Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 271 (2019) 113676

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Duplex real-time RT-PCR assay for detection and subgroup-specific identification of human respiratory syncytial virus

Lijuan Wang[a], Pedro A. Piedra[b,c], Vasanthi Avadhanula[b,c], Edison L. Durigon[d], Ann Machablishvili[e], María-Renée López[f], Natalie J. Thornburg[g], Teresa C.T. Peret[g,*]

[a] IHRC, Contracting Agency to the Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, GA, United States
[b] Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States
[c] Department of Pediatrics, Baylor College of Medicine, Houston, TX, United States
[d] Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[e] National Center for Disease Control and Public Health, Tbilisi, Georgia
[f] Center for Health Studies, Research Institute, Universidad del Valle de Guatemala, Guatemala City, Guatemala
[g] Respiratory Viruses Branch, Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, GA, United States

ARTICLE INFO

Keywords:
Human respiratory syncytial virus
HRSV
HRSV subgroups
Real-time RT-PCR
Respiratory viruses

ABSTRACT

Human respiratory syncytial virus (HRSV) is a leading cause of acute respiratory illness in young children worldwide. Reliable detection and identification of HRSV subgroup A and B infections are essential for accurate disease burden estimates in anticipation of licensure of novel HRSV vaccines and immunotherapies. To ensure continued reliability, molecular assays must remain current with evolving virus strains. We have developed a HRSV subgroup-specific real-time RT-PCR (rRT-PCR) assay for detection and subgroup identification using primers and subgroup-specific probes targeting a conserved region of the nucleoprotein gene combined in a single duplex reaction using all genome sequence data currently available in GenBank. The assay was validated for analytical sensitivity, specificity, reproducibility, and clinical performance with a geographically diverse collection of viral isolates and respiratory specimens in direct comparison with an established pan-HRSV rRT-PCR reference test. The assay was sensitive, reproducibly detecting as few as 5–10 copies/reaction of target RNA. The assay was specific, showing no amplification with a panel of 16 other common respiratory pathogens or predicted by *in silico* primer/probe analysis. The duplex rRT-PCR assay based on the most current available genome sequence data permits rapid, sensitive and specific detection and subgroup identification of HRSV.

1. Introduction

HRSV is a major cause of severe acute respiratory illness in infants and young children worldwide (Nair et al., 2010). Two HRSV subgroups, A and B, and multiple genotypes within each subgroup have been described (Mufson et al., 1985; Peret et al., 2000). In natural human infections, protection between homologous subgroup viruses is more pronounced than between heterologous subgroups (Mufson et al., 1987), a finding confirmed in animal challenge studies (Johnson et al., 1987). Some studies have suggested that infection with HRSV subgroup A viruses yield more severe infections than subgroup B (Hall et al., 1990; Laham et al., 2017; Walsh et al., 1997), although other studies have reported no significant differences (Devincenzo, 2004; Fodha et al., 2007). Molecular assays that discriminate between HRSV subgroups would facilitate studying the prevalence of subgroups, potential differences in clinical presentations, and immune responses to subgroup which may eventually help better inform the development of HRSV vaccines and immunotherapies (Vandini et al., 2017).

Reliable methods for detection of HRSV may be useful for clinical management and accurate disease burden estimates. Whereas antigen-based HRSV assays have proven useful in the clinical setting for testing infants and young children, molecular diagnostic methods are more sensitive generally and particularly with older children and adults that more often present with low viral loads and later into their illnesses (Mahony, 2008). Given their inherent design advantages and wide general use, real-time RT-PCR (rRT-PCR) assays for HRSV that both detect and discriminate between the two HRSV subgroups are available (Kuypers et al., 2004; Liu et al., 2016; Perkins et al., 2005; van Elden et al., 2003). However, a weakness inherent to all molecular assays is their susceptibility to pathogen strain variation that evolve overtime

* Corresponding author.
E-mail address: TPeret@cdc.gov (T.C.T. Peret).

https://doi.org/10.1016/j.jviromet.2019.113676
Received 1 March 2019; Received in revised form 6 June 2019; Accepted 7 June 2019
Available online 07 June 2019

Table 1
Primer/probe sequences for HRSV real-time RT-PCR assays.

HRSV rRT-PCR Assay	Gene target	Genome location [a]	Name	Primer or Probe	Sequence (5'-3')
Duplex	Nucleocapsid	1141-1162	HRSV-F	Forward primer	ATGGCTCTTAGCAAAGTCAAGT
		1239-1262	HRSV-R	Reverse primer	TGCACATCATAATTRGGAGTRTCA
		1171-1204	HRSV A-P[b]	Probe	ACACTCAACAAAGA"T"CAACTTCTRTCATCCAGCA
		1171-1204	HRSV B-P[c]	Probe	ACATTAAATAAGGA"T"CAGCTGCTGTCATCCAGCA
Pan (Fry et al., 2010)	Matrix	3255-3278	HRSV-pan-F	Forward primer	GGCAAATATGGAAACATACGTGAA
		3311-3338	HRSV-pan-R	Reverse primer	TCTTTTTCTAGGACATTGTAYTGAACAG
		3281-3307	HRSV-pan-P[d]	Probe	CTGTGTATGTGGAGCCTTCGTGAAGCT

[a] Primer nucleotide numbering was based on human RSV A2 strain. Probe nucleotide numbering was based on human RSV A2 and B1 strains (GenBank accession numbers KT992094 and AF013254, respectively).
[b] Probe labeled with 5′ reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with Black Hole Quencher (BHQ) 1. A terminal 3′ phosphate is added to prevent probe extension by *Taq* polymerase.
[c] Probe labeled with 5′ CAL Fluor Red 610 and quenched internally at a modified "T" residue with BHQ2. A terminal 3′ phosphate is added as above.
[d] Probe labeled with 5′-FAM and 3′-BHQ1.

leading to primer/probe mismatches and potential false negative results (Suss et al., 2009; Whiley and Sloots, 2005, 2006). To remain viable, molecular assays must "evolve" with the pathogen. Instructive of this, Kamau et al. (2017) recently described their failure to detect HRSV among some immunofluorescence assay (IFA) positive specimens using a widely used multiplex respiratory pathogen rRT-PCR assay (Gunson et al., 2005) that acquired HRSV primer/probe sequences from an assay reported in 2003 by van Elden et al. (2003). The investigation revealed that the IFA-positive-rRT-PCR-negative samples collected during 2014/15 and 2015/16 HRSV epidemic seasons in Kilifi, Kenya, contained a new HRSV B strain with polymorphisms in the rRT-PCR probe region that hindered annealing. The authors noted that failure to detect these strains could have adversely affected studies that used this assay (Anderson et al., 2013; Choudhary et al., 2013; Gimferrer et al., 2015). To address this, we have developed and validated a novel duplex rRT-PCR assay using the most current HRSV genome sequence data available in GenBank. This assay will allow sensitive and specific detection and subgroup identification of HRSV, including possible dual infections.

2. Methods

2.1. Clinical specimens and virus isolates

Three-hundred and thirty-four HRSV positive respiratory samples were available for assay development. Specimens were originally submitted to CDC to support public health surveillance or outbreak response and had been previously tested for multiple respiratory pathogens. Specimens continuously stored at −70 °C included i) 41 mixed respiratory specimens from children and adults with acute respiratory infections in the U.S. from 2017 to 2018; ii) 190 nasopharingeal aspirates from young children (< 24 months) hospitalized with severe acute respiratory infections in Brazil from 2008 to 2010; iii) 58 combined nasopharyngeal and oropharyngeal swab specimens in viral transport media obtained from mostly children presenting at health clinics with acute respiratory illness in Kenya (2006 to 2008) (Kim et al., 2012); and iv) 21 and 24 respiratory swab specimens collected in Guatemala (2015–2016) and Tiblisi, Georgia (2015–2017), respectively. All specimens were previously tested for human RNase P to monitor for extract integrity and absence of rRT-PCR inhibitors. Nineteen genetically diverse HRSV isolates were also available for testing.

2.2. Primer/probe design and synthesis

A total of 959 HRSV whole genome sequences available on GenBank through February 2016 (730 HRSV A and 229 HRSV B) were downloaded and aligned using MAFFT implemented in Geneious 10.0.9 (https://www.geneious.com/). Primers/probe sets were selected using Primer Express v2.0 software (Thermo Fisher Scientific, Waltham, MA USA) and by visual inspection following real-time hydrolysis probe assay design guidelines (Rodriguez et al., 2015). Potential interference from sequence secondary structure was assessed in the target region using the Mfold web server (Zuker, 2003). Primer/probes targeting conserved regions in the HRSV nucleoprotein (N) gene were designed to discriminate between HRSV subgroups A and B and be compatible in a duplex reaction. Primer/probes were synthesized by the CDC Biotechnology Core Facility and probes were HPLC-purified. To minimize overlap in dye emission spectra, the HRSV A probe was labeled at the 5′-end with 6-carboxyfluorescein (FAM) and the HRSV B probe was labeled at the 5′-end with CAL Fluor Red 610. Probes were internally quenched with Black Hole Quencher 1 or 2 and 3′-phosphate end-labeled to prevent probe extension by *Taq* polymerase. Primer and probe sequences are listed in Table 1.

2.3. RNA extraction and real-time RT-PCR assays

Total nucleic acid was extracted from 200 µl of each clinical specimen using NucliSens® easyMAG® or 100 µl of virus isolate using miniMAG® extraction systems following manufacturer's instructions (bioMerieux, Inc., Durham, NC). Extracts were stored at −70 °C until use. The duplex rRT-PCR assay for subgrouping HRSV was developed following amplification conditions previously described for the CDC reference rRT-PCR pan-HRSV assay that does not distinguish between HRSV subgroups (Fry et al., 2010). Briefly, the assay was performed in 25 µl reactions containing 0.2 µM forward and reverse primers, 0.05 µM HRSV A probe, 0.05 µM HRSV B probe, and 5 µl of extracted RNA on an Applied Biosystems 7500 Fast Dx real-time PCR instrument (Thermo Fisher Scientific) using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems/Life Technologies). Thermocycling conditions consisted of 10 min at 45 °C for reverse transcription, 10 min at 95 °C for activation of the *Taq* polymerase, and 45 cycles of 15 s at 95 °C and 1 min at 55 °C. Each run included one viral template control (see below) and one non-template control. A specimen was considered positive for HRSV A or B if a well-defined fluorescence curve crossed the auto threshold setting within 45 cycles. Specificity of the duplex HRSV rRT-PCR was evaluated by testing other respiratory pathogens (RNA and DNA genomes) also using the AgPath-ID™ One-Step RT-PCR kit which has been validated for simultaneous testing of RNA and DNA pathogens (Weinberg et al., 2013).

2.4. Viral template control

A HRSV positive RNA control template was synthesized and cloned into pUC57 by GenScript® USA Inc. (http://www.genscript.com/). The control template contained primer/probe sequences for duplex HRSV A and B, pan-HRSV and RNP rRT-PCR assays arranged 5′→ 3′ in one continuous sequence capped with 5′-T7 and 3′-SP6 promoter sequences.

Table 2
Duplex HRSV rRT-PCR assay limits of detection with RNA transcripts.

Predicted no. of transcript copies/reaction	No. of positive tests/no. of transcript replicates (%)		
	HRSV A	HRSV B	pan-HRSV
50	16/16 (100)	16/16 (100)	16/16 (100)
10	16/16 (100)	16/16 (100)	16/16 (100)
5	14/16 (87.5)	16/16 (100)	14/16 (87.5)
2.5	11/16 (68.8)	8/16 (50)	9/16 (56.3)
1.25	6/16 (37.5)	9/16 (56.3)	9/16 (56.3)

The insert sequence was confirmed and run-off RNA transcripts were prepared using the MEGAscript T7 kit and purified using the MEGAclear kit (Thermo Fisher Scientific). Transcript quantitation was performed on a Qubit 4 Fluorometer (Thermo Fisher Scientific).

3. Results

3.1. Assay selection

After sequence selection and oligonucleotide synthesis, multiple primer/probe sets were compared for optimal performance against four representative HRSV A and B isolates. Primer/probe concentrations were determined by cross-titrations adjusted to achieve comparable Ct values with the reference pan-HRSV assay. A single primer pair targeting the nucleoprotein gene with two subgroup discriminating probes performed in a single duplex reaction were selected for further study.

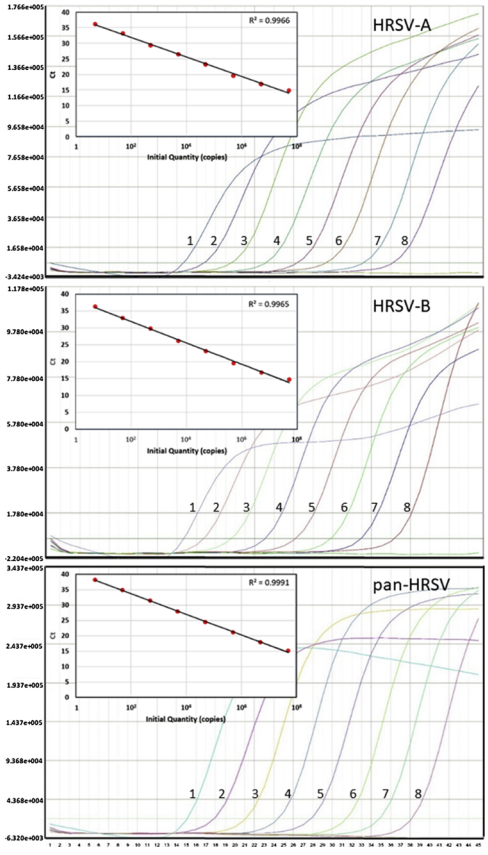

Fig. 1. Amplification plots and standard curves of serial 10-fold dilutions ranging from 5×10^7 (curve 1) to 5×10^0 (curve 8) copies/reaction for RNA transcripts analyzed by duplex HRSV and pan-HRSV rRT-PCR assays. Plot inserts show calculated linear correlation coefficients (R2) for each assay.

The duplex assay was then evaluated against 15 diverse HRSV isolates by staff who were blinded to subgroup. All 15 isolates were correctly identified by the duplex assay as well as detecting both HRSV A and B in mixed reactions (Supplementary Table 1).

3.2. Assay analytical sensitivity and specificity

To estimate the analytical sensitivity of the rRT-PCR assays, serial 2-fold dilutions of the RNA transcript were prepared in 10 mM Tris-EDTA buffer containing 50 ng/µl yeast tRNA and tested in 16 replicates. The limit of detection for HRSV A and B that yielded 100% replicate positives ranged from 5 to 10 RNA transcript copies per reaction, identical with the pan-HRSV assay (Table 2). Linear amplification was achieved over a 8-log dynamic range, from 5×10^0 to 5×10^7 copies per reaction (Fig. 1). The specificity of the duplex HRSV rRT-PCR assay was evaluated with high concentration total nucleic acids extracted from a diverse collection of other respiratory pathogens, including adenovirus, human metapneumovirus, rhinovirus, parainfluenza viruses, influenza viruses, coronaviruses, human bocavirus, *Mycoplasma pneumoniae* and *Streptococcus pneumoniae* (Supplementary Table 2). No false positive results were obtained with these samples. In addition, the HRSV primer/probe sequences were evaluated by *in silico* BLASTn analysis queries. No combined homologies with human genome, other respiratory tract pathogens or commensals were observed that would predict potential false positive results.

3.3. Assay reproducibility

Assay reproducibility was assessed using three RNA transcript concentrations (5×10^5, 5×10^3 and 5×10^1 copies/reaction) representing the range of HRSV Ct values found with most positive clinical specimens (see below). Intra-assay variation was estimated from four replicates each of the three transcript concentrations tested in a single run. Inter-assay variation was estimated from four identical replicates tested twice on two separate days. The intra-assay coefficient of variation (CV) ranged from 0.35% to 1.31%; inter-assay CV ranged from 0.45% to 1.51% (Table 3).

3.4. Assay clinical evaluation

Assay clinical performance was assessed with 334 respiratory specimens that previously tested positive for HRSV. Specimens were

Table 3
Duplex HRSV and pan-HRSV rRT-PCR assays reproducibility with RNA transcripts.

	Assay	Copies/ reaction	Number of replicates	Mean Ct	SD	CV (%)
Intra-assay	HRSV A	5×10^5	4	19.73	0.15	0.78
		5×10^3	4	26.47	0.26	0.97
		5×10^1	4	32.97	0.32	0.98
	HRSV B	5×10^5	4	19.66	0.12	0.59
		5×10^3	4	26.41	0.20	0.78
		5×10^1	4	33.07	0.43	1.31
	pan-HRSV	5×10^5	4	21.09	0.07	0.35
		5×10^3	4	27.84	0.10	0.35
		5×10^1	4	34.76	0.14	0.41
Inter-assay	HRSV A	5×10^5	8	19.52	0.27	1.39
		5×10^3	8	26.19	0.40	1.51
		5×10^1	8	33.18	0.31	0.94
	HRSV B	5×10^5	8	19.90	0.26	1.31
		5×10^3	8	26.41	0.15	0.58
		5×10^1	8	33.01	0.32	0.96
	pan-HRSV	5×10^5	8	21.08	0.09	0.45
		5×10^3	8	27.56	0.32	1.17
		5×10^1	8	34.31	0.48	1.41

SD, standard deviation; CV, coefficient of variation.

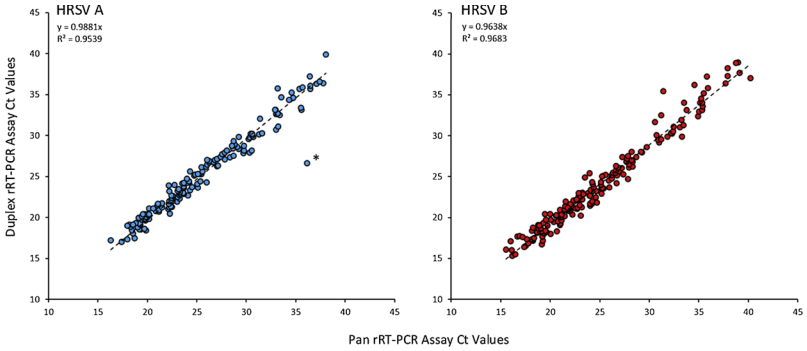

Fig. 2. Comparison of duplex HRSV and pan-HRSV rRT-PCR assays with 156 HRSV A and 178 HRSV B single positive clinical specimens. Linear regression lines fitted to cycle threshold (Ct) data with regression equations and coefficients of determination (R^2) insets. Outlier sample (*) selected for retesting.

simultaneously retested by the duplex and pan-HRSV rRT-PCR assays. HRSV was detected in all specimens and correctly identified as subgroup A (152) or B (177) as confirmed by RT-PCR and sequencing (Supplementary Table 3). Five patients were identified with dual HRSV A and B detections by the duplex assay that were confirmed by sequencing (Supplementary Table 4). Ct values obtained by duplex and pan assays were correlated with each other (HRSV A, $R^2 = 0.954$; B, $R^2 = 0.968$). However, one sample from Brazil showed a ~9.5 Ct difference between the pan (Ct = 26.66) and duplex subgroup A assays (Ct = 36.15) (Fig. 2) that was confirmed on retesting. Sequencing revealed a single base substitution at the 3′-terminis of the duplex assay reverse primer (T → A) that would predict diminished amplification efficiency. A review of more recent HRSV genomes deposited in GenBank following study completion found 31 sequences that contained two single base substitutions in the primer/probe region of the duplex assay that would also predict compromised performance; one at the 3′-terminis of the forward primer and the other near the middle of RSV B probe (*e.g.*, MF973158). A review of sequences obtained from clinical specimens in our study found two (8221 and 8266) with identical substitutions (unpublished result). Interestingly, Ct values obtained by the duplex and pan assays for these specimens were nearly identical, suggesting that the two substitutions had no deleterious effect on the duplex assay.

4. Discussion

In this study, we developed and validated a duplex rRT-PCR assay for detection and subgroup-specific identification of HRSV using the most currently available sequences in the GenBank. Our duplex assay proved sensitive and specific and correctly identified HRSV subgroups from a diverse collection of HRSV positive clinical specimens with comparable performance to our previously reported pan HRSV reference assay (Fry et al., 2010). To achieve long-term efficacy of our assay in the face of continued HRSV genome evolution, we targeted conserved regions of the nucleoprotein gene. The nucleoprotein gene offers the added advantage of location nearer the 3′-promoter where an enhanced transcription gradient yields increased potential targets and theoretically improved test sensitivity (Cowton et al., 2006). Primer/probe design also included degeneracy at mixed base alleles and long, internally quenched probes that exhibit lower background fluorescence and are more tolerant to sequence polymorphisms.

Our duplex rRT-PCR assay offers several important advantages over previous assay designs. Earlier assays were handicapped by the limited sequence data available for primer/probe design and sequences that were available were often from archaic highly passaged culture isolates not representative of naturally occurring HRSV strains (Kuypers et al., 2004; Perkins et al., 2005). We were able to take advantage of the now greatly expanded HRSV genomic data made available by the J. Craig Venter Institute Genomic Center for Infectious Diseases (http://gcid.jcvi.org/projects/gcid/viral/aim1/project) and others. Moreover, our assays were validated with newer commercial single-step rRT-PCR enzyme kits that offer improved amplification efficiency and reduced set-up times not previously available.

We chose to target the HRSV nucleocapsid gene for assay development because it is relatively conserved compared to other genome regions. Other assays that targeted the G glycoprotein gene (Tan et al., 2012) that shows the highest sequence variability are subject to increased risk of primer/probe mismatch destabilization. In another, older assay (Perkins et al., 2005), melting temperatures of rRT-PCR probes were lower than their corresponding primers, which would result in a lower percentage of probe bound to the target during each amplification cycle, compromising assay sensitivity (Rodriguez et al., 2015). To exploit short regions of homology in the HRSV genome for primer/probe targeting, some assays used short probes with 3′-minor groove binder (MGB) moieties to increase the stability of the probe–target hybrid (Kuypers et al., 2004; You et al., 2017). However, MGB probes were originally designed to detect single nucleotide polymorphisms and are inherently susceptible to base mismatch destabilization (Whiley and Sloots, 2006; Yao et al., 2006).

Commercial molecular assays with HRSV packaged individually or as part of larger respiratory pathogen panels and certified for *in vitro* diagnostic use by U.S. or international agencies are a seemingly attractive alternative (Reddington et al., 2013). However, commercial assays are also vulnerable to potential variant dropout, are costly, often depend on dedicated equipment and oligonucleotide sequences used are typically proprietary and inaccessible to end users to assess compatibility with currently circulating HRSV strains. Moreover, when primer/probe changes are needed to accommodate new genetic variants, regulatory requirements can delay implementation. Finally, where some HRSV subgrouping assays require up to 11 primer/probes to perform (Perkins et al., 2005), our assay only requires four oligonucleotides for subgroup identification (or seven total if combined with our pan rRT-PCR assay).

The recent study by Kamau and collaborators (Kamau et al., 2017) identified a HRSV subgroup B variant that failed detection by an established rRT-PCR assay (Gunson et al., 2005). Sequencing identified mismatched bases in the probe's central region disrupting probe-template annealing. The authors designed a replacement assay to correct this problem, but did not provide validation data documenting assay performance. These variants were discovered because the hospitalized children were routinely screened for HRSV by IFA, a method less susceptible to virus strain variation. However, routine IFA screening is impractical and would be less effective for testing adults where the method would lack the requisite sensitivity. Even with updated sequences, we also encountered a single HRSV strain that was poorly amplified with our duplex assay. Sequencing revealed a single nucleotide mismatch with the 3′-end of the reverse primer. Although generally more tolerant than probes (Suss et al., 2009; Whiley and Sloots, 2005), primer mismatches can also diminish target amplification (Blais et al., 2015). Although occasional HRSV variants that elude molecular detection would not be expected to greatly impact HRSV disease burden estimates, HRSV variants can quickly replace competing strains as occurred with the novel "BA" virus that emerged in 1999 and is now the

most common HRSV B lineage detected worldwide (Trento et al., 2003, 2006). Simultaneous testing of specimens with both our duplex and pan rRT-PCR assays would reduce the risk of undetected HRSV variant dropout (Blais et al., 2015).

In conclusion, we developed a duplex rRT-PCR assay for rapid, sensitive and specific detection and simultaneous subgroup identification of HRSV using currently available genome sequence data in GenBank. This assay can be applied to outbreak investigations and surveillance studies to obtain baseline information regarding circulating HRSV subgroups and serve as a reference standard for other assays to achieve comparable HRSV disease burden estimates.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Ethics approval

All participants or participant guardians provided informed consent. Ethics approval for the surveillance activities for respiratory diseases and specimen retention was obtained from the collaborative sites.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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.jviromet.2019.113676.

References

Anderson, T.P., Werno, A.M., Barratt, K., Mahagamasekera, P., Murdoch, D.R., Jennings, L.C., 2013. Comparison of four multiplex PCR assays for the detection of viral pathogens in respiratory specimens. J. Virol. Methods 191, 118–121.

Blais, J., Lavoie, S.B., Giroux, S., Bussieres, J., Lindsay, C., Dionne, J., Laroche, M., Giguere, Y., Rousseau, F., 2015. Risk of misdiagnosis due to allele dropout and false-positive PCR artifacts in molecular diagnostics: analysis of 30,769 genotypes. J. Mol. Diagn. 17, 505–514.

Choudhary, M.L., Anand, S.P., Heydari, M., Rane, G., Potdar, V.A., Chadha, M.S., Mishra, A.C., 2013. Development of a multiplex one step RT-PCR that detects eighteen respiratory viruses in clinical specimens and comparison with real time RT-PCR. J. Virol. Methods 189, 15–19.

Cowton, V.M., McGivern, D.R., Fearns, R., 2006. Unravelling the complexities of respiratory syncytial virus RNA synthesis. J. Gen. Virol. 87, 1805–1821.

Devincenzo, J.P., 2004. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. Pediatr. Res. 56, 914–917.

Fodha, I., Vabret, A., Ghedira, L., Seboui, H., Chouchane, S., Dewar, J., Gueddiche, N., Trabelsi, A., Boujaafar, N., Freymuth, F., 2007. Respiratory syncytial virus infections in hospitalized infants: association between viral load, virus subgroup, and disease severity. J. Med. Virol. 79, 1951–1958.

Fry, A.M., Chittaganpitch, M., Baggett, H.C., Peret, T.C., Dare, R.K., Sawatwong, P., Thamthitiwat, S., Areerat, P., Sanasuttipun, W., Fischer, J., Maloney, S.A., Erdman, D.D., Olsen, S.J., 2010. The burden of hospitalized lower respiratory tract infection due to respiratory syncytial virus in rural Thailand. PLoS One 5, e15098.

Gimferrer, L., Campins, M., Codina, M.G., Martin Mdel, C., Fuentes, F., Esperalba, J., Bruguera, A., Vilca, L.M., Armadans, L., Pumarola, T., Anton, A., 2015. Molecular epidemiology and molecular characterization of respiratory syncytial viruses at a tertiary care university hospital in Catalonia (Spain) during the 2013–2014 season. J. Clin. Virol. 66, 27–32.

Gunson, R.N., Collins, T.C., Carman, W.F., 2005. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. J. Clin. Virol. 33, 341–344.

Hall, C.B., Walsh, E.E., Schnabel, K.C., Long, C.E., McConnochie, K.M., Hildreth, S.W., Anderson, L.J., 1990. Occurrence of groups A and B of respiratory syncytial virus over 15 years: associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. J. Infect. Dis. 162, 1283–1290.

Johnson Jr., P.R., Olmsted, R.A., Prince, G.A., Murphy, B.R., Alling, D.W., Walsh, E.E., Collins, P.L., 1987. Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. J. Virol. 61, 3163–3166.

Kamau, E., Agoti, C.N., Lewa, C.S., Oketch, J., Owor, B.E., Otieno, G.P., Bett, A., Cane, P.A., Nokes, D.J., 2017. Recent sequence variation in probe binding site affected detection of respiratory syncytial virus group B by real-time RT-PCR. J. Clin. Virol. 88, 21–25.

Kim, C., Nyoka, R., Ahmed, J.A., Winchell, J.M., Mitchell, S.L., Kariuki Njenga, M., Auko, E., Burton, W., Breiman, R.F., Eidex, R.B., 2012. Epidemiology of respiratory infections caused by atypical bacteria in two Kenyan refugee camps. J. Immigr. Minor. Health 14, 140–145.

Kuypers, J., Wright, N., Morrow, R., 2004. Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children. J. Clin. Virol. 31, 123–129.

Laham, F.R., Mansbach, J.M., Piedra, P.A., Hasegawa, K., Sullivan, A.F., Espinola, J.A., Camargo Jr., C.A., 2017. Clinical profiles or respiratory syncytial virus subtypes A and B among children hospitalized with bronchiolitis. Pediatr. Infect. Dis. J. 36, 808–810.

Liu, W., Chen, D., Tan, W., Xu, D., Qiu, S., Zeng, Z., Li, X., Zhou, R., 2016. Epidemiology and clinical presentations of respiratory syncytial virus subgroups a and B detected with multiplex real-time PCR. PLoS One 11, e0165108.

Mahony, J.B., 2008. Detection of respiratory viruses by molecular methods. Clin. Microbiol. Rev. 21, 716–747.

Mufson, M.A., Belshe, R.B., Orvell, C., Norrby, E., 1987. Subgroup characteristics of respiratory syncytial virus strains recovered from children with two consecutive infections. J. Clin. Microbiol. 25, 1535–1539.

Mufson, M.A., Orvell, C., Rafnar, B., Norrby, E., 1985. Two distinct subtypes of human respiratory syncytial virus. J. Gen. Virol. 66 (Pt 10), 2111–2124.

Nair, H., Nokes, D.J., Gessner, B.D., Dherani, M., Madhi, S.A., Singleton, R.J., O'Brien, K.L., Roca, A., Wright, P.F., Bruce, N., Chandran, A., Theodoratou, E., Sutanto, A., Sedyaningsih, E.R., Ngama, M., Munywoki, P.K., Kartasasmita, C., Simoes, E.A., Rudan, I., Weber, M.W., Campbell, H., 2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 375, 1545–1555.

Peret, T.C., Hall, C.B., Hammond, G.W., Piedra, P.A., Storch, G.A., Sullender, W.M., Tsou, C., Anderson, L.J., 2000. Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. J. Infect. Dis. 181, 1891–1896.

Perkins, S.M., Webb, D.L., Torrance, S.A., El Saleeby, C., Harrison, L.M., Aitken, J.A., Patel, A., DeVincenzo, J.P., 2005. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. J. Clin. Microbiol. 43, 2356–2362.

Reddington, K., Tuite, N., Barry, T., O'Grady, J., Zumla, A., 2013. Advances in multiparametric molecular diagnostics technologies for respiratory tract infections. Curr. Opin. Pulm. Med. 19, 298–304.

Rodriguez, A., Rodriguez, M., Cordoba, J.J., Andrade, M.J., 2015. Design of primers and probes for quantitative real-time PCR methods. Methods Mol. Biol. 1275, 31–56.

Suss, B., Flekna, G., Wagner, M., Hein, I., 2009. Studying the effect of single mismatches in primer and probe binding regions on amplification curves and quantification in real-time PCR. J. Microbiol. Methods 76, 316–319.

Tan, L., Lemey, P., Houspie, L., Viveen, M.C., Jansen, N.J., van Loon, A.M., Wiertz, E., van Bleek, G.M., Martin, D.P., Coenjaerts, F.E., 2012. Genetic variability among complete human respiratory syncytial virus subgroup A genomes: bridging molecular evolutionary dynamics and epidemiology. PLoS One 7, e51439.

Trento, A., Galiano, M., Videla, C., Carballal, G., Garcia-Barreno, B., Melero, J.A., Palomo, C., 2003. Major changes in the G protein of human respiratory syncytial virus isolates introduced by a duplication of 60 nucleotides. J. Gen. Virol. 84, 3115–3120.

Trento, A., Viegas, M., Galiano, M., Videla, C., Carballal, G., Mistchenko, A.S., Melero, J.A., 2006. Natural history of human respiratory syncytial virus inferred from phylogenetic analysis of the attachment (G) glycoprotein with a 60-nucleotide duplication. J. Virol. 80, 975–984.

van Elden, L.J., van Loon, A.M., van der Beek, A., Hendriksen, K.A., Hoepelman, A.I., van Kraaij, M.G., Schipper, P., Nijhuis, M., 2003. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J. Clin. Microbiol. 41, 4378–4381.

Vandini, S., Biagi, C., Lanari, M., 2017. Respiratory syncytial virus: the influence of serotype and genotype variability on clinical course of infection. Int. J. Mol. Sci. 18.

Walsh, E.E., McConnochie, K.M., Long, C.E., Hall, C.B., 1997. Severity of respiratory syncytial virus infection is related to virus strain. J. Infect. Dis. 175, 814–820.

Weinberg, G.A., Schnabel, K.C., Erdman, D.D., Prill, M.M., Iwane, M.K., Shelley, L.M., Whitaker, B.L., Szilagyi, P.G., Hall, C.B., 2013. Field evaluation of TaqMan Array Card (TAC) for the simultaneous detection of multiple respiratory viruses in children with acute respiratory infection. J. Clin. Virol. 57 (July (3)), 254–260.

Whiley, D.M., Sloots, T.P., 2005. Sequence variation in primer targets affects the accuracy of viral quantitative PCR. J. Clin. Virol. 34, 104–107.

Whiley, D.M., Sloots, T.P., 2006. Sequence variation can affect the performance of minor groove binder TaqMan probes in viral diagnostic assays. J. Clin. Virol. 35, 81–83.

Yao, Y., Nellaker, C., Karlsson, H., 2006. Evaluation of minor groove binding probe and Taqman probe PCR assays: influence of mismatches and template complexity on quantification. Mol. Cell. Probes 20, 311–316.

You, H.L., Chang, S.J., Yu, H.R., Li, C.C., Chen, C.H., Liao, W.T., 2017. Simultaneous detection of respiratory syncytial virus and human metapneumovirus by one-step multiplex real-time RT-PCR in patients with respiratory symptoms. BMC Pediatr. 17, 89.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.