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Pathogen transcriptional profile in nasopharyngeal aspirates of children with acute respiratory tract infection

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ABSTRACT

Background: Acute respiratory tract infections (ARI) present a significant morbidity and pose a global health burden. Patients are frequently treated with antibiotics although ARI are most commonly caused by virus, strengthening the need for improved diagnostic methods.

Objectives: Detect viral and bacterial RNA in nasopharyngeal aspirates (NPA) from children aged 6–23 months with ARI using nCounter.

Study design: A custom-designed nCounter probeset containing viral and bacterial targets was tested in NPA of ARI patients.

Results: Initially, spiked control viral RNAs were detectable in ≥ 6.25 ng input RNA, indicating absence of inhibitors in NPA. nCounter applied to a larger NPA sample ($n = 61$) enabled the multiplex detection of different pathogens: RNA viruses Parainfluenza virus (PIV 1–3) and RSV A-B in 21%, Human metapneumovirus (hMPV) in 5%, Bocavirus (BoV), CoV, Influenza virus (IV) A in 3% and Rhinovirus (RV) in 2% of samples, respectively. RSV A-B was confirmed by Real Time PCR (86.2–96.9% agreement). DNA virus (AV) was detected at RNA level, reflecting viral replication, in 10% of samples. Bacterial transcripts from *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* were detected in 77, 69, 26, 8, 3 and 2% of samples, respectively.

Conclusion: nCounter is robust and sensitive for the simultaneous detection of viral (both RNA and DNA) and bacterial transcripts in NPA with low RNA input (<10 ng). This medium-throughput technique will increase our understanding of ARI pathogenesis and may provide an evidence-based approach for the targeted and rational use of antibiotics in pediatric ARI.

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1. Background

Acute respiratory tract infection (ARI) accounts for both high mortality and morbidity in children <5 years old [1]. In developing countries, 75% of all acute morbidities were assigned as ARI but identification of the etiological agent in these cases is not common [2]. Upper respiratory tract infections are most commonly

caused by virus and only 10% of cases are attributed to bacteria [3]; nonetheless, most patients are treated with antibiotics [1].

Respiratory viruses can be diagnosed by culture, antibody-based and nucleic acid-based detection methods. Polymerase chain reaction (PCR) is the most widespread technique for pathogen identification [4]; however, simultaneous detection of different pathogens by multiplex PCR demands reaction standardization. To this end, nCounter (NanoString Technologies), a digital mRNA expression profiling method, can overcome these drawbacks: it identifies genetic material using two probes, one anchored to the genetic material and the other anchored to a singular bar code that identifies the target by specific hybridization. Upon bar code

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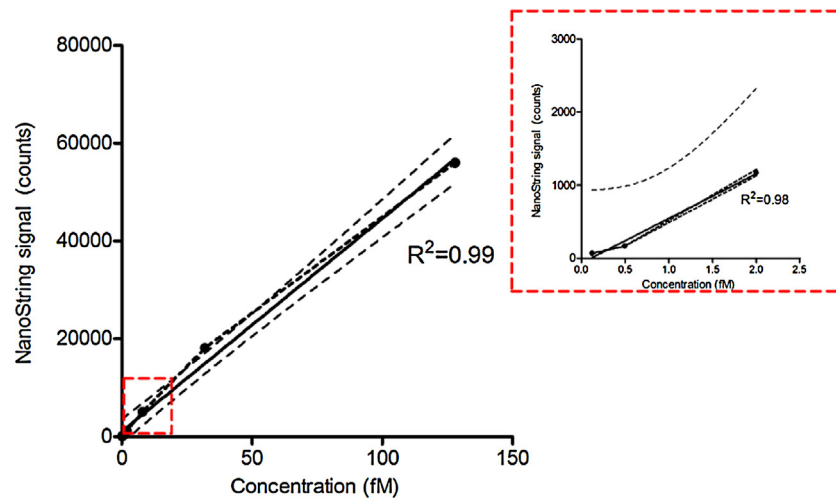


Fig. 1. Quantification of the positive control RNAs by nCounter.

Number of counts for exogenous positive control RNAs (background-subtracted) plotted against the different concentrations tested (0.125–128 fM). The correlation coefficients (R^2 values) of a linear fit to the mean ($n=61$) are indicated for the entire concentration range tested and, in the insert, for the concentrations <0.25 fM.

hybridization, presence and frequency of the target is revealed and multiple barcodes in the same reaction allow multiplex analysis [5].

2. Objectives

We designed a codeset for quantification of viral (RV, RSV A and B, IV A and B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV, CoV) and bacterial (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*) transcripts in one multiplex reaction employing nasopharyngeal aspirates (NPA) from children presenting ARI.

3. Study design

3.1. Clinical samples

Our study complied with the guidelines of the Declaration of Helsinki and was approved by the institutional review board of the Universidade Federal da Bahia (UFBA) (#067/2009). Written informed consent was obtained from all legal guardians. This was a prospective cohort study involving a sample of children aged 6–23 months, seen at UFBA's Emergency Unit between 2009 and 2011 [6]. Community-dwelling patients fulfilling inclusion criteria (fever, cough, sneeze or nasal obstruction for a period up to seven days and without previous episode of wheezing) were enrolled and NPA were collected. Control samples (nasopharyngeal wash) were obtained from individuals ($n=7$) undergoing routine outpatient visits. After collection, samples were placed in Nuclisens lysis buffer (Biomerieux) and frozen at -70°C .

3.2. RNA extraction and nCounter analysis

Viral and bacterial detection in NPA was performed using nCounter transcriptomic analysis (NanoString Technologies), allowing us to simultaneously assay for viral and bacterial targets. Total RNA (10–50 ng), obtained from a subset of randomly selected NPAs, was extracted using Qiagen's RNEasy, following manufacturer's instructions, and was subsequently hybridized against probes targeting RV, RSVA-B, IV A-B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV and CoV (229A and OC43), *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus*, *C. pneumoniae* and *M.*

pneumoniae (Supplementary Table 1), synthesized by NanoString Technologies. Hybridization was also performed with control viral and bacterial RNA pools [kindly donated by K. Bruyninckx, UZ Leuven], in three different concentrations (6.25, 12.5 and 25 ng for viral RNA and 1.5, 3 and 6 ng for bacterial RNA).

3.3. Real-Time PCR

RSV A and B were independently quantified by Real-Time PCR, as described [7].

3.4. Data analysis

Raw data were pre-processed using both nSolver 2.0 software (Nanostring Technologies) and the NanoStringNorm R package [8]. Preprocessing sequentially corrects for three factors: technical variation, background and sample content. First, using a set of exogenous positive control RNAs present in each sample (see also Fig. 1), technical variation is accounted for by adjusting the counts in each sample to the geometric mean of counts for positive control counts in all samples. Subsequently, a background correction is performed by subtracting the maximum count value of the negative control probes in a sample from each probe output within the sample. Transcripts counts that are negative after background correction are set to 1. Each sample is then normalized for RNA content by adjusting the counts to the geometric mean of 15 housekeeping genes (Supplementary Table 2). Finally, the data were log₂ transformed. Full details of each step can be found in the supplementary information of Waggott et al. [8]. Network data were designed by association matrix and the graph file was created using Gephi. Nodes were aligned by Force Atlas algorithm and the centrality was calculated by the betweenness. Statistical analyses were performed using Graphpad Prism version 5.0.

4. Results

4.1. Pathogen detection in NPAs by nCounter transcriptomics

The digital nCounter profiling technology captures and counts specific nucleic acid molecules in a complex mixture [5]. Briefly, unique pairs of capture and detection probes are designed against each gene of interest. The capture probe consists of a sequence complementary to a target pathogen mRNA and a short common

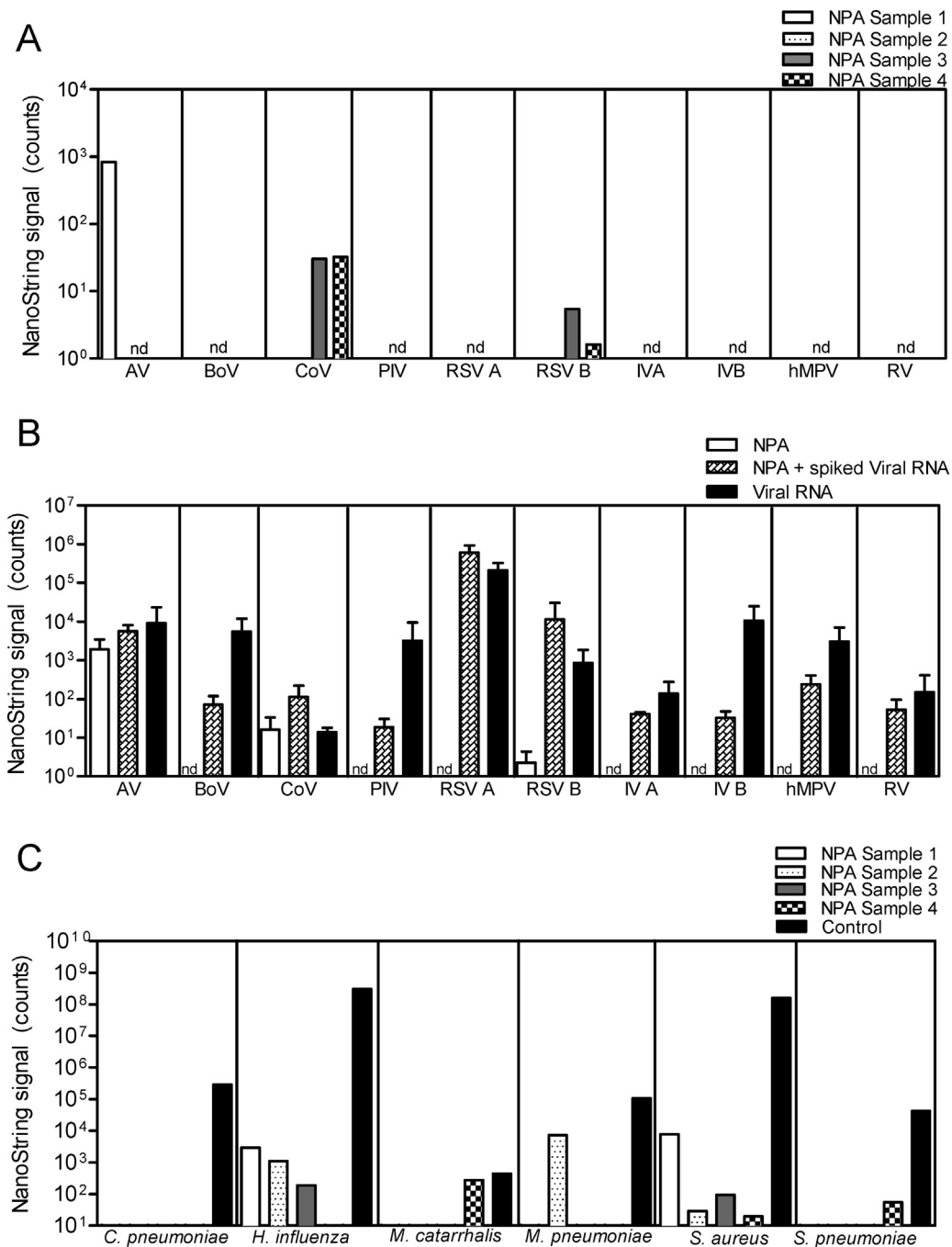


Fig. 2. nCounter profile of pathogen transcripts in nasopharyngeal aspirates (NPA) from patients with ARI.

NPA ($n=4$) was submitted to RNA extraction and, subsequently, to nCounter analysis for the detection of (A) Adenovirus (AV), Bocavirus (BoV), Coronavirus (CoV), Parainfluenza virus (PIV), Respiratory Syncytial virus A and B (RSV A and B), Influenza virus A and B (IV A and B), Human metapneumovirus (hMPV) and Rhinovirus (RV). Data are shown as the number of counts (transcripts), for each target. (B) Pooled NPA RNA ($n=4$) were hybridized alone (NPA) or in the presence of control viral RNA (NPA + spiked viral RNA). Black bars represent hybridization with control viral RNA alone. Data are shown as the mean number of counts (transcripts), for each target \pm SD. (C) Detection of *Chlamydomphila pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* in each individual NPA sample. Black bars reflect hybridization with control bacterial RNA (pooled). Data are shown as the number of counts (transcripts), for each target.

sequence coupled to an affinity tag (biotin). The reporter probe also contains the complementary sequence but is coupled to a color-coded tag, providing the detection signal. The color code in each tag is unique, allowing the accurate discrimination of the different targets, in a complex mixture. Capture and reporter probes are mixed with total RNA in a single hybridization reaction, resulting in the formation of tripartite structures (target bound to the specific reporter and capture probes). Unhybridized probes are washed away and remaining complexes are exposed to streptavidin. Tar-

gets bound to the specific probes are immobilized on a solid surface that is scanned; the unique reporter probe then identifies each target and the level of expression is measured by the digital readout of transcript counts. The reaction occurs in the absence of nucleic acid amplification and/or enzymatic reactions. To estimate target concentration, nCounter uses a series of positive controls covering a range of known concentrations (0.1–100 fM, comparable to 0.2–200 copies per cell). Based on the counts obtained for these known concentrations, the concentration of mRNA transcripts can

be estimated. This estimate is done by plotting the counts obtained for the positive controls versus the concentrations tested and by fitting a regression line through the average (Fig. 1). The regression line allows the quantification of mRNA transcript concentration. The correlation between the counts for positive controls and the concentration was 0.99 and 0.98 for the lower concentration range.

Next, we assessed the potential of nCounter as a diagnostic technique for ARI. In pilot experiments, we used RNA obtained from NPA of four patients and the set of probes custom-designed for detection of viral [RV, RSV A-B, IV A-B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV and CoV (229A and OC43)] and bacterial (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus*, *C. pneumoniae*, and *M. pneumoniae*) targets (Supplementary Table 1). We detected the presence of AV, CoV and RSVB (Fig. 2A), whereas BoV, PIV, RSV A, IV A-B, hMPV and RV were detected in none of the four samples initially tested. Detection was also positive in NPA spiked with known viral RNA (6.25–12.5–25 ng), indicating absence of inhibitors in RNA isolated from NPA (Fig. 2B). In addition, use of tRNA (an RNA carrier) to ARI samples did not improve viral detection (Supplementary Fig. 1), confirming that even NPA containing low input RNA (<10 ng) is suitable. Bacterial RNA (*H. influenzae*, *M. catarrhalis*, *M. pneumoniae*, *S. aureus*, and *S. pneumoniae*) was also detected in both patients and control NPA (Fig. 2C).

For comparison purposes, we performed RSV A and B detection by Real-Time PCR. Agreement between nCounter and Real-Time PCR was 86.2% for RSV A (kappa=0.231) and 96.9% for RSV B (kappa=0.82) (Table 1). RNA counts detected by nCounter were significantly correlated to the number of copies detected by Real-Time PCR for both RSV A ($r=0.25$, Spearman; $p=0.049$) and RSV B ($r=0.83$; Spearman $p=0.001$).

Table 1

RSVA and RSVB agreement between Real Time PCR and nCounter results in NPA samples from ARI patients.

nCounter	Real-Time PCR					
	RSVA			RSVB		
	Positive	Negative	Total	Positive	Negative	Total
Positive, n	2	4	6	5	0	5
Negative, n	5	54	59	2	58	60
Total	7	58	65	7	58	65

4.2. Pathogen network in NPA samples of children with ARI

We then expanded nCounter analysis to a set of 61 randomly selected NPA and included seven healthy control samples (Fig. 3). Using this extended group, we detected RV (2% of samples), BoV (3%), CoV (229E and OC43, 3%), IV A (3%), hMPV (5%), PIV (genotypes 1 and 3, 21%) and RSV A and B (21%). In addition, detection of DNA virus AV (types 2 and 5) was confirmed at the RNA level in 10% of samples, indicating ongoing (intracellular) viral replication. Transcripts for *C. pneumoniae*, *M. catarrhalis*, *M. pneumoniae* and *S. pneumoniae* were detected in 2, 3, 8 and 26% of samples, respectively. *H. influenzae* and *S. aureus* transcripts were ubiquitous (69 and 77% of samples, respectively) and were also detected in healthy controls (28 and 100% of samples, respectively). Using this data set, we observed bacterial presence in the majority of children (55.1%), whereas in 43.5% of children, both viruses and bacteria were present (Fig. 4A). A low percentage of children (1.5%) presented viruses only. Upon observation of the number of transcripts per type of pathogen (Fig. 4B), AV 2 and RSB (A and B) showed

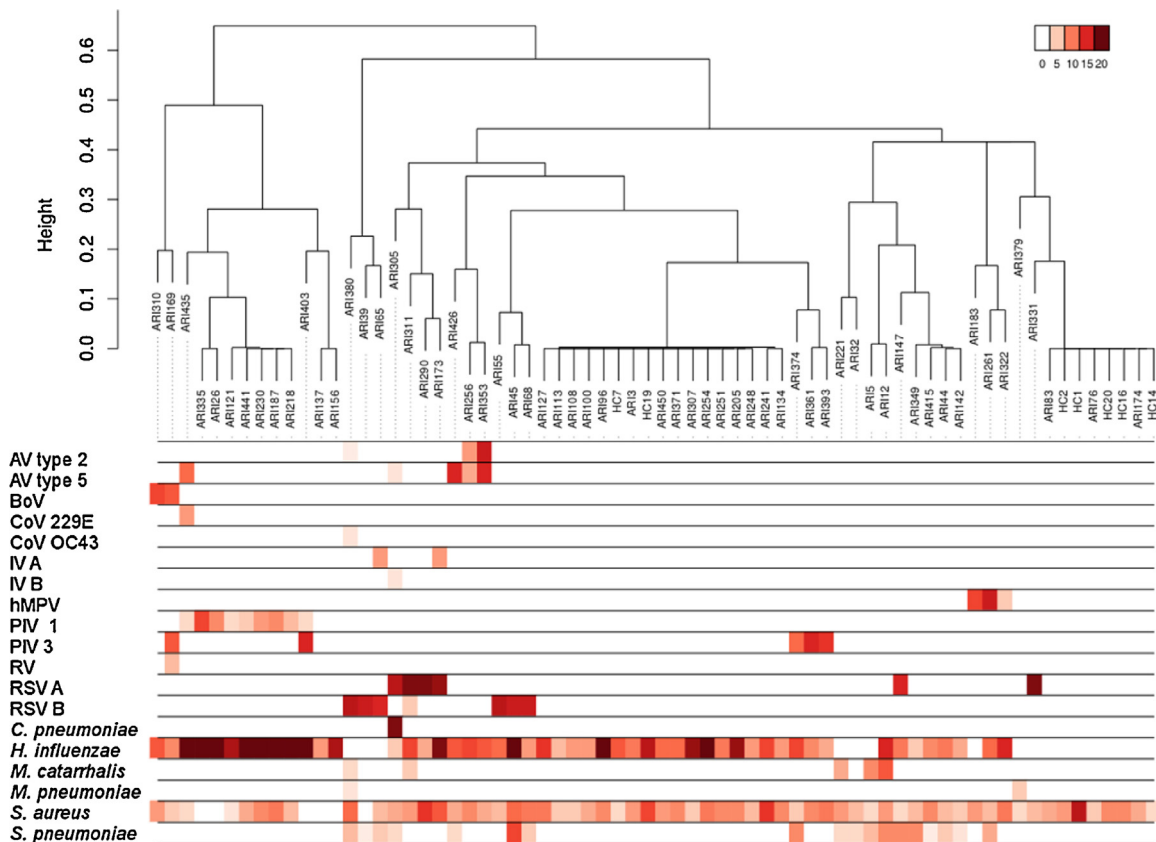


Fig. 3. Heat map dendrogram of pathogen detection by nCounter in children with ARI.

RNA from ARI patients ($n=61$) and from healthy controls ($n=7$) were hybridized against probes for adenovirus (AV), *Bocavirus* (BoV), *Coronavirus* (CoV), Influenza virus (IV), *Human metapneumovirus* (hMPV), parainfluenza virus (PIV), Rhinovirus (RV), Respiratory Syncytial Virus (RSV), *C. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *M. pneumoniae*, *S. aureus* and *S. pneumoniae*. Samples were clustered by complete linkage of spearman correlation and color intensity indicates probe counts in each sample. The processed data were log₂-transformed.

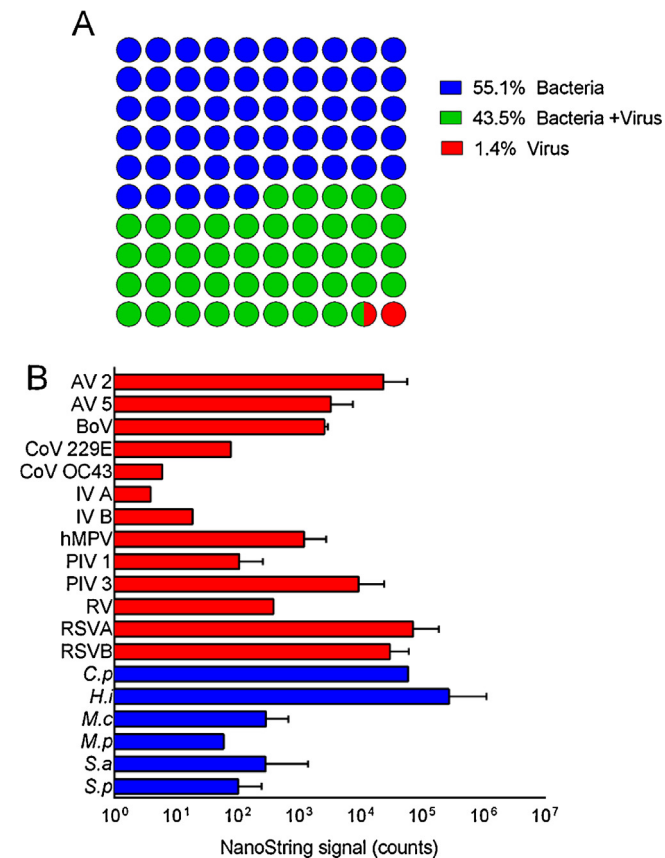


Fig. 4. Microbe counts (transcripts) in NPA of children with ARI, determined by nCounter.

(A) Chart showing the percentages of samples ($n=61$) presenting counts (transcripts) for bacteria (blue), viruses (red) or both viruses and bacteria (green). (B) Bar plot representing the mean number of counts (transcripts) detected for each pathogen in NPA samples ($n=61$). Adenovirus (AV), Bocavirus (BoV), Coronavirus (CoV), Influenza virus (IV), Human metapneumovirus (hMPV), parainfluenza virus (PIV), Rhinovirus (RV), Respiratory Syncytial Virus (RSV), C.p, *C. pneumoniae*; H.i, *H. influenzae*; M.c, *M. catarrhalis*; M.p, *M. pneumoniae*; S.a, *S. aureus* and S.p, *S. pneumoniae*.

the highest mean transcript numbers ($>10,000$), comparing the 61 samples. The mean number of transcripts detected for AV 5, BoV and PIV 3 was $<10,000$. CoV (229E and OC43), IV (A and B), hMPV, PIV 1 and RV presented the lowest mean number of transcripts (≤ 1000). For bacteria, the mean transcript number of *C. pneumoniae*, across the 61 NPA samples, was comparable to that detected for AV and RSV ($\sim 10,000$). For the other bacterial targets (*H. influenzae*, *M. catarrhalis*, *M. pneumoniae*, *S. aureus* and *S. pneumoniae*), the mean number of transcripts was ≤ 1000 .

Network analysis also revealed the complex interactions that occur between individual viruses and bacteria detected in NPA (Fig. 5). For example, the strongest links were observed for bacteria: *H. influenzae*/*S. aureus* and, secondly, *H. influenzae*/*S. aureus* or *H. influenzae*/*S. pneumoniae*. Comparing virus and bacteria, PIV1/*H. influenzae* showed a strong interaction as did RSV (A and B)/*S. aureus*. Of note, the interactions of CoV 229E were different from those observed for CoV OC43.

5. Discussion

Our results demonstrate that nCounter is a robust medium-throughput technique for detection of both viral and bacterial RNA transcripts in ARI patients. The sensitivity and reproducibility of nCounter has already been compared with Openarray[®] [9], Affymetrix microarray [10] and TaqMan PCR [5]. In the latter,

nCounter was equally reproducible and sensitive regarding detection of differentially modulated genes; however, nCounter detected low abundant transcripts, not detected by microarray. nCounter was also successfully used for detection of pathogen RNA transcripts in clinical samples [11], and an important advantage is transcript detection without the need for purification or amplification, yielding a rapid indicator of pathogen replication.

Transcriptomic profiling by nCounter allowed simultaneous detection of both viral and bacterial transcripts in ARI patients. We detected the presence of AV, BoV, CoV, IV, hMPV, PIV, RV and RSVA-B, which are associated with respiratory tract infections [1]. To our knowledge, this is the first description of detection of a pathogenic DNA virus (Adenovirus type 2 and 5) at the RNA level in clinical samples. In contrast to a standard DNA PCR, which quantifies DNA in (mostly extracellular) viral particles, nCounter enables demonstration of ongoing (intracellular) viral replication, which we hypothesize to be more relevant to ARI pathogenesis. We also, simultaneously detected transcripts for *C. pneumoniae*, *M. catarrhalis* and *S. pneumoniae*, which are commonly isolated from the respiratory tract [12], as is *H. influenzae*. We observed a good agreement between Real-Time PCR and nCounter for RSV (A and B) detection, reinforcing the possibility of using the latter as an alternative method for pathogen detection. Of note, RSVA strains present a high variability in the target used for detection (G protein domain) [13], also in Brazil [6], which could explain the weaker correlation observed for nCounter and Real-Time PCR, as compared to RSVB.

The healthy upper respiratory tract is colonized by commensals and potential pathogens kept in check by the immune system. There is evidence that viral respiratory infections can stimulate an increase in bacterial load and this association can lead to secondary complications [14]. Preterm infants with RSV and bacterial co-infection were hospitalized for longer periods and more frequently admitted to the intensive care unit, compared to children infected with RSV alone [15]. In our samples, the majority of ARI patients presented *H. influenzae* and *S. aureus*, which, together with *S. pneumoniae* and *M. catarrhalis* are the usual opportunistic pathogens co-detected with RSV [16–18]. Indeed, transcripts for all these agents were detected in our NPA samples from children with ARI. Network analysis showed strong links between PIV 1 and *H. influenzae* and between RSV B and *S. pneumoniae*, for example. Both *S. pneumoniae* [19] and RSV activate the inflammasome [20]; *S. pneumoniae* triggers NF- κ B activation leading to an inflammatory response whereas, RSV infection results in a strong interferon response. The possible overlap between the signaling pathways triggered by each pathogen may synergize and amplify the inflammatory signal during co-infection. This exacerbated immune response could accentuate damage in situ and increase morbidity. Given this scenario, knowing the effective pathogen load, as shown here by nCounter, can contribute towards understanding the contribution of each pathogen to disease severity. As a proof-of-principle, our group has already used nCounter to simultaneously quantify both human and retroviral transcripts; thus, revealing intact IFN signaling in HTLV-1 infection [21].

We conclude that nCounter can be used as an alternative medium-throughput method to identify, in a single reaction, the presence of multiple pathogens in NPA from ARI patients. Given that respiratory infections are one of the most common reasons for visits to physicians, improved diagnostic methods such as nCounter can contribute to better patient management. Moreover, a transcriptomic approach involving pathogen detection, and as discussed above, immune response transcripts will enable the identification of biomarkers in ARI clinical samples, even those presenting low RNA yield (10–50 ng) such as NPA. This approach, combined with long-term clinical follow-up, will increase our understanding of ARI pathogenesis, disease progression (wheez-

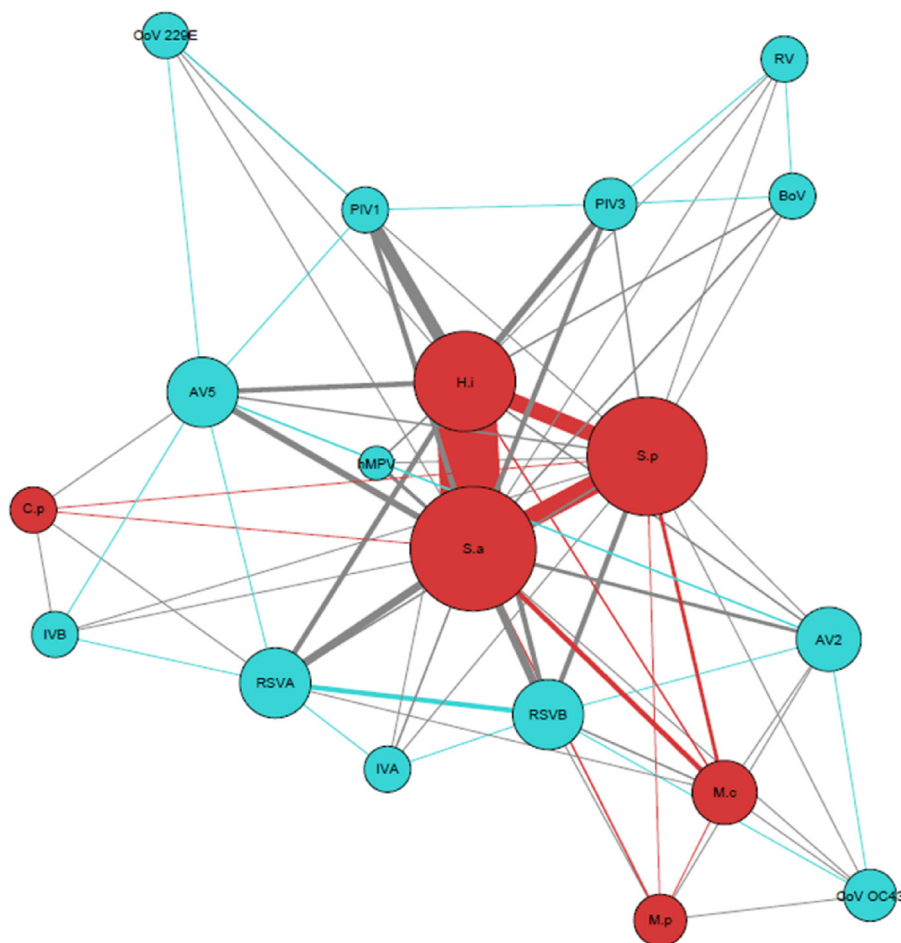


Fig. 5. Network distribution of viral and bacterial agents in NPA samples of children with ARI.

Each system component [Virus; RV, RSV-A-B, IV A-B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV and CoV (229A and OC43) and bacteria (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus*, *C. pneumoniae* and *M. pneumoniae*)] is indicated by a blue or red node, respectively and is a partner of the network. Each line is a link between two nodes and its color represents interactions with similar category circles (blue or red) or with a different category (grey). The width of each line is a measure of the strength of the link between two nodes. The circles are represented according to the ForceAtlas2 scheme.

ing, asthma) and, more importantly, provide an evidence-based approach for the targeted and rational use of antibiotics in pediatric ARI.

Competing interests

Authors declare they have no conflicts of interest

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Ethical approval

Our study complied with the guidelines of the Declaration of Helsinki and was approved by the institutional review board of the Universidade Federal da Bahia (UFBA) (#067/2009). Written informed consent was obtained from all legal guardians.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcv.2015.06.005>

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