background

Respiratory syncytial virus (RSV) is one of the most common etiological agents of acute respiratory infections (ARI) among children such as bronchiolitis and pneumonia [1,2]. RSV can be divided into two subgroups (A and B) that commonly produce annual epidemics characterized by the circulation of several genotypic strains [3]. The seasonality of RSV-A and RSV-B can be markedly different [4]. Virus-specific molecular assays such as real-time polymerase chain reaction (RT-PCR) are now considered the gold standard in the diagnosis of viral respiratory tract infections. They are rapid, relatively inexpensive and offer increased sensitivity and specificity over prior techniques such as viral culture and direct immunofluorescence, but are sometimes (multiple) detection of different pathogens remains challenging. Upon comparison of four different commercial assays, Salez et al. [5] found considerable variation in sensitivity (20–100%), positive predictive value (50–100%) and Youden Index (0.20–1.00) between assays for fourteen respiratory viruses. Moreover, RNA content measure in all RT-PCR protocols requires enzymatic amplification, which can be considered a major limitation, given the highly variable RNA quantity and quality in NPA [6].

A digital method of mRNA expression quantification, nCounter (NanoString Technologies), can overcome these disadvantages. It identifies by specific hybridization of the genetic material using two probes of 50 nucleotides each (both adjacent on the RNA target), one capture probe and one reporter probe anchored to a barcode [7,8]. Our group and others have previously shown that nCounter detection is highly sensitive and robust for samples with degraded RNA, such as nasopharyngeal aspirates (NPA) and tissue biopsies [8,9,11,12].

RSV-A and RSV-B had been previously detected by nCounter in NPA in a small sample of patients (n = 65) [8]. However, a formal validation of the detection of RSV-A and RSV-B by this new method is lacking.

2. Objective

We aimed to evaluate the accuracy of nCounter (NanoString Technologies) to identify and quantify RSV-A and RSV-B in NPA of children with ARI (aged 6–23 months), using real-time PCR as the reference method.

3. Study design

3.1. Patients selection

This cross-sectional study evaluated community-dwelling children with ARI attending the Pediatric Emergency Room of the Federal University of Bahia Hospital, in Salvador, Northeastern Brazil, between September 2009 and October 2013. Inclusion criteria were children aged from 6 to 23 months with report of fever, sneeze, running nose, nasal blockage, or cough for up to seven days. Children transferred from other hospitals or reporting a previous episode of wheeze were excluded. Clinical and demographic data as well as NPA were collected at enrolment.

3.2. Samples collection

NPA samples were collected by using the following protocol: the distance between the entrance of the nostril and the ear lobe was measured as an estimate of the distance from the entrance of the nostril to the nasopharynx; an aseptic plastic sputum catheter was inserted into the nostril until reaching the nasopharynx; negative pressure was applied and approximately 2 mL of nasal secretions were collected and deposited in a sterile tube with 1 mL of Nuclisens Lysis Buffer (Biomerieux, Boxtel, The Netherlands) and kept frozen at −70 °C until further processing.

3.3. RNA extraction and nCounter digital quantification

Total RNA was extracted using RNEasy (Qiagen’s, Hilden, Germany) following manufacturer’s instructions, and was subsequently hybridized against probes targeting RSV A–B, as part of a customized codeset containing other viral and bacterial pathogens and human target genes [8], designed by NanoString Technologies. For nCounter analysis, each sample was mixed with eight positive control RNAs in fixed amounts (0.2–2000 fg), as well as six negative control RNAs. Laboratory procedures and analysis were performed at the Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, (KU Leuven, Belgium), where researchers were blinded to clinical data and to the results of the other method.

3.4. RSV-A and RSV-B quantification by real-time PCR

The presence and viral load of RSV-A and RSV-B in the extracted RNA was measured by singleplex real-time PCR using subgroup-specific primers and probes (adapted from Zlateva et al.) [13]. The primers and probe for RSV-A are located in the F-gene of RSV-A, whereas the primers and probe for RSV-B are located in the N-gene of RSV-B (Table 1). Real-time qPCR reactions were performed using EuroScript reverse transcriptase and Reaction buffer of the OneStep RT qPCR MasterMix kit (Eurogentec,) with a concentration of 250 nM of each primer for RSV-A and 375 nM of each primer for RSV-B, and 125 nM of probe. To a reaction volume of 20 μL, 5 μL of RNA (extracted sample RNA or diluted cRNA for standard curve) or water (negative control) was added. Reactions were run on an ABI7500 Fast PCR instrument. Quantification of the RSV-A or RSV-B viral load was done by inference on a standard curve, generated using a dilution series containing 10^3–10^8 copies per reaction of RSV-A or B cRNA standards. For generation of cRNA, the same genomic region that is used for the real-time PCR was first amplified by reverse transcriptase PCR, after which the PCR product was transcribed to cRNA using T7 MEGAshortscript. Quantification of cRNA was done by Nanodrop measurement.

3.5. Bioinformatic analysis

Raw data were processed using nSolver 2.0 software (NanoString Technologies) sequentially correcting three factors: technical variation between samples and experiments (positive control RNAs), background correction (negative control RNAs) and RNA content by adjusting the counts (geometric mean) for the 3 human housekeeping genes (G6PD, GAPDH, GUSB), followed by normalization using logarithmic transformation (base 2). The nCounter probe sequences were compared with globally circulating RSV-A and RSV-B strains from the NR (non-redundant) database using BLAST software [14]. For visualization, 50 sequences were selected, and multiple alignments were performed with Muscle software [15]. Real-time PCR primers were aligned using Primer Blast software [16].

Table 1

<table>
<thead>
<tr>
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<th>Primers and probes used for real-time PCR quantification of RSV.</th>
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<tbody>
<tr>
<td>RSV-A</td>
<td>FW primer</td>
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<td></td>
<td>REV primer</td>
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<tr>
<td></td>
<td>MGB probe</td>
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<tr>
<td>RSV-B</td>
<td>FW primer</td>
</tr>
<tr>
<td></td>
<td>REV primer</td>
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<td></td>
<td>MGB probe</td>
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</table>
3.6. Statistical analysis

Categorical variables were presented as absolute number (percentage). 95% Confidence Interval (CI) was included for each of the reported parameters: sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Sensitivity was calculated as the number of cases with virus detected by nCounter among the number of cases with virus detected by RT-PCR. Specificity was calculated as the number of cases without the virus detected by nCounter among the number of cases without the same virus detected by RT-PCR. Positive predictive value was calculated as the number of cases with virus detected by both nCounter and RT-PCR in the numerator and the number of cases with the same virus detected by nCounter in the denominator. Negative predictive value was calculated as the number of cases without virus by both nCounter and RT-PCR in the numerator and the number without this virus as defined by nCounter in the denominator.

Accuracy was calculated as the sum of cases with or without the virus as defined by both nCounter and RT-PCR in the numerator and the total number of studied cases in the denominator. All cases had RT-PCR performed and only one case did not have nCounter performed and was therefore excluded. Correlation between both assays was calculated using non-parametric (Spearman) correlation (GraphPad Prism 6.0), as viral loads did not follow a normal distribution.

4. Results

Among 1154 evaluated children, 504 (43.7%) reported at least one previous episode of wheezing, 16 (1.4%) did not have NPA collected successfully, 1 (0.9%) did not have sufficient NPA for nCounter performance, 11 (1.0%) came from other hospitals and 63 (5.4%) did not consent (Fig. 1). Thus, this study group comprised 559 cases. The mean age was 11.4 ± 4.5 months and 286 (51.2%) were female. The most frequent complaints were cough (86.2%), running nose (86.9%), fever (81.6%), and sneeze (77.3%). On physical examination, the most frequent findings were fever (40.3%), rochini (36.9%), and tachypnea (23.6%).

Overall, RSV was detected by RT-PCR in 139 (24.9%) cases, RSV-A in 74 (13.2%) cases, and RSV-B in 67 (12.0%) cases (Fig. 1). Two (1.4%) were co-infected. Digital quantification by nCounter, the new diagnostic method, detected RSV in 122 (21.8%) samples, RSV-A in 63 (11.3%) and RSV-B in 63 (11.3%) (Fig. 1). Co-infections were detected in 4 (3.3%) cases. Interestingly, detection of RSV-A and RSV-B by any method occurred in 158 (28.3%) cases, indicating both methods might be complementary in detecting the complete RSV epidemic.

The validation of nCounter as a qualitative measure (presence vs. absence), considering RT-PCR as the reference standard is shown in Table 2A for RSV-A and in Table 2B for RSV-B. The sensitivity of nCounter was 74.3% (95%CI:63.3%–82.9%) for RSV-A and 77.6% (95%CI:66.3%–85.9%) for RSV-B; the specificity was 98.4% (95%CI:96.8%–99.2%) for RSV-A and 97.8% (95%CI:96.0%–98.8%) for RSV-B; the positive predictive value was 87.3% (95%CI:76.9%–93.4%) for RSV-A and 82.5% (95%CI:71.4%–90.0%) for RSV-B and the negative predictive value was 96.1% (95%CI:94.1%–97.5%) for RSV-A and 96.9% (95%CI:95.1%–98.2%) for RSV-B. Overall, accuracy was 95.2% (95%CI:93.1%–96.7%) for RSV-A and 95.3% (95%CI:93.3%–96.9%) for RSV-B. Moreover, quantification of both RSV-A and RSV-B viral RNA was significantly correlated between nCounter and RT-PCR, as shown in Fig. 2A and B. Again, using RT-PCR as a reference, a significant correlation (Spearman r = 0.44, p = 8 × 10⁻⁵) was found in RSV-A-positive samples (n = 74), between quantitative detection by nCounter (measured as normalized counts) and RT-PCR (measured as copies/ml) (Fig. 2A).

Similarly, in RSV-B RT-PCR-positive samples (n = 67), a significant correlation (Spearman r = 0.73, p = 3 × 10⁻¹²) was found between RSV-B quantitative detection by nCounter and RT-PCR (Fig. 2B). A similarly significant result was obtained when selecting only nCounter-positive samples or samples positive by any method (data not shown).

Since a small but considerable number of samples (27 for RSV-A, 26 for RSV-B) were detected by a single method only, a portion of the RSV epidemic might remain undetected depending on the method used. Therefore, we performed a comparative gene alignment for the sequences targeted by primers and probes in RT-PCR vs. nCounter using a total of 100 RSV-A and RSV-B strains representing the global epidemic. As shown in Fig. 3, a nearly perfect alignment with nCounter probes (> 98%) was obtained for RSV-B, whereas a slightly lower (> 95%) alignment was observed for RSV-A, which might partially explain the higher correlation coefficient for RSV-B (r = 0.73) vs. RSV-A (r = 0.44) when comparing both methods. In agreement with Houspie et al. 2013 [3] and Zlateva et al. 2007 [17], we found that the degenerated RT-PCR primers for both RSV-A and RSV-B performed equally well in gene alignment with globally circulating strains (Supplementary Fig. S1 and Fig. S2).

To further explore if discrepancies between nCounter and RT-PCR might be due to technical variation or genetic differences within RSV-A and RSV-B strains, we were able to perform a confirmatory experiment in a subset of 35 (mostly discordant) samples, for which sufficient RNA was available for replication. Of those, 10 samples were retested with the original RT-PCR protocol, showing concordant results in 8/10 samples (80%) compared with the first analysis by RT-PCR (one false positive for each RSV-A and RSV-B, data not shown). In contrast, replication of ten samples by nCounter resulted in 100% concordance for RSV-A and RSV-B. Another 15 samples were retested with an independent third assay, a real-time PCR targeting a different gene region (polymerase gene for both RSV-A and RSV-B [18]). Of those, 9/15 samples were concordant for RT-PCR (six false positive for RSV-A in the first analysis) and 10/15 for nCounter (three false positive for RSV-B and one false negative for each RSV-A and RSV-B, data not shown). However, all false positives were characterized by a low viral load, in either RT-PCR (659 ± 996 copies/ml) or nCounter (1.5 ± 2.0 counts), such that 13/15 samples (86.7%) were concordant in all three assays at viral loads > 1000 copies/ml (both RT-PCR protocols) or > 10 counts (nCounter). Only 2/15 samples (13.3%) with a moderate/high viral load were repeatedly discrepant (one in both RT-PCR assays: 0 vs. 3712 copies/ml, and one between both RT-PCRs and nCounter: 0 copies/ml vs. 35,546 counts), hinting at possible genetic differences between the samples. Considering the results of the third independent assay, sensitivity of the nCounter assay increased most for RSV-A, from 74.3% to 83.1% (95%CI:71.3%–89.5%) and to a lesser extent for RSV-B, from 77.6% to 78.8% (95%CI:67.5%–86.9%), accompanied by a rise in diagnostic accuracy to 96.4% and 95.5%, respectively. Taken together, this confirmatory experiment suggests technical variation might explain a minor portion of discrepant results for RT-PCR but not nCounter, whereas a larger part of discrepancies might be due to low viral load, as well as possible genetic variation between viral strains.

5. Discussion

Using real-time PCR as a reference, nCounter was shown to detect RSV-A and RSV-B simultaneously with high accuracy, both qualitatively and quantitatively. To the best of our knowledge, this is the first study to assess the diagnostic accuracy of nCounter for the detection of RSV-A and B in NPA collected from a large sample of children with ARI. Considering the high specificity of nCounter, its absence of cross-hybridization to other viruses [8], as well the use of internal positive and negative control RNAs for each individual sample, we believe the additional 19 samples (8 RSV-A and 11 RSV-B, detected only by nCounter, Tables 2A and 2B) represent genuine RSV infections, which would have gone undetected if only RT-PCR was used for diagnosis. Replication experiments indeed indicate a larger technical variation for RT-PCR, either using the same or an independent protocol, in contrast to nCounter. This could be explained by a greater possibility of
contamination between samples, as well as the lack of internal positive and negative controls to correct for RNA quality e.g. the presence of enzymatic inhibitors in the case of RT-PCR. Of note, real-time PCR primers and probes were designed [3] and updated (this study) to fully capture the Belgian RSV epidemic, whereas nCounter probes were designed for and tested in Brazilian RSV samples [8]. In addition, primers and probe are located in the F-gene for RSV-A and in the N-gene for RSV-B, whereas the nCounter probes are located in the hypervariable region of the G protein for both RSV-A and RSV-B. It is reassuring that a third independent assay targeting the polymerase gene resolved most discrepancies between RT-PCR and nCounter, increasing both sensitivity and accuracy for the latter. However, it will be necessary to regularly check whether probes still match with circulating strains, in order to update the custom nCounter probe set when necessary. Our alignment of nCounter probes shows 95% and 98% identity, respectively, for RSV-A and RSV-B (Fig. 3), with a striking similarity between Brazilian and Belgian samples, as well as other strains circulating in Latin America, Europe, North America (USA) and Asia (South Korea, Saudi Arabia). Regarding the global spread of RSV-A and RSV-B epidemics, we believe that our study demonstrates that digital

Fig. 1. Flow-chart of recruitment of children with Acute Respiratory Infection and Venn diagram of RSV-A and RSV-B investigation by RT-PCR and by nCounter.

Table 2A
Diagnostic accuracy of nCounter to detect RSV-A.

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<tr>
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<th>RT-PCR</th>
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<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>nCounter</td>
<td>Yes</td>
<td>55 (74.3%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>19 (25.7%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>74 (13.2%)</td>
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Table 2B
Diagnostic accuracy of nCounter to detect RSV-B.

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<tr>
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<th>RT-PCR</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>nCounter</td>
<td>Yes</td>
<td>52 (77.6%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>15 (22.4%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>67 (12.0%)</td>
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quantification of respiratory viruses by nCounter will be useful in large epidemiological studies worldwide. Considering the clinical and research application of nCounter vs. RT-PCR assays, both turnaround time and cost should be considered, as well as the total number of samples to be processed. For a batch of 12–24 samples, total turnaround time is 24–36 h (30 min hands-on time) vs. 4 h (1.5 h hands-on time), for nCounter (full respiratory panel) vs. in-house RT-PCR (for RSV-A and RSV-B only), respectively. Cost per sample corresponds to 55 euro for nCounter and 50–70 euro for in-house and confirmatory RT-PCR protocols, which compare favorably to most routinely used commercial assays (65–140 USD/sample, although list prices obviously vary worldwide). RT-PCR protocols are usually the method of choice when sample sizes are small and fast diagnosis is preferred, e.g. emergency rooms. On the other hand, we propose nCounter technology as a valid and cost-effective option with larger sample sizes, e.g. cohort studies, or when less hands-on time is preferred, e.g. core laboratories, due to its high-throughput capacity.

Some limitations of our study must be highlighted. First of all, this was a single-center study. Therefore, studies conducted at other sites are necessary. Secondly, the 95% CI precision for sensitivity and positive predictive value was wide, which implies the evaluation of these parameters requires a sample larger than the one presented here. Finally, the exclusion of children with a previous episode of wheezing may have selected a group of previously healthy children different from children with ARI typically seen in emergency rooms. This exclusion was due to the cross-sectional study design, within a prospective cohort study on the development of recurrent wheezing. Nevertheless, our study has several strengths. First, patients were recruited under daily surveillance in a specialized Pediatric Emergency Department, during the entire study period, when all patients who might fulfill the inclusion criteria were evaluated. Second, the 95% CI precision was narrow for specificity, negative predictive value, and accuracy. Third, the data collection period lasted 4 years and 1 month, which argues against a possible seasonality bias. In addition, nCounter technology has been shown to allow accurate mRNA expression quantitation using low amounts of total RNA and is able to detect as little as 0.5 fM of mRNA transcripts. This advantage could reduce the amplification bias offered by the RT-PCR method.

In conclusion, digital RNA quantification of RSV-A and RSV-B by nCounter is highly accurate (> 95%), using real-time PCR as a reference. Its robustness, high-throughput multiplex capacity and detection of cases undetected by real-time PCR indicate its suitability for large-scale epidemiological studies.

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Ethical approval

The study was approved by the Ethics Committee from the Federal University of Bahia (n° 067/2009). Parents or legal guardians signed written informed consent before enrollment of each study participant.

Competing interests

Authors declare they have no conflicts of interest.

Acknowledgements

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Fig. 3. Alignment of nCounter probes (100 bp) with global strains of RSV-A (A) and RSV-B (B).
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.jcv.2018.07.003.

References


