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A simplified, amplicon-based method for whole genome sequencing of human respiratory syncytial viruses

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ABSTRACT

Background: Human Respiratory Syncytial Virus (RSV) infections pose a significant risk to human health worldwide, especially for young children. Whole genome sequencing (WGS) provides a useful tool for global surveillance to better understand the evolution and epidemiology of RSV and provide essential information that may impact on antibody treatments, antiviral drug sensitivity and vaccine effectiveness.
Objectives: Here we report the development of a rapid and simplified amplicon-based one-step multiplex reverse-transcription polymerase chain reaction (mRT-PCR) for WGS of both human RSV-A and RSV-B viruses.
Study design: Two mRT-PCR reactions for each sample were designed to generate amplicons for RSV WGS. This new method was tested and evaluated by sequencing 206 RSV positive clinical samples collected in Australia in 2020 and 2021 with RSV Ct values between 10 and 32.
Results: In silico analysis and laboratory testing revealed that the primers used in the new method covered most of the currently circulating RSV-A and RSV-B. Amplicons generated were suitable for both Illumina and Oxford Nanopore Technologies (ONT) NGS platforms. A success rate of 83.5% with a full coverage for the genome of 98 RSV-A and 74 RSV-B was achieved from all clinical samples tested.
Conclusions: This assay is simple to set up, robust, easily scalable in sample preparation and relatively inexpensive, and as such, provides a valuable addition to existing NGS RSV WGS methods.

1. Introduction

Human Respiratory Syncytial Virus (RSV) belongs to the *Pneumoviridae* family and is a common cause of severe respiratory tract infections in infants, young children, older adults and immunocompromised individuals [1,2]. Despite the significant public health impact and global economic burden of RSV infections [3,4], there are no vaccines or effective treatments currently available. The only prevention currently is an expensive monoclonal antibody prophylaxis for young children [5–7].

RSV is divided into two major types RSV-A and RSV-B [8]. Most genetic studies of RSV are focused on the attachment glycoprotein (*G*) gene, which is the most variable region and has been commonly used for RSV genotyping [9]. In contrast, the F protein (which is responsible for fusion of viral and host cell membranes and syncytium formation) is highly conserved between strains and is the target of most vaccine and

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Table 1
The information for primers used in the one-step multiplex RT-PCR.

Multiplex Tube	Primer Name	Primer Sequence (5′- 3′)[b]	Position of first 5′ base[a]	Amplicon length (bp)	Final concentration (μM)
Tube 1	1–1F	ACGCGAAAAAATGCGTACTACAAAC	1		0.167
	1–1R	CTG**M**ACCATAGGCATTCATAAACA	1898	1898	0.167
	1–3F	GCTATGGCAAGACT**Y**AGGAATG	2926		0.25
	1–3R	TTGAG**R**TCTAACACTTTGCTGGT	6304	3379	0.25
	1–5F	TGATGCATCAATATCTCAAGTCA	7181		0.6
	1–5R	G**R**CCTAT**D**CCTGCATACTC	11,116	3936	0.6
Tube 2	2–2F	ATGGGAGA**R**GT**R**GCTCCAGAATA	1562		0.2
	2–2R	CGTGTAGCTGT**R**TG**Y**TTCCAA	4004	2443	0.2
	2–4F	AGCAAATT**Y**TGGCC**Y**TA**Y**TTTAC	4334		0.6
	2–4R1	CTCATAGCAACACATGCTGATTG	7967	3634	0.3
	2–4R2	GAGTTTGCTCATGGCAACACAT	7974	3641	0.3
	2–6F	TGGACCAT**W**GAAGC**Y**ATATCA	10,912		0.32
	2–6R	AGTGTCAAAAACTAAT**R**TCTCGT	15,265	4354	0.32

[a]Primer nucleotide numbering was based on a human RSV-B strain (GISAID accession number 2,584,506).
[b]Degenerate bases are highlighted in bold.

monoclonal antibody treatments currently being developed [10]. The mutations acquired during evolution may lead to changes in various aspects of virus biology, such as infectivity, antigenicity, drug resistance and potentially virulence [11,12]. Therefore, there is an ongoing need for effective monitoring of RSV viral evolution and spread throughout human society.

Among different Next generation sequencing (NGS) approaches, the PCR amplicon-based NGS is a sensitive method for sequencing small genomes such as RSV for population-scale viral surveillance [13]. One challenge associated with this method is the generation of PCR amplicons that can cover all variants. Several singleplex reverse-transcription (RT)-PCR amplicon-based NGS methods have been used for RSV whole-genome sequencing (WGS) including a commonly used method that requires at least four separate reactions per genome [14,15]. While the use of multiplex PCR (mPCR) to amplify multiple DNA fragments in the same reaction provides a simpler solution to reducing the number of reactions [16], it often leads to a decreased sensitivity, due to complex primer-primer interactions and competition for usage of reagents [17]. Therefore, the establishment of optimal primer pools for mPCR can be challenging and may require many sets of primers as seen with the ARTIC protocol for COVID-19 WGS [18,19]. The development of amplicon-based WGS should ideally be suitable for multiple NGS platforms, especially in developing countries where the burden of RSV is greatest. One such system that is currently widely used is the ONT's MinION system, which is a portable and low-cost NGS that can significantly simplify the sequencing workflow and shorten the turnaround time for sequencing [20].

In April 2019, the World Health Organization (WHO) initiated phase 2 of their Global RSV Surveillance program to study the circulation and impact of RSV worldwide. One of the elements of this study was to increase the sequencing capacity for RSV viruses [21], especially in developing countries. In order to meet the increasing demand for RSV sequencing, we endeavoured to develop a simplified one-step multiplex RT-PCR (mRT-PCR) method for RSV WGS.

2. Methods and materials

2.1. Clinical specimens

Samples used in this study were de-identified respiratory samples (including nasal swabs, nasopharyngeal swabs, nasal washes or nasopharyngeal aspirates) that were positive for RSV by routine clinical laboratory molecular testing at Queensland and Victorian (Australia) laboratories collected between December 2020 and April 2021. An aliquot of these samples was shipped to the center as part of the WHO RSV surveillance program and samples were stored at -80 °C until further analyzed.

2.2. RNA extraction and real-time RT-PCR assay

Viral RNA was extracted from 200 μl of clinical sample using QIAamp 96 Virus QIAcube HT (QIAGEN) according to the manufacturer's instruction. The CDC Respiratory Syncytial Virus Real-time RT-PCR panel (RSV_RUO-01) [22], was used for RSV subtype detection with TaqPath 1-Step Multiplex Master Mix (No ROX) on Applied Biosystems 7500 Real-Time PCR System (ABI) as instructed. Reactions were run using the following cycling conditions: one cycle of 25 °C for 2 min, followed by one cycle of 53 °C for 10 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 1 min. The resulting cycle threshold (Ct) values were rounded to the nearest whole number.

2.3. RSV amplicon generation by one-step mRT-PCR for RSV WGS

SuperScript IV One-Step RT-PCR System (Invitrogen) was used for RSV whole genome amplification with two separate mRT-PCR reactions using six pairs of primers designed for this study (Table 1 and Figure S1). Briefly, 50 μL reaction containing 5.1 μl of primer pool one or 5.6 μl primer pool two, 9.9 μl or 9.4 μl of template RNA, 25 μl of 2 x Platinum SuperFi RT-PCR Master Mix and 0.5 μl of SuperScript IV RT Mix. Thermocycling conditions consisted of 10 min at 55 °C for reverse transcription, 2 min at 98 °C to inactivate the reverse transcriptase enzyme followed by 40 cycles of 98 °C for 10 s, 55 °C for 10 s and 72 °C for 3 min, with a final extension step at 72 °C for 5 min. The sizes of mRT-PCR products were evaluated and amplicons were quantified on a TapeStation device (Agilent). Equal amounts of RT-PCR products from two reactions per sample were pooled together for downstream NGS library preparation and sequencing.

2.4. Library preparation and sequencing on an Illumina platform

Two hundred ng of mRT-PCR products from each of two reactions per genome were pooled for the Illumina NGS library preparation. Pooled amplicons were cleaved into small fragments and tagged with adaptor sequences followed by indexing according to manufacturer's instruction. After library preparation, there were 31 to 48 sample libraries pooled together for sequencing on each Illumina iSeq flow cell. The pooled libraries were quantified by the Qubit dsDNA High Sensitivity assay (Thermo Fisher Scientific). The sizes of pooled libraries were measured with High Sensitivity D1000 ScreenTape and reagents on a TapeStation device (Agilent). Based on the results from the Qubit and TapeStation measurements, pooled libraries were diluted to 100 pM in Illumina RSB buffer, spiked with 1% PhiX, and sequenced on an Illumina iSeq sequencer using paired-end runs with 300 cycles (2 × 150 bp).

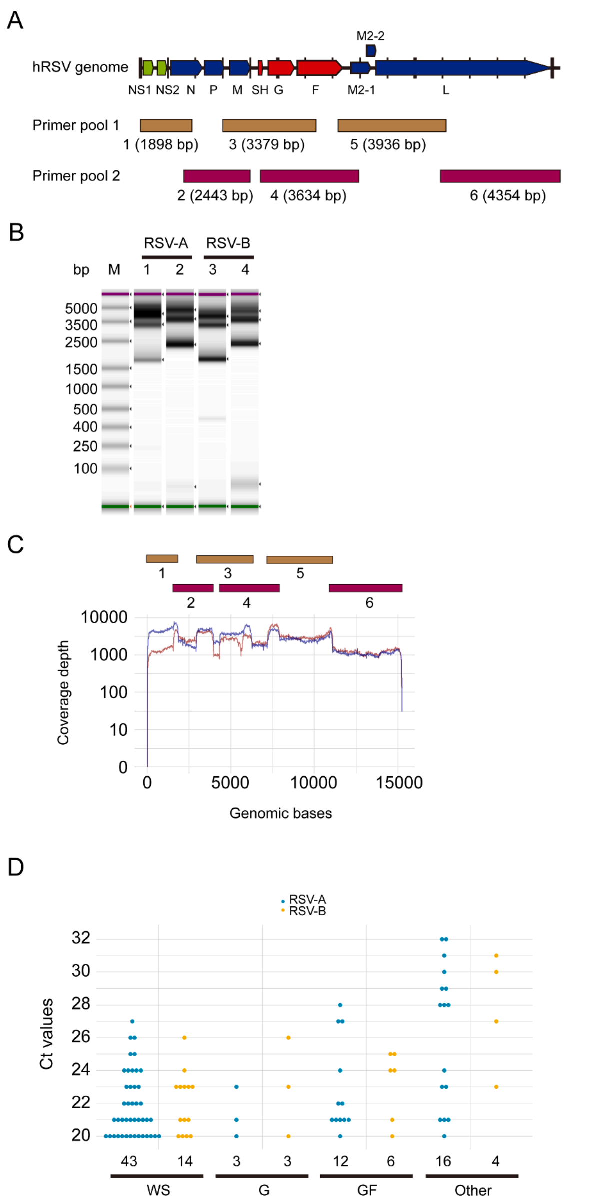

Fig. 1. One-step multiplex RT-PCR for RSV WGS. (A) Schematic overview of the 6 amplicons designed to cover the RSV genome and the encoded proteins; NS1: non-structure protein 1, NS2: non-structure protein 2, N: nucleoprotein, P: phosphoprotein, M: matrix protein, SH: small hydrophobic protein, G: glycoprotein, F: fusion protein, M2–1: Matrix protein 2–1, M2–2: Matrix protein 2–2, L: large polymerase. One PCR reaction contains pool 1 primers that generate three fragments (numbered as 1, 3 and 5) ranging from about 1898 to 3936 bp, while the second PCR reaction has pool 2 primers that generate another three fragments (numbered as 2, 4 and 6) of sizes between 2443 and 4354 bp based on a human RSV-B strain (GISAID accession number 2,584,506). (B) Representative Tapestation images of two-pool PCR products of viral RNA from one RSV-A and one RSV-B positive clinical samples. Molecular marker (M); mRT-PCR products of pool 1 (lane 1) and pool 2 (lane 2) reactions from hRSV/A/Australia/QLD-RBWH114/2021; mRT-PCR products of pool 1 (lane 3) and pool 2 (lane 4) from hRSV/B/Australia/QLD-RBWH289/2021. (C) Coverage depth of sequenced RSV-A (Dark red) and RSV-B (Dark blue) in genomic positions covered by six overlapping amplicons. (D) Sensitivity of RSV genome sequencing in clinical samples with varied Ct values. Dot plot showing the outcomes of RSV-A and RSV-B samples sequenced by NGS according to their RT-PCR Ct values; whole-genome sequence obtained (WS), *G* gene only obtained (G), *G* and *F* genes obtained (GF), and partial sequence without *G* gene obtained (Other); the total number of samples for each RSV type and sequencing outcome are indicated, respectively.

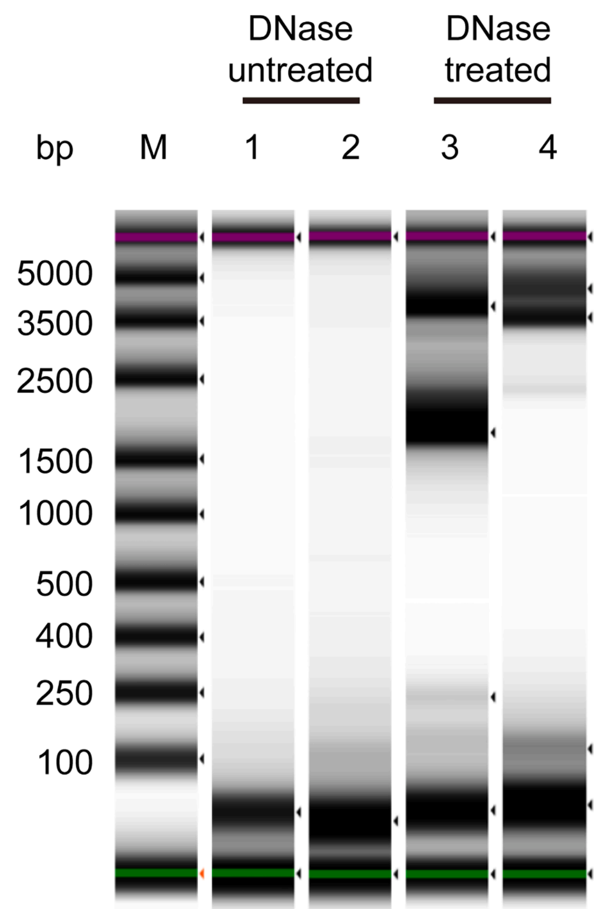

Fig. 2. DNase treatment improved assay sensitivity. RSV amplicons (pool 1 and 2) generated from a RSV-B positive clinical sample hRSV/B/Australia/QLD-RBWH239/2021 without and with DNase treatment.

2.5. Other methods

Methods for DNase treatment of viral RNA for mRT-PCR, library preparation and sequencing on an ONT platform, and data analyses are described in the Supplementary Information.

3. Results

3.1. Development of one-step mRT-PCR for RSV WGS

As shown in Fig. 1A, six amplicons in two mRT-PCR tubes that fully covered the whole RSV genome were amplified from both RSV-A (hRSV/A/Australia/QLD-RBWH114/2021) and RSV-B (hRSV/B/Australia/QLD-RBWH289/2021) respiratory clinical samples with relatively similar efficiency and the six amplicons were easily distinguishable by size using electrophoresis (Fig. 1B). The amplicons from two reactions were combined in an equal amount and used for library preparation for iSeq100 NGS. Results revealed that both RSV-A and RSV-B genomes were fully sequenced with relatively even coverage (Fig. 1C).

3.2. Performance of RSV mRT-PCR in clinical samples

Two hundred and sixteen RSV positive respiratory clinical samples with cycle thresholds (Ct) values between 10 and 32 were selected for RSV mRT-PCR testing; Among 115 samples with Ct values below 20 and tested for amplicon generation, 50 RSV-A and 55 RSV-B samples had all six bands clearly detectable and were selected for WGS. As expected, all these samples had RSV whole genomes fully sequenced.

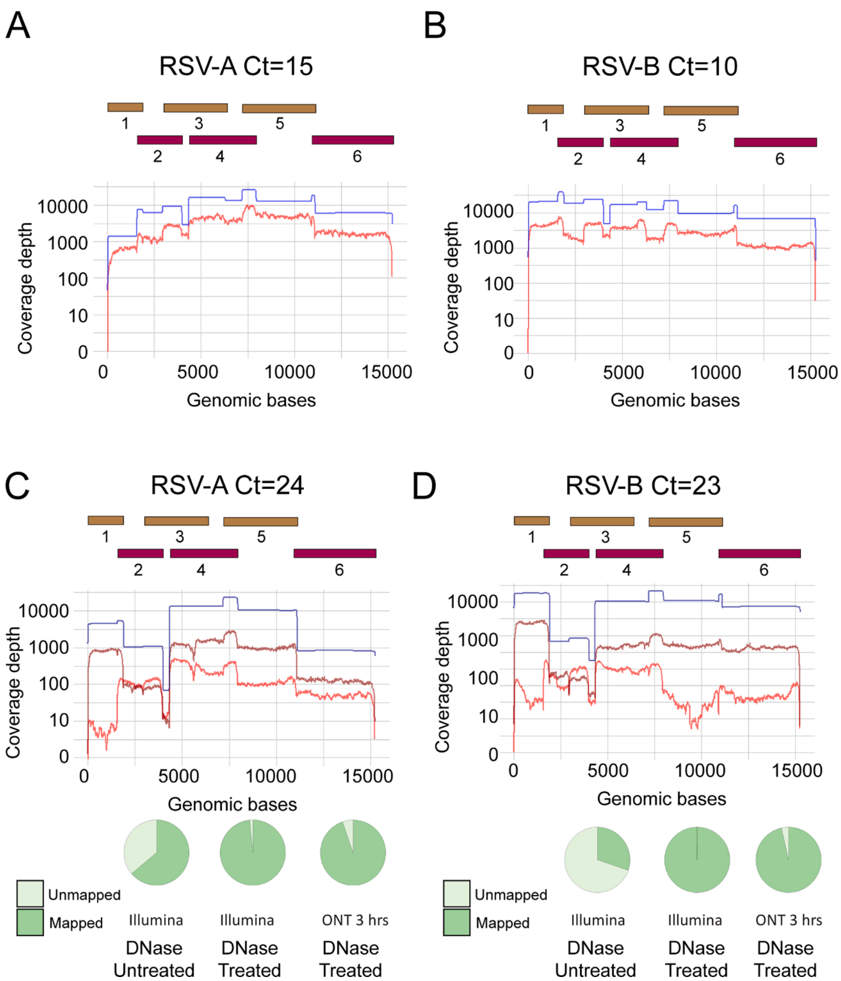

Fig. 3. Coverage depths comparison of NGS data generated from Illumina and ONT platforms. High coverage depths of a RSV-A (A) and a RSV-B (B) samples with high viral loads (low Ct values) sequenced on Illumina (red) and ONT (blue) platforms at each nucleotide position. A RSV-A (C) and a RSV-B (D) sample with low viral loads (high Ct values) were treated or not treated with DNase and run on the two platforms. Illumina sequencing without DNase treatment shown in red and after treatment shown in dark red. ONT sequencing after DNase treatment shown in dark blue. The pie charts show the NGS reads that were RSV specific (green) and nonspecific (light green) from both iSeq100 and MinION platforms with or without DNase treatment. No data was obtained for samples of low viral loads without DNase treatment due to failure in generating enough amount of NGS library for ONT sequencing.

Next, we assessed the sensitivity of this new mRT-PCR assay for sequencing RSV clinical samples with relatively higher Ct values than those tested above. The mRT-PCR amplicons from 101 RSV samples with Ct values ranging from 20 to 32 were used for Illumina NGS, regardless of whether all six amplicons were visible or not upon TapeStation analysis. Whole-genome sequences (WS) were obtained from 57 out of 101 samples with Ct value up to 27 (56.4%) (Fig. 1D), of these, all six PCR amplicons were only obvious in 27 samples, with at least one amplicon not detectable by TapeStation from the other 30 samples. This result suggested that NGS can still work well even with weak PCR fragments that are not visible on a TapeStation System. In addition, 24 samples generated partial RSV genomes, importantly 18 with complete *G* and *F* genes (GF) and 6 with the complete *G* gene only.

3.3. DNase treatment of samples with lower viral load improved NGS results

Analyses of the sequenced samples indicated the presence of human genome DNA in the extracted viral RNA as shown in Figure S2, A-C. Particularly, the samples with low RSV viral abundance were more significantly affected by human genome contamination than those with high RSV viral loads in this assay (Figure S2D).

To improve sensitivity of the mRT-PCR NGS in samples with lower viral loads, we used DNase treatment on the purified viral RNA in samples with a RSV Ct value of 23 and above, and compared mRT-PCR and NGS results on these samples with and without DNase treatment. Thirty-one RSV-A and 17 RSV-B positive clinical samples were chosen for the DNase treatment comparison. Notably, DNase treatment of viral RNA significantly improved the efficiency of mRT-PCR, and RSV amplicons were more prominent upon TapeStation examination (Fig. 2, S3A). Furthermore, WS were obtained from 60.4% of the samples (29 samples) including 18 RSV-A and 11 RSV-B, while GF sequences were obtained from a further 12 viruses (25%) and one sample had the complete *G* gene only. In total, 42 samples with Ct values of up to 32 generated useful sequence data, amounting to 87.5% of the samples tested (Figure S3B), with only 6 samples (12.5%) failing to generate useful sequences with complete *G* gene. In contrast, without DNase treatment, WS could only be successfully generated from 21 samples (43.8%) including 14 RSV-A and 7 RSV-B, plus 8 samples with GF sequences (16.7%) and 3 with only *G* sequences (6.3%) generated amounting to a total success rate of 66.7% (Figure S3C) while 16 (33.3%) that did not produce useful sequence data. Hence, the DNase treatment improved the success rate of useful sequence generation by 20.8%.

NGS data analysis of all 48 samples indicated that the mean percentage of RSV specific reads from all samples was significantly increased from 25.6% to 88.9% after DNA removal (Figure S3D), whereas human reads abundance dropped from 60.9% to 5.7% as estimated by Kraken 2 (Figure S3E) [23].

All WS generated in this study were analyzed phylogenetically and showed that the RSV-A genomes clustered into 2 main clades, exclusively with other Australian viruses and had some heterogeneity but all fell into the ON1 clade (Figure S4) while the RSV-B genomes clustered into 3 main clades, also exclusively with other Australian viruses and again with a degree of heterogeneity but all within the BA clade (Figure S5).

3.4. Sequencing of samples on both Illumina and ONT platforms

The same mRT-PCR products from two RSV positive clinical samples with high RSV viral loads (hRSV/A/Australia/QLD-RBWH060/2021 with a Ct of 15; hRSV/B/Australia/QLD-RBWH289/2021 with a Ct of 10) were used for sequencing on both Illumina and ONT platforms. As shown in Fig. 3A and 3B, both platforms generated data with good coverage throughout the whole genome.

We further evaluated if DNase treatment could improve sequencing quality with ONT platform and compared the results obtained with the Illumina system. Both DNase treated and untreated viral RNA from two clinical samples with relatively low RSV viral loads (hRSV/A/Australia/QLD-RBWH191/2021 with a Ct of 24 and hRSV/B/Australia/QLD-RBWH239/2021 with a Ct of 23) were used for sequencing comparison. We found DNase treatment also improved the NGS performance on ONT platform as well as the Illumina platform (Fig. 3C and 3D). Notably, without DNase treatment, two samples failed to generate enough amount of NGS library for ONT sequencing, even though more PCR product was used for ONT library preparation than for the Illumina sequencing.

4. Discussion

To the best of our knowledge, previously published amplicon-based RSV WGS approaches require at least 4 different singleplex PCR amplification reactions following the cDNA synthesis from viral RNA with separate reverse transcription reactions that takes significant time to process a large number of samples [14,15,24]. The mRT-PCR method described herein provides a convenient, cost-effective and more scalable way for sequencing the RSV A or B whole genomes. It only requires two tubes and a one-step RT-PCR reaction to generate overlapping PCR fragments from viral RNA and therefore significantly reduces handling time and reduces the risk of contamination. Traditional mPCR-tiling amplicon methods for WGS normally have primers designed to generate several amplicons of similar size for achieving comparable PCR amplification efficiencies in the same tube, making it impossible to tell if all amplicons have been successfully amplified without sequencing them. However, this mRT-PCR method generated amplicons are of easily distinguishable sizes, making the identification of all segments amplified very easy through electrophoresis, giving the user a good indication whether WS can be likely achieved for each sample.

By introducing an optional DNase treatment step for original specimens with lower viral loads, we were able to improve the WGS success rate. Even if the WS couldn't be obtained from some samples, the *G* gene sequence was obtained in many of these samples along with the *F* gene in some. The *G* and *F* genes are the most informative genes with the *G* gene being the more variable and being used for both evolutionary analysis as well as for classification systems that are important for RSV surveillance, while the F protein is the main target for vaccine development and for prophylactic monoclonal antibody preventatives and hence requires close scrutiny. While previous studies have shown that similar phylogenetic topologies to WS can be obtained with sequences from just the F, G, or G-ectodomain of RSV [25], other important viral characteristics can only be obtained by whole genome sequencing. Some studies have also demonstrated advantages in using DNase treatment for enhancing virus detection in serum and plasma samples by boosting the specific fragment generation with the sequence-independent single primer amplification (SISPA) for sequencing [26]. Recently, DNase treatment on RNA extracted from clinical samples also demonstrated improved RSV detection with SISPA followed by NGS [27].

The method described here can be used to prepare libraries for different sequencing-platforms such as Illumina or ONT. According to a recent study of ONT sequencing of SARS-CoV-2, long amplicons (~2 and ~2.5-kb) PCR-tiling clearly showed better performance in coverage variation and overall quality of the final sequence consensus when compared to short amplicons (~400-bp) [28]. Thus, our method with long amplicons for RSV WGS is also suitable for ONT in addition to Illumina sequencing.

Fig. 4. Schematic diagram of the RSV whole genome sequencing workflow. A decision tree showing the different processing paths depending on the viral load in the clinical samples.

Overall, we have demonstrated a new method and workflow for NGS of RSV whole genomes directly from clinical respiratory samples without the need for typing that is based on a one-step mRT-PCR amplicon based approach (Fig. 4). For optimum NGS results, we suggest the pre-treatment of RNA with DNase prior to mRT-PCR for samples with RSV Ct values between 23 (approximate copy number (CN) of 600,000) and 28 (CN=~1500) for RSV-A, and CN=~60,000 and CN=~180 respectively for RSV-B, according to the standard curves previously published [22]. Downstream NGS could be done with either the short-read Illumina platforms or the long-read ONT sequencing platform. With its robust performance, faster and more scalable preparation, this protocol is a valuable addition to existing RSV WGS methods.

Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2023.105423.

References

[1] L.L. Han, J.P. Alexander, L.J. Anderson, Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden, J. Infect. Dis. 179 (1999) 25–30.
[2] H. Nair, D.J. Nokes, B.D. Gessner, M. Dherani, S.A. Madhi, R.J. Singleton, et al., Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis, Lancet 375 (2010) 1545–1555.
[3] Y. Li, X. Wang, D.M. Blau, M.T. Caballero, D.R. Feikin, C.J. Gill, et al., Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in children younger than 5 years in 2019: a systematic analysis, Lancet 399 (2022) 2047–2064.
[4] M. Tin Tin Htar, M.S. Yerramalla, J.C. Moisi, D.L. Swerdlow, The burden of respiratory syncytial virus in adults: a systematic review and meta-analysis, Epidemiol. Infect. 148 (2020) e48.
[5] J.S. Eden, C. Sikazwe, R. Xie, Y.M. Deng, S.G. Sullivan, A. Michie, et al., Off-season RSV epidemics in Australia after easing of COVID-19 restrictions, Nat. Commun. 13 (2022) 2884.
[6] R. Barr, C.A. Green, C.J. Sande, S.B. Drysdale, Respiratory syncytial virus: diagnosis, prevention and management, Ther. Adv. Infect. Dis. 6 (2019), 2049936119865798.
[7] B. Resch, Product review on the monoclonal antibody palivizumab for prevention of respiratory syncytial virus infection, Hum. Vaccin. Immunother. 13 (2017) 2138–2149.
[8] M.A. Mufson, C. Orvell, B. Rafnar, E. Norrby, Two distinct subtypes of human respiratory syncytial virus, J. Gen. Virol. 66 (Pt 10) (1985) 2111–2124.
[9] J.C. Munoz-Escalante, A. Comas-Garcia, S. Bernal-Silva, D.E. Noyola, Respiratory syncytial virus B sequence analysis reveals a novel early genotype, Sci. Rep. 11 (2021) 3452.
[10] E. Andreano, I. Paciello, M. Bardelli, S. Tavarini, C. Sammicheli, E. Frigimelica, et al., The respiratory syncytial virus (RSV) prefusion F-protein functional antibody repertoire in adult healthy donors, EMBO Mol. Med. 13 (2021) e14035.
[11] C. Efstathiou, S.H. Abidi, J. Harker, N.J. Stevenson, Revisiting respiratory syncytial virus's interaction with host immunity, towards novel therapeutics, Cell Mol. Life Sci. 77 (2020) 5045–5058.
[12] C. Griffiths, S.J. Drews, D.J. Marchant, Respiratory syncytial virus: infection, detection, and new options for prevention and treatment, Clin. Microbiol. Rev. 30 (2017) 277–319.
[13] C.J. Houldcroft, M.A. Beale, J. Breuer, Clinical and biological insights from viral genome sequencing, Nat. Rev. Microbiol. 15 (2017) 183–192.
[14] S.A. Schobel, K.M. Stucker, M.L. Moore, L.J. Anderson, E.K. Larkin, J. Shankar, et al., Respiratory Syncytial Virus whole-genome sequencing identifies convergent evolution of sequence duplication in the C-terminus of the G gene, Sci. Rep. 6 (2016) 26311.
[15] M.E. Bose, J. He, S. Shrivastava, M.I. Nelson, J. Bera, R.A. Halpin, et al., Sequencing and analysis of globally obtained human respiratory syncytial virus A and B genomes, PLoS ONE 10 (2015), e0120098.
[16] J. Quick, N.D. Grubaugh, S.T. Pullan, I.M. Claro, A.D. Smith, K. Gangavarapu, et al., Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples, Nat. Protoc. 12 (2017) 1261–1276.
[17] E.M. Elnifro, A.M. Ashshi, R.J. Cooper, P.E. Klapper, Multiplex PCR: optimization and application in diagnostic virology, Clin. Microbiol. Rev. 13 (2000) 559–570.
[18] S.S. Brown, Y.W. Chen, M. Wang, A. Clipson, E. Ochoa, M.Q. Du, PrimerPooler: automated primer pooling to prepare library for targeted sequencing, Biol Methods Protoc. 2 (2017) bpx006.
[19] A.W. Lambisia, K.S. Mohammed, T.O. Makori, L. Ndwiga, M.W. Mburu, J. M. Morobe, et al., Optimization of the SARS-CoV-2 ARTIC network V4 primers and whole genome sequencing protocol, Front. Med. (Lausanne) 9 (2022), 836728.
[20] Y. Wang, Y. Zhao, A. Bollas, Y. Wang, K.F. Au, Nanopore sequencing technology, bioinformatics and applications, Nat. Biotechnol. 39 (2021) 1348–1365.
[21] A.K. Todd, A.M. Costa, G. Waller, A.J. Daley, I.G. Barr, Y.M. Deng, Rapid detection of human respiratory syncytial virus A and B by duplex real-time RT-PCR, J. Virol. Methods 294 (2021), 114171.
[22] L. Wang, P.A. Piedra, V. Avadhanula, E.L. Durigon, A. Machablishvili, M.R. Lopez, et al., Duplex real-time RT-PCR assay for detection and subgroup-specific identification of human respiratory syncytial virus, J. Virol. Methods 271 (2019), 113676.
[23] D.E. Wood, J. Lu, B. Langmead, Improved metagenomic analysis with Kraken 2, Genome Biol. 20 (2019) 257.
[24] L. Wang, T.F.F. Ng, C.J. Castro, R.L. Marine, L.C. Magana, M. Esona, et al., Next-generation sequencing of human respiratory syncytial virus subgroups A and B genomes, J. Virol. Methods 299 (2022), 114335.
[25] S. Goya, M. Galiano, I. Nauwelaers, A. Trento, P.J. Openshaw, A.S. Mistchenko, et al., Toward unified molecular surveillance of RSV: a proposal for genotype definition, Influenza Other Respir. Viruses 14 (2020) 274–285.
[26] T. Allander, S.U. Emerson, R.E. Engle, R.H. Purcell, J. Bukh, A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species, Proc. Natl. Acad. Sci. U S A 98 (2001) 11609–11614.
[27] S. Goya, L.E. Valinotto, E. Tittarelli, G.L. Rojo, M.S. Nabaes Jodar, A.L. Greninger, et al., An optimized methodology for whole genome sequencing of RNA respiratory viruses from nasopharyngeal aspirates, PLoS ONE 13 (2018), e0199714.
[28] B. Brejova, K. Borsova, V. Hodorova, V. Cabanova, A. Gafurov, D. Fricova, et al., Nanopore sequencing of SARS-CoV-2: comparison of short and long PCR-tiling amplicon protocols, PLoS ONE 16 (2021), e0259277.