Human rhinovirus and respiratory syncytial virus genotypes in Sudden Unexpected Death in Infancy (SUDI) cases at Tygerberg Hospital, Cape Town, South Africa

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Research Article

Human Rhinovirus and Respiratory Syncytial Virus Genotypes in Sudden Unexpected Death in Infancy (SUDI) Cases at Tygerberg Hospital, Cape Town, South Africa

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Abstract: Infant mortality remains a major global concern. Sudden unexpected death in infancy (SUDI) is reported globally and in the Western Cape of South Africa it accounted for 40% of all infant deaths between 2012 and 2016. Research highlighting molecular typing of respiratory viruses in SUDI cases is lacking. A total of 116 PCR-positive human rhinovirus (HRV) and respiratory syncytial virus (RSV) swab samples collected from the lungs and trachea of SUDI cases admitted to Tygerberg Medico-legal Mortuary in Cape Town between 2015 and 2019 were included and underwent sequencing and phylogenetic analysis. Three distinct HRV species were identified; HRV-A (n=28), HRV-C (n=11), and HRV-B (n=4), including eight HRV-A, seven HRV-C and one HRV-B genotypes. For RSV, both RSV-A (n=5) and -B (n=5) were detected, but no RSV-A. After amino acid alignment indicating 20 amino acid duplication and nine substitutions, two BA9 genotypes were confirmed. This study describes the first molecular and phylogenetic characterisation of respiratory viruses in SUDI cases in South Africa. The rapid decline in viral viability in post-mortem samples and the retrospective study design employed for the current study did not allow for any correlations between viral genotypes and cause of death and future prospective studies should investigate temporality and specific viral strains associated with clinical disease severity.

Keywords: respiratory infection; human rhinovirus; respiratory syncytial virus; genotypes; SUDI / SIDS; infant death; Sanger sequencing; phylogenetic analysis

1. Introduction

Lower respiratory tract infection (LRTI) is a major barrier to the United Nations Millennium Development Goal to reduce global under-five child mortality [1]. Human rhinovirus (HRV) and respiratory syncytial virus (RSV) are among the common causes of LRTIs in infants and are associated with wheezing and childhood asthma [2–11].

HRV was first discovered in the 1950s as a causative agent for the common cold (12,13], and is still regarded as a leading cause of infant hospitalisation [5,14,15]. Primary infection occurs through inhalation of aerosolised droplets or physical inoculation after contact with fomites, followed by a 2-day incubation period [16]. Viral replication and infection occur in the epithelial cells of the upper and lower respiratory tracts [4,17]. Children serves as the main reservoir and infection is four times more common in children than in adults [5,12,14,18]. HRV is a member of the family Picornaviridae and the genus *Enterovirus*. These viruses are positive sense (ps), single-stranded (ss) RNA viruses with a 7 200 base pair (bp) genome, consisting of structural and non-structural regions, flanked by 5' and 3' untranslated regions (UTR). HRV is classified by genotype into HRV-A (80 serotypes), HRV-B (32 serotypes), and HRV-C (57 serotypes), and by cell surface receptor differences into Major and Minor groups. Over 160 genotypes exist [16,19]. Genotype differentiation of HRV is commonly

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achieved by reverse transcriptase polymerase chain reaction (RT-PCR) targeting the 5'UTR and VP4/2 followed by sequence analysis [20–27].

RSV was first identified in 1956 in a chimpanzee during an outbreak of coryza. The virus was later detected in humans which remain the only host [9,28]. Similar to HRV, RSV is also regarded as a leading global cause of LRTI and hospitalisations in infants [8,29–34]. Mortality may be lower in infants in developed countries; however, regardless of the socioeconomic status, infants under the age of six months have the same high risk of childhood asthma and recurrent wheeze when infection occurs during peak RSV season [9,35]. RSV used to be a subfamily within the Paramyxoviridae family [36], but in 2016 it was reclassified into the family of Pneumoviridae, genus *Orthopneumovirus*. The virus is a non-segmented, negative sense (ns) enveloped ssRNA virus with an approximate genome length of 15 000 bp [28]. Subtypes are based on sequence variations of the second hypervariable region (HVR), or HRV2, of the RSV G-gene [37]. Immune pressure, inherent lack of proof-reading capabilities, and duplication events in the RSV G-gene ectodomain enabled the detection of the Buenos Aires (BA) and the Ontario genotypes (ON) in 1999 and 2010 respectively [31,38,39]. The ON1 and BA strains within RSV-A and -B respectively, are differentiated by nucleotide duplications in the genome [31]. The relationship between genotype variation and clinical severity is still unclear. RSV-A and -B co-circulate with one predominating subtype each, with ON and BA genotypes rapidly becoming the predominant subtypes across the globe [28].

All three HRV species circulate within South Africa, commonly infecting young children. Similar to Jordan, Hong Kong, and Taiwan, HRV-A and -C predominate circulation in South Africa [4,6,16,40]. A previous South African study of 220 children presenting with wheeze at Red Cross War Memorial Children's Hospital, Cape Town, showed that all three HRV genotypes were detected, with HRV-C (52%) predominating, followed by HRV-A (37%), and -B (11%). More than 70% of these children were under the age of 2 years [4]. Another South African study [42] confirmed this HRV-species distribution, but neither studies investigated any relationship between HRV genotype and disease severity [4,42]. More recently a study conducted in Mali, South Africa, and Zambia, found a higher prevalence of HRV-A in infants than HRV-B and HRV-C [25]. Clinical and molecular epidemiology data in low- and middle-income countries in Africa are limited and the specific role of HRV in childhood respiratory disease needs elucidation [4,25].

Both RSV-A and -B subtypes circulate in South Africa and are associated with LRTI [44,45] and infants comprise the majority of hospitalisation as a result of RSV [46]. The Drakenstein Child Health Study is an ongoing birth cohort longitudinal study on 1 143 infants in the Western Cape, South Africa. Of these, only 13% developed RSV-associated LRTI, while 60% did not develop any LRTI. Infants with RSV-associated LTRI had a ten-fold higher incidence of recurrent wheezing (43%) than those with no prior infection (3%). The results of this study further showed that LRTI and hospitalisation as a result of RSV were most common in winter and infants younger than 6 months of age, becoming less common with increasing age [11].

Globally, respiratory viral infections is a major burden in infants [15,32,47–49]. HRV, RSV, human CoV, and human adenovirus are commonly detected in infants with respiratory illness. Sudden unexpected death in infancy (SUDI) cases, defined as sudden and unexpected death in an infant with no clinical signs suggesting possible illness up to two days prior to death, show the same viral patterns than live infants [50–55]. All SUDI cases in South Africa must undergo a full medico-legal investigation at the respective Medico-legal Mortuaries and if no cause of death can be confirmed after an extensive autopsy, review of medical history, and death scene investigation, such cases are classified as sudden infant death syndrome (SIDS) [56–58]. Thus, SIDS is a subset of SUDI [59,60]. Both are regarded as leading causes of infant mortality [60,61].

While viral screening is often included in the investiation protocol in SUDI cases in South Africa [50,57–59], this will be the first report to describe HRV and RSV genotypes in SUDI cases admitted to Tygerberg Hospital in South Africa.

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2. Materials and Methods

2.1. Ethical Considerations

This study was approved by the Health Research Ethics Committee (HREC) of Stellenbosch University, Cape Town, South Africa (HREC Registration number: N12/02/007).

2.2. Sample Collection and Analysis

During autopsy, flocculated FLOQSwabs™ (Copan Flock technologies, Italy) samples were collected from the trachea and left lower lobes of the lungs of SUDI cases between 2015 and 2019 and screened with the Seegene Allplex™ RV Essential multiplex real-time RT-PCR assay (Seegene, South Korea) as per manufacturer's instructions for a panel of respiratory viruses. All samples positive for HRV or RSV were included in this study.

Samples underwent automated RNA extraction using Microlab STAR Hamilton (Hamilton Company, USA) with the NucleoMag Pathogen Kit (Macherey-Nagel, Germany) as per the manufacturer's guidelines. A one-step, nested RT-PCR assay was optimised for specific gene amplification of HRV and RSV.

2.3. Sequencing and Phylogenetic Analysis

Primers for genotyping and sequencing were selected from the literature for HRV [22,62,63] and manufactured by Integrated DNA Technologies (USA). Detailed methodolocy is attached as Supplementary Data.

Phylogenetic analysis was performed by assembling patient consensus sequences with De Novo Assemble tool of forward and reverse sequences in Geneious Prime® 2021.1.1. The Basic Local Alignment Search Tool (BLAST) within Geneious Prime was used to identify reference sequences for each nucleotide sequence. For HRV, Coxsackie virus (M16560) and Poliovirus 1 (V01149), and for RSV, Bovine orthopneumovirus (AF092942), listed by the International Committee on Taxonomy of Viruses website, were selected as outgroups for phylogenetic analysis. All files were uploaded to Multiple Alignment using Fast Fourier Transformation version 7 software for multiple sequence alignment.

The HRV phylogenetic tree was constructed using the Maximum Likelihood Method in Randomised Axelerated Maximum Likelihood (RAxML) program with 1000 bootstrap replicates with values over 70% shown. The General Time Reversible substitution model with gamma distributed rate variation and invariant sites (GTR + G + I). The tree was rooted with Coxsackievirus B1 (M16560) and Poliovirus 1 (V01149), and represent the outgroups in this phylogenetic analysis, as both viruses are closely related to the in-group (HRV) as per the ICTV website. Blue shading indicates HRV-A species, purple shading indicated HRV-B species, and pink shading indicates HRV-C species. The Samples successfully genotyped of each species are shaded in darker colour and are labelled with the respective genotype following the tip label, where appropriate.

The RSV phylogenetic tree was constructed using the Maximum Likelihood Method in Randomised Axelerated Maximum Likelihood program with 1000 bootstrap replicates with values over 70% shown. The Hasegawa-Kishino-Yano model with gamma distributed rates (HKY + G) was used. The tree was rooted with Bovine orthopneumovirus (AF092942), and represents the outgroup selected for phylogenetic analysis. Blue shading indicates RSA-A genotype and purple shading indicated RSV-B genotype. The Samples successfully subtyped of each genotype are shaded in darker colour and are labelled with the respective subtyped following the tip label, where appropriate.

3. Results

Previous prospective viral screening during 2015/2016 and 2018/2019 produced 106 positive HRV and 56 postive RSV samples. Since different PRC kits were utilised during previous studies and in order to obtain Ct values, all positive samples were retrieved and retested with the Seegene

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Allplex™ RV Essential multiplex real-time RT-PCR assay. Samples with Ct values below 40 for HRV and 30 for RSV were included in manual PCR.

Of these, 64/106 (60%) and 15/56 (27%) were positive for HRV and RSV respectively. Ct values were below 40 for 60 HRV-positive samples and below 30 for 10 RSV-positive samples. The remaining four HRV-positive and five RSV-positive samples were not included in the manual PCR, because the Ct values were outside the RSV manual PCR amplification limits. Two of the selected RSV-positive samples produced Ct values of 29.30 and 27.99, which were higher than the detection limit previously determined by the RSV manual PCR assay, but these samples were included, and amplification was successful.

PCR products were obtained from a total of 60 HRV and 10 RSV positive samples which successfully amplified, of which 43 HRV and all 10 RSV samples were successfully sequenced.

HRV samples produced three distinct species , HRV-A (n=28), followed by -C (n=11), and -B (n=4). In total, eight HRV-A (A28, A80, A10, A82, A43, A56, A11, and A56), one -B (B84), and seven -C (C22, C19, C24, C35, C29, C38, and C2) genotypes were identified. HRV-A was detected in all seasons, while -C was detected in the cooler seasons (autumn and winter), and -B was only detected in winter.

RSV-A and RSV-B were confirmed in five samples each. None of the RSV-A sequences were assigned as ON1, and three samples were assigned BA9 after phylogenetic analysis. RSV-A was detected in samples from all seasons, except summer, while RSV-B was detected throughout the year. Some samples from the same SUDI case clustered together and had a bootstrap value of 100 (HRV samples 4A and 10B and 1 and 7, and RSV samples 5 and 9).

Phylogenetic trees for HRV and RSV are presented as Figures 1 and 2.

All RSV-A deduced amino acid sequences were aligned and compared to prototype ON1 strain (Accession number: JN257693; ON67-1210A), which is characterised by a nucleotide insertion of a 72-nucleotide duplication in the second hypervariable region of the RSV G-gene, otherwise a 23 amino acid duplication (QEETLHSTTSEGYLSPSQVYTTS) beginning at amino acid 261 to 283 and 285 to 307. None of the RSV-A samples presented with this duplication or matching N-glycosylation sites.

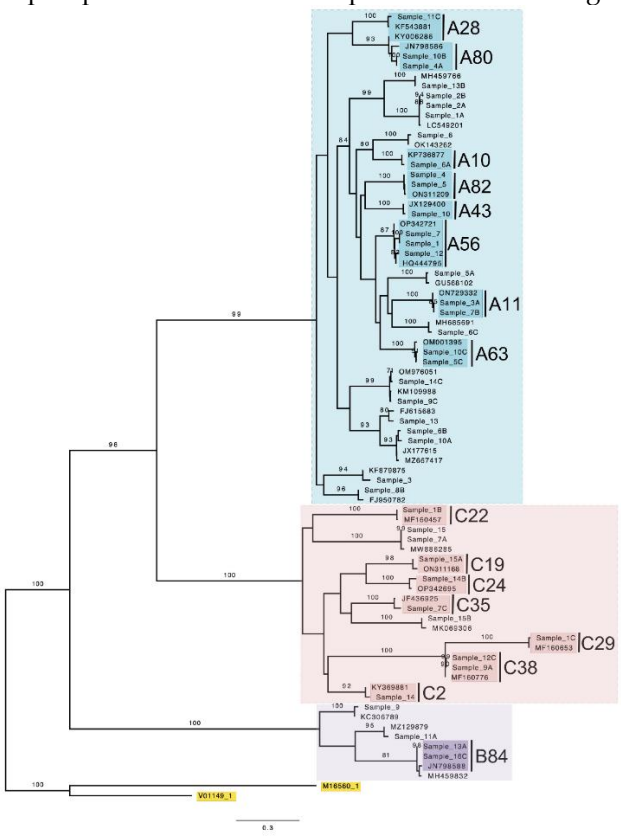

Figure 1. Phylogeny of HRV 5′UTR and capsid coding region sequences of samples from SUDI cases and reference sequences obtained from NCBI BLAST.

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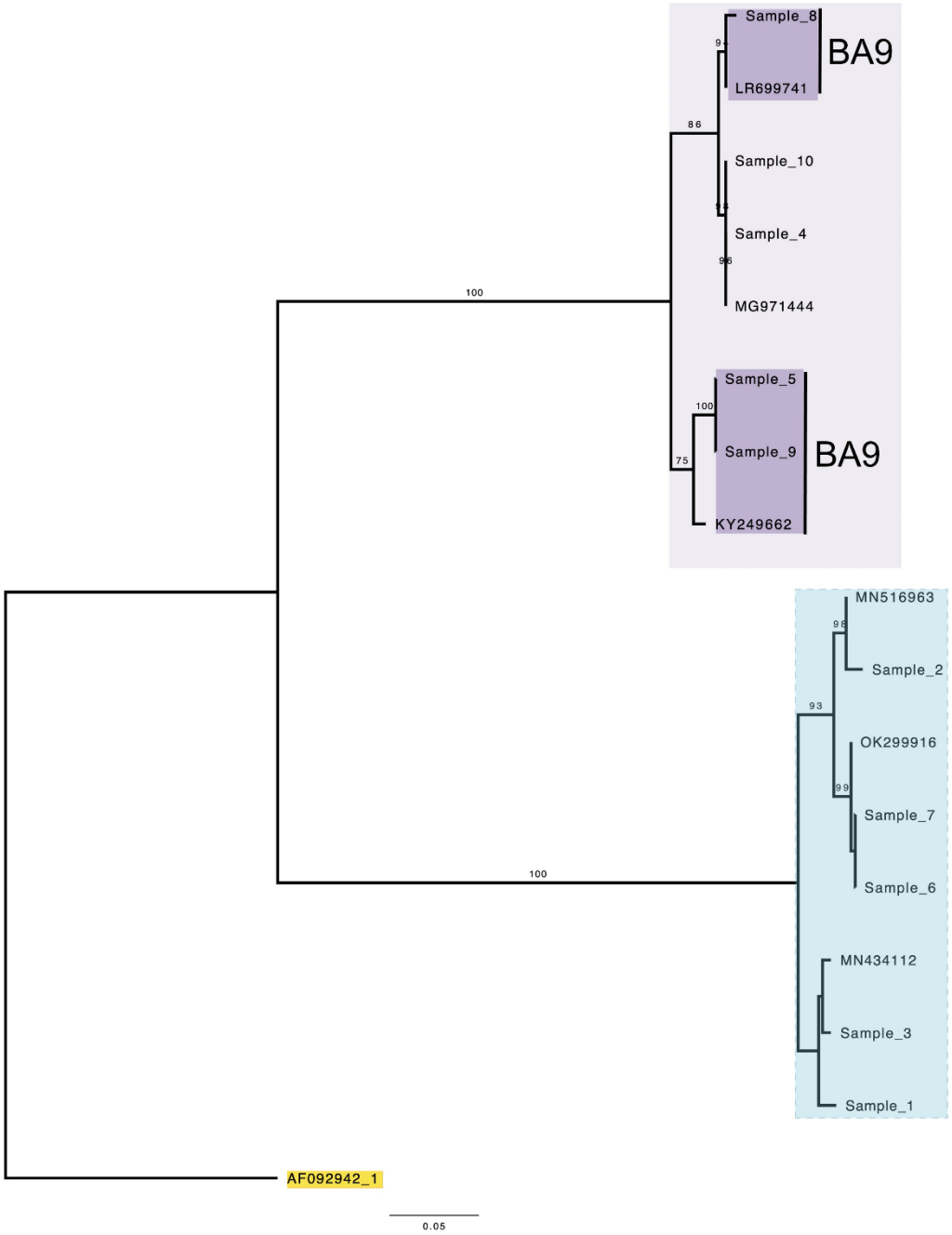

Figure 2. Phylogeny of RSV G-gene sequences of samples from SUDI cases and reference sequences obtained from NCBI BLAST.

BA9 genotypes display a characteristic 60 nucleotide duplication, otherwise a duplication of 20 amino acids (TERDTSTSQSTVLDTTTSKH) beginning at position 240 to 259 and 260 to 279 of the RSV G-gene. Though sample 8 was closely related to LR699741—genotype BA9—and Samples 5 and 9 diverge from KY249662—also genotype BA9—as depicted in phylogenetic analysis following amino acid alignment of all RSV-B sequences obtained in this study to sequences of prototype BA1 (Accession number: AY333364; BA/4128/99B) and the inclusion of a BA9 subtype (Accession number: DQ227395; BA/100/04), only samples 9, and 5 displayed the insertion of 20 amino acid duplication, and amino acid substitutions K218T, L223P, K225I, V251T, D253N, S267P, H287Y, S288L, and E292G. All samples showed S247P, and four showed L286P substitutions. Additionally, outside of the second hypervariable region, all samples had I200T substitution. Two NST N-Glycosylation sites are reported within the RSV-B second hypervariable region of the G- gene, at codons 296 to 298 and 310

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to 312. N-Glycosylation sites were determined by scores over 0.5. Sample 9 retained both N-Glycosylation sites, at codon 296 (0.52) and 310 (N-Glyc: 0.57). Sample 5 also retained both N-Glycosylation sites at codons 296 (0.51) and 310 (0.56). Sample 8 matched the first glycosylation site (N-Glyc: 0.59) with N296L substitution.

Deduced amino acid alignment of RSV G-gene of RSV-A and RSV-B samples are presented as Figures 3 and 4. RSV-A sample sequences were aligned and are shown relative to the sequence of RSV ON1 (Accession number: JN257693; ON67-1210A), while RSV-B samples sequences were aligned and are shown relative to sequence of prototype RSV-BA1 (Accession number: AY333364; BA/4128/99B) and BA9 (Accession number: DQ227395; BA/100/04).

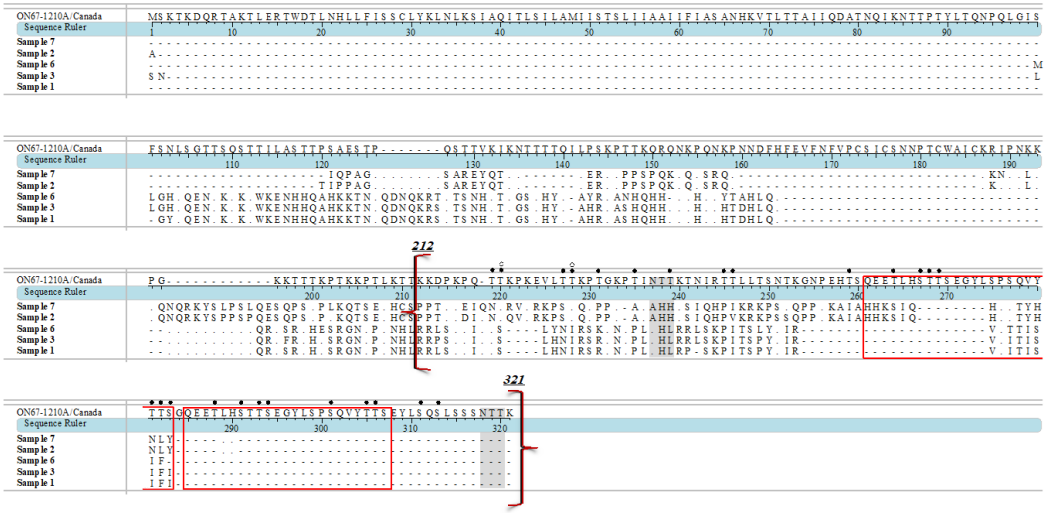

Figure 3. Deduced amino acid alignment of RSV G-gene of RSV-A samples. Sample sequences were aligned and are shown relative to the sequence of RSV ON1 (Accession number: JN257693; ON67-1210A). The amino acid numbers of the sequence ruler (in blue) within the orange braces correspond to the positions of 212 and 321 of the second hypervariable region of the G-gene of the RSV ON1 sequence. The red outlined rectangular boxes depict the 23 amino acid duplication (QEETLHSTTSEGYLSPSQVYTTS) of the 24 amino acid insertion, characteristic to RSV ON1. Dots represent matching amino acid to reference sequence (RSV ON1). Dashes represent missing amino acid. Grey shading depicts predicted N-glycosylation sites.

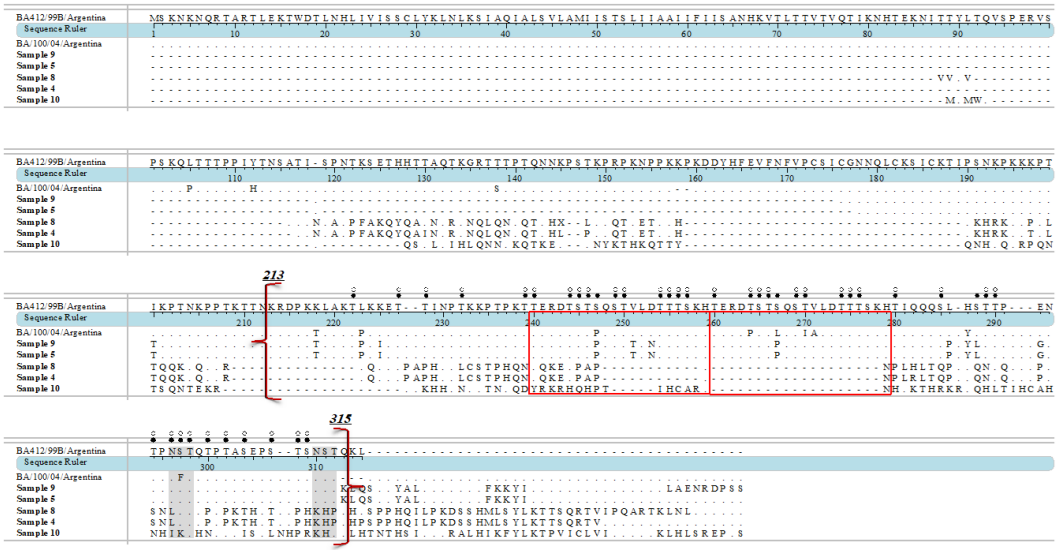

Figure 4. Deduced amino acid alignments of RSV G-gene sequences of RSV-B sample. Samples sequences were aligned and are shown relative to sequence of prototype RSV-BA1 (Accession number: AY333364; BA/4128/99B) and BA9 (Accession number: DQ227395; BA/100/04). The amino acid numbers of the sequence ruler (in blue) within the orange braces correspond to the positions of

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213 and 315 of the second hypervariable region of the G-gene of the RSV-BA1 sequence. The red outlined rectangular boxes depict the 20 amino acid duplication (TERDTSTSQSTVLDTTTSKH) insertion, characteristic to RSV-BA. Dots represent matching amino acid to reference sequence (RSV-BA1). Dashes represent missing amino acid. Grey shading depicts predicted N-glycosylation sites.

4. Discussion

This study used Sanger sequencing to describe the different HRV and RSV genotypes in retrospective respiratory tract samples collected from SUDI cases admitted to the Tygerberg Medico-legal Mortuary in Cape Town between 2015 and 2019. The literature describes genotypic characterisation of HRV-A, -B, and -C, as well as RSV-A and -B in infants and children admitted for hospital care in South Africa [4,44,64]; however, this is the first study to genotype respiratory viruses in SUDI cases at Tygerberg Medico-legal Mortuary, and South Africa.

4.1. Human Rhinovirus

HRV genotypes HRV-A10, -A11, A28, -A43, -A56, -A63 and -A80 and -A82 were all confirmed in SUDI samples from this study. HRV-B84, -C2, -C19,- C22, -C24, -C29, -C35 and -C38 were also detected.

The results agree with the literature that describes HRV-A10, HRV-A11 and HRV-A28 in infants with acute respiratory illness [65]. Although HRV-A10 is less frequently reported than HRV-A28, HRV-A56, and HRV-A80, it is commonly detected in infants presenting with respiratory disease in other sub-Saharan Africa countries [25,66,67]. Furthermore HRV-A80 was also detected in Tunisia and in China in hospitalised infants presenting with severe and acute respiratory tract infection during the same time period as the current study [68,69]. Certain HRV genotypes, such as HRV-A82 and HRV-A43, are not exclusively detected in infants, but also in adolescents and adults, who may transmit the virus to infants when in close contact [70], such as bed-sharing. Since most infants in this study had reported a history of bed-sharing where the infant is in close contact with other individuals for prolonged periods of time. If such individuals present with HRV or any other viral infection, it may increase the risk of viral transmission.

The samples from this study were collected before the COVID-19 pandemic and were not tested for severe acute respiratory syndome-2 (SARS-CoV-2), but HRV-A82 was commonly reported in children and adolescents presenting with respiratory disease during the COVID-19 pandemic [71,72]. Genotype HRV-A43 has also been described to reinfect children, adults and the elderly with acute respiratory infections and subsequent prolonged viral shedding [73–75]. It is therefore postulated that HRV may either play a contributory role in infant death, or the detection during post-mortem analysis may not infer causation of SUDI, but rather transmission from close-contact with other infected individuals. This hypothesis is supported by a study from the Salt River Mortuary, Cape Town, that has identified the consequences of overcrowded living conditions and infection-related causes of death in SUDI cases [76]. Finally, genotypes HRV-A28, -A56 and -A80 were among the most frequently reported in clinical studies reporting on HRV diversity Asia and Africa between 2013 and 2021 [16]. Only Genotype HRV-A63 has been detected in another SUDI case in the literature [77]. This may be attributed to the lack of SUDI investigations assessing viral genotypes in forensic settings in South Africa and globally.

Only one HRV-B genotype (-B84) was detected in this study, which agrees with the reported trend by molecular epidemiology research in Tanzania, Nigeria, Kenya, Mozambique and South Africa [25,67,78–80]. HRV-B84 was one of the top 25 genotypes reported by seven studies from Africa [16]. This species is not associated with severe disease and parents might therefore not seek medical attention and laboratory testing, suggesting that the true burden may be underrepresented [78]. The low detection of HRV-B in this study might therefore also not be a true representation of its diversity in SUDI cases in this population.

HRV-C is commonly detected in children with respiratory disease [4]. HRV-C2 and -C38 were among the top three identified in Kenya from 2007 to 2018 in children presenting with severe acute respiratory infection, and -C2 was previously detected in Amsterdam between 2007 and 2012 [16,81].

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Genotype -C22 was also detected in children in Nigeria and Tanzania suspected of respiratory illness, and hospitalised children in Sweden [16,67,78].

The burden of HRV infection in infants living in Africa and hospitalised in the Cape Town Metropole has previously been well described [4,42,67]. The findings of this study suggest that certain HRV genotypes were present in the SUDI cases at the time of death, but the studies to date were not designed to assess a causal relationship between PCR-results and infant death. Since quantification of viral loads in post-mortem samples proves to be challenging and unreliable due to decrased viral viability post-mortem, the mechanisms by which infection may lead to death, especially in infants, may be supplemented by biomarkers of infection and sepsis, and genotype identification in the presence of clinical features of respiratory disease to further elucidate the role of HRV genotypes in infant death [15,82,83].

4.2. Respiratory Syncytial Virus

RSV-A remains the most common and diverse group in South Africa, China and Saudi Arabia [64,84–87]. Despite a high prevalence of ON1 in South Africa between 2015 and 2017, none of the RSV-A samples from this study closely related to ON1.

RSV-B has been reported to predominate in eight other countries between 2017 and 2018, possibly due to virulence and circulation of specific strains at the time [84] and was first detected in South Africa in 2006 during a nosocomial outbreak [37,85,88,89]. RSV-BA9 was first identified in Japan during the 2006 and 2007 epidemic season and is commonly detected in infants presenting with LRTI [84,90–93].

In 2021, RSV-BA9 was detected in an outbreak in China in hospitalised neonates presenting with respiratory failure [94]. One study sample closely related to RSV-BA9 first isolated from a male infant in Spain as per the GenBank record. RSV-BA9 has previously been detected in infants and other regions of South Africa, however recently published amino acid substitutions are limited [37,44,64,87,95]. The amino acid substitutions noted in RSV-BA9 this study were characteristics of RSV-BA9 strains reported in the literature [92,96,97]. However, substitutions I281T and P291L differentiating between sub-genotypes RSV-BA9a and RSV-BA9b were not noted in this study [93], although two samples had commonly reported RSV-BA9 K218T, L233P, and H287Y substitutions [98–100]. S247P, also a common substitution of RSV-BA9 strains, was present in all samples, while L223P was only noted in two sampels. S247P substitution has been reported from Taiwan, Panama, Thailand, and other countries [92,100–103]. Furthermore, 247P with L223P and H287Y substitutions detected in these two samples were noted in strains identified in Central African Republic between 2015 and 2018 [104].

In the RSV-B duplication site, D253N amino acid substitution was reported in RSV seasonal epidemics in Shanghai, China and multiple studies in India from children presenting with respiratory disease from children presenting with LRTI [91,105,106]. Additionally, the substitution may lead to a possible third N-glycosylation site [107], but this was not present in the current study. Interestingly, E292G substitution in the above-mentioned two samples were reported in RSV-BA10 detected in children admitted for acute respiratory infection in Vietnam and is confirmed by other publications in RSV-BA10 genotypes [108,109]. One additional sample did not have any amino acid duplication insertion, but it had T254I substitution, which was reported as a characteristic of RSV-BA9 [92]. The literature suggsts that this substitution in combination with mutation K314R, might have been involved in the BA9 predominance in 2015/2016 in Taiwan [100]. When RSV-B sequences from this study were compared to DQ227395 (BA/100/04), the A269V substitution was confirmed, which is in agreement with reported RSV strains in children in China during the COVID-19 pandemic [110].

4.3. Strengths and Limitations

This was the first study in South Africa to investigate specific viral genotypes of the most common respiratory viruses in SUDI cases and this forms the foundation for continued research to further identify and describe distribution patterns, viral diversity, severity of disease, and possible

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associations between the identified risk factors and genotypes for the most prevalent respiratory viruses in infants and children.

The main limitations of this study were varying success in retrieval, and amplification of previously identified PCR-positive HRV and/or RSV samples, confirmatory PCR retesting and successful sequencing of amplified PCR products.

Limited resources and research capacity at the Tygerberg Medico-legal Mortuary resulted in intermittend sample collection periods, challenging assessment of the distribution and true seasonality patterns of the viruses commonly found in SUDI cases. Financial constraints also only allowed for Sanger sequencing to be used, but future studies should employ more second- or third-generation sequencing platforms, such as Illumina or Oxford Nanopore Technology.

The most important limitation is the fact that viral viability decreases significantly with increasing post-mortem interval, which is typically between 3 and 10 days at the Tygerberg Medico-legal Mortuary and this does not allow for quantification of viruses or assessing temporality in formulating the cause of death.

5. Conclusions

This study described specific viral genotypes in post-mortem samples collected from SUDI cases in Cape Town, South Africa and was able to confirm the presence of specific HRV and RSV genotypes with known morbidity in infants and children from the literature during the same time period than the current study. Due to ethical constraints and the invasive nature of sample collection, it is not possible to include a post-mortem infant control group and results were compared to published data of infants and children with respiratory disease where HRV and/or RSV were confirmed. Rapidly decreasing viral viability after death makes quantification difficult and the direct association between viruses and active infection cannot be assessed. Other aspects, such as macro- and microscopic investigation of tissue, need to be included and considered in the formulation of the cause of death of these infants.

Future studies should therefore include prospective investigation of other markers of infection or sepsis, such as cytokines and acute phase proteins. Known sociodemographic risk factors should also be assessed and considered in the formulation of the cause of death in SUDI cases.

Author Contributions: Conceptualization, Protocol and Methodology, Institutional Review Application and Approval, Funding acquisition, Resources, Provided training and supervision, Project administration, Visualisation, Overseeing laboratory work, results, data analysis and interpretation, Writing – review, editing and submission of manuscript – C.D.B.; Methodology, Data analysis and interpretation, writing—original draft preparation, H.D.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Stellenbosch University Health Research Ethics Committee [HREC] (Registration Number N12/02/007, first registration 07/03/2012, with annual renewal).

Informed Consent Statement: HREC approved a waiver of consent under Protocol N12/02/007. Consent is provided for by Section 3(a) of the Inquests Act (58 of 1959) and the Criminal Procedure Act (1977) as all SUDI cases must be subjected to a full post-mortem investigation according to the Act (58 of 1959), as it is regarded as unnatural deaths, and samples are collected to ascertain the cause(s) of death.

Data Availability Statement: No publicly archived datasets were analyzed or generated during the study. Datasets are available on request from the authors.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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