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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:94.1%–97.5% RSV-A), and 96.9% (95%CI:95.1%–98.2% RSV-B). Accuracy was 95.2% (95%CI:93.1%–96.7%) for RSV-A and 95.3% (95%CI:93.3%–96.9%) for RSV-B, while both methods significantly correlated for RSV-A ($r = 0.44$, $p = 8 \times 10^{-5}$) and RSV-B ($r = 0.73$, $p = 3 \times 10^{-12}$) 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.

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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 virus culture and direct immunofluorescence, but simultaneous (multiplex) 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]. Unique barcoding and gene-specific nucleic acid target capture allows up to 800 different targets to be simultaneously detected in a single experiment [9]. In addition, nCounter uses standardized internal control RNAs (8 negative and 6 positive) in each sample, allowing for efficient RNA quality control, as well as precise quantification of both host and viral transcripts up to (sub-)femtomolar level [7–10]. 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 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 trancriptase 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
Primers and probes used for real-time PCR quantification of RSV.

RSV-A	FW primer	AFFW	5’ CTGTGATAGARTTCCAACAAAAGAACA 3’
	REV primer	AFRV	5’ AGTTACACCTGCATTAACACTAAATTCC 3’
	MGB probe	AFTP	FAM 5’ CAGACTACTAGAGATTACC 3’ MGB
RSV-B	FW primer	BNFW	5’ GGCTCCAGAATATAGGCATGATTC 3’
	REV primer	BNRV	5’ TGGTTATTASAAGRGCAGCTATACAYAGT 3’
	MGB probe	BNTP	FAM 5’ TATCATCCCACAGTCTG 3’ MGB