

Clinical and biological consequences of respiratory syncytial virus genetic diversity

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Abstract: Respiratory syncytial virus (RSV) is one of the most common etiological agents of global acute respiratory tract infections with a disproportionate burden among infants, individuals over the age of 65, and immunocompromised populations. The two major subtypes of RSV (A and B) co-circulate with a predominance of either group during different epidemic seasons, with frequently emerging genotypes due to RSV's high genetic variability. Global surveillance systems have improved our understanding of seasonality, disease burden, and genomic evolution of RSV through genotyping by sequencing of attachment (G) glycoprotein. However, the integration of these systems into international infrastructures is in its infancy, resulting in a relatively low number (~2200) of publicly available RSV genomes. These limitations in surveillance hinder our ability to contextualize RSV evolution past current canonical attachment glycoprotein (G)-oriented understanding, thus resulting in gaps in understanding of how genetic diversity can play a role in clinical outcome, therapeutic efficacy, and the host immune response. Furthermore, utilizing emerging RSV genotype information from surveillance and testing the impact of viral evolution using molecular techniques allows us to establish causation between the clinical and biological consequences of arising genotypes, which subsequently aids in informed vaccine design and future vaccination strategy. In this review, we aim to discuss the findings from current molecular surveillance efforts and the gaps in knowledge surrounding the consequence of RSV genetic diversity on disease severity, therapeutic efficacy, and RSV–host interactions.

Keywords: clinical outcomes, genetic diversity, genotype, molecular surveillance, respiratory syncytial virus, therapeutic design, whole-genome sequencing

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Introduction

Respiratory syncytial virus (RSV) is one of the leading causes of acute respiratory tract infections (ARTIs) worldwide, with most of the disease burden occurring in pediatric,¹ elderly,² and immunocompromised³ populations. There are estimated to be over 33 million cases of RSV infection every year, with many children experiencing their first case of RSV by the age of 2.⁴ While most cases cause only mild to moderate symptoms, susceptible populations may experience more severe clinical manifestations, including pneumonia and respiratory failure. In addition, infants can experience serial infections over the course of their lives and into adulthood

as a result of waning antibody response to RSV.^{5–7} With no available vaccine and a single monoclonal antibody treatment (palivizumab) that is costly and restricted to use in high-risk infants, there is an overwhelming need to identify new antibody-based and antiviral therapeutics for the treatment of severe disease.⁸

Despite its global significance, the disease burden of RSV has been difficult to quantify due in large part to a lack of surveillance and reporting, particularly in resource-restricted countries.⁹ Even less is understood about how the ongoing evolution of RSV is impacting the epidemiology of the virus, the clinical manifestation of disease, and

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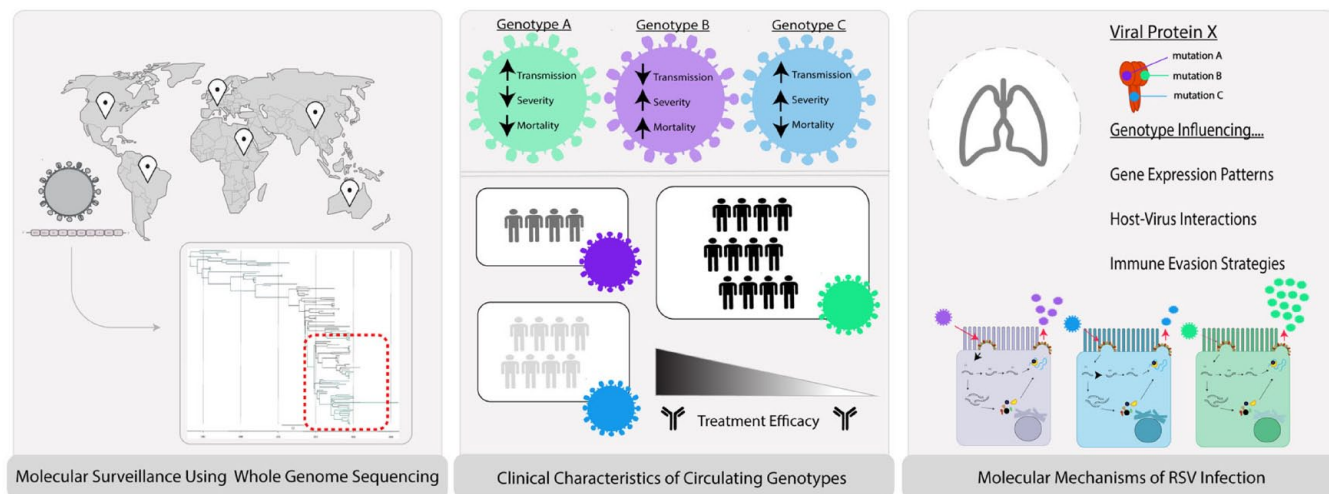


Figure 1. Representation of molecular surveillance pipeline. Sample collection and preparation of clinical samples from RSV positive populations from global locations in surveillance systems give rise to the ability to track RSV seasonality and rise in emerging genotypes. When performing phylogenetic analysis of the RSV whole-genome sequences, certain circulating genotypes may present differential clinical characteristics, such as clinical severity, transmission, and mortality. These genotypes (depicted in green, purple, and blue) may have varying infectivity, resulting in varying population size with a wide range of treatment efficacy. When observing the molecular underpinnings of each genotype, mutations within RSV open reading frames (ORFs) unique to defined genotypes may influence gene expression, host–pathogen interactions, and immune evasion strategies.

therapeutic efficacy. There are currently two major antigenic groups that are known to circulate in the human population, RSV-A and RSV-B, but studies comparing the clinical severity of disease between these groups have failed to reach consensus.¹⁰ Variants within each antigenic group have been described through RSV genotyping and attachment glycoprotein G sequencing, but there is a lack of available sequence information to clearly define subtypes and determine their impact on disease.^{11–13} As a point of comparison, there are currently over 9.6 million whole-genome sequences of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) available in the Global Initiative on Sharing All Influenza Data (GISAID) database since 2020, but only about 2200 RSV whole-genome sequences are currently available despite RSV’s identification in 1955. To further complicate these assessments, the public health measures implemented to mitigate the coronavirus disease 2019 (COVID-19) pandemic have had a suppressive effect on RSV cases, decreasing overall case counts and disrupting the previously observed seasonal patterns.^{14,15} Predictive models suggest a resurgence of RSV once COVID-19 mitigation measures lapse, but it is unclear what new epidemiological patterns and genotypes may emerge.¹⁴ In an international longitudinal observation study, rebound RSV

cases were identified across 11 countries after easing nonpharmaceutical interventions (NPIs) such as re-opening academic institutions, showcasing the need for molecular surveillance in real-time to track potential increases in potential population susceptibility.¹⁶

In this review, we will evaluate and discuss what is known and what remains to be discovered about the molecular epidemiology of RSV and the clinical and biological significance of RSV genetic diversity. We will address this topic in three sections: (1) RSV global surveillance and viral evolution, (2) the impact of RSV genetic diversity on clinical outcomes and therapeutics, and (3) the functional consequences of these genetic changes on RSV biology (Figure 1). Finally, we discuss new tools, techniques, and approaches that can inform future research on RSV toward development of safe, effective, and broadly applicable therapeutics.

Methods

G and WGS sequences from GenBank

To identify the number of RSV genome sequences and G sequences publicly available and deposited, we downloaded both the accession numbers

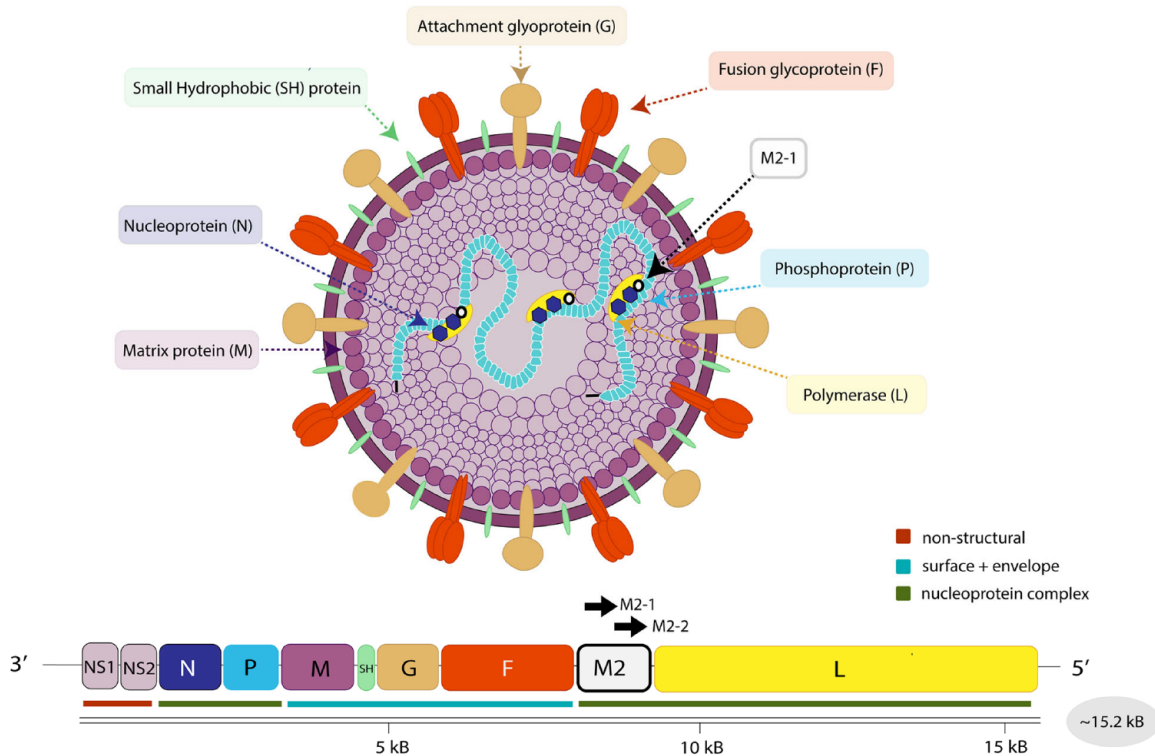


Figure 2. Schematic of RSV Virion Structure and Genome. The RSV RNA genome consists of 10 genes of 15.2 kb length that encode 11 proteins. These proteins can be categorized into ribonucleocapsid proteins [phosphoprotein (P), nucleoprotein (N), RNA polymerase (L), M2-1, and M2-2], nonstructural proteins (NS1 and NS2), three surface proteins [small hydrophobic protein (SH) attachment glycoprotein (G), and fusion protein (F)], and matrix protein (M) which surrounds the envelope and nucleocapsid.

and corresponding collection_date information from NCBI GenBank during April 2020. To identify RSV G sequences, the following search terms were used:

((('Human orthopneumovirus'[Organism] OR human respiratory syncytial virus[All Fields] AND attachment[All Fields] AND glycoprotein[All Fields]) AND 'Human orthopneumovirus'[porgn] NOT mutant[All Fields] NOT attenuated[All Fields] NOT chimeric[All Fields] NOT recombinant[All Fields] NOT unverified [All Fields] NOT pangolin[All Fields] AND (viruses[filter] AND [PROP] AND [filter] AND [filter])).

Of 2203 of these obtained genome sequences, 2172 sequences had collection date information between 2000 and 2022; the number of sequences per year were plotted. To identify complete genome RSV sequences, the following search terms were used:

(((((('Respiratory syncytial virus'[Organism] OR ('Respiratory syncytial virus'[Organism] OR Respiratory Syncytial Virus[All Fields])) OR 'Respiratory syncytial virus'[Organism]) NOT attenuated[All Fields]) NOT mutant[All Fields]) NOT unverified[All Fields]) NOT chimeric[All Fields]) AND ('Human orthopneumovirus'[Organism] OR ('Human orthopneumovirus'[Organism] OR Human orthopneumovirus[All Fields])) AND complete[All Fields] AND (viruses[filter] AND biomol_genomic[PROP] AND is_nucore[filter] AND ('14000'[SLEN]: '17000'[SLEN])) NOT pangolin[All Fields] NOT unverified[All Fields]) AND 'Human orthopneumovirus'[porgn] AND (viruses[filter] AND biomol_genomic[PROP] AND ddbj_emb_l_genbank[filter] AND is_nucore[filter] AND ('15000'[SLEN]: '15300'[SLEN]))).

Of the 19,214 sequences obtained, 10,890 sequences had collection date information

between 2000 and 2022; the number of sequences per year were plotted.

RSV lifecycle

RSV was first isolated in 1957 at Johns Hopkins University in Baltimore, Maryland, from a child with bronchopneumonia and other respiratory tract infection complications.¹⁷ RSV is an enveloped virus with a single-stranded negative-sense ribonucleic acid (RNA) genome recently reclassified in 2016 from subfamily *Paramyxoviridae* to the *Pneumoviridae* family within the *Orthopneumovirus* genus.¹⁸ The RSV genome is approximately 15.2 kb in length and contains 10 open reading frames (ORFs) that encode nine structural and two nonstructural proteins important for pathogenicity and immune evasion (Figure 2). The RSV envelope is comprised of three surface proteins (G, F, and SH) that orchestrate binding, fusion, and entry into ciliated bronchial epithelial cells¹⁹ as well as several immune cell subsets (i.e. dendritic cells,²⁰ regulatory B cells,²¹ T cells²²). The heavily glycosylated G protein is responsible for host cell attachment through binding to one or more surface receptors and attachment factors. It is additionally secreted in a soluble form as a means to mediate immune evasion through antibody binding as a decoy.²³ After virion attachment, the trimeric transmembrane F protein then mediates the fusion of RSV viral envelope and the host membrane following a proteolytically enabled conformational change.²⁴ RSV entry is thought to be pH-independent with direct entry occurring at the plasma membrane, though macropinocytosis has also been postulated as an entry mechanism.²⁵ Although not fully characterized, the small hydrophobic (SH) protein has been proposed to function as a pentameric ion channel²⁶ viroporin with both anti-apoptotic and anti-inflammatory effects through suppression of tumor necrosis factor (TNF) signaling.²⁷ Matrix (M) protein coats the inner envelope surface, are critical for viral assembly, and are thought to inhibit host cell transcription.²⁸ Inside the viral envelope, phosphoprotein (P) tethers the RNA-dependent RNA polymerase (L) to the nucleoprotein (N) RNA complex, forming the core machinery necessary for viral transcription and replication. The RNA-dependent RNA polymerase (RdRp) complex is responsible for both viral mRNA production as well as genome replication.

After fusion, this complex is released into the cytoplasm where replication, transcription, and viral assembly occur.^{29,30} Overlapping ORFs in the M2 gene encode two separate factors, M2-1 and M2-2, involved in transcription and RNA genome replication, respectively.^{31,32} The nonstructural proteins NS-1 and NS-2 play important roles in suppressing innate immune induction, interferon-stimulated gene (ISG) expression,^{33,34} and dendritic cell maturation.³⁵ Ultimately, progeny viruses are assembled and released starting at 10–12 hours post-infection (hpi) with peak viral release around 20 hpi.³⁶

RSV genotypes

The two antigenic groups of RSV, RSV-A and RSV-B, were originally distinguished through the use of monoclonal antibodies^{37,38} and complementary deoxyribonucleic acid (cDNA) probes³⁹ on several circulating strains in 1985. The advent of deep sequencing approaches subsequently allowed for the broader characterization of RSV diversity.⁴⁰ Within these antigenic groups, there have been 37 identified genotypes documented for RSV-B⁴¹ and 13 distinct genotypes documented for RSV-A.^{42,43} The evolutionary rates for both subtypes differ from one another, with RSV-A proposed to have a lower rate at 1.48×10^{-3} nucleotide substitutions/site/year as opposed to RSV-B at 1.92×10^{-3} substitutions/site/year.⁴⁴ However, in other studies comparing diversity within antigenic groups, RSV-A was shown to have higher consensus diversity and lower intra-host variation as opposed to RSV-B.⁴⁵ Across the genome, the highest nucleotide substitution rates occur in the attachment glycoprotein (G), which is subject to substantial evolutionary pressure both as a determinant of viral transmission and as a primary target of the humoral immune response.⁴⁶ These selective pressures have been thought to drive the convergent evolution of short duplication events in G, which have been observed in both RSV-A (genotype ON1) and RSV-B (genotype BA1). Given the strong diversity in this region, conventional methods to genotype RSV have relied on targeted sequencing of the G ORF.^{13,47} Generally, most current molecular epidemiology efforts rely on targeted sequencing of G, and specifically of the second hypervariable region, for phylogenetic analysis and classification of viral genotypes.⁴⁸

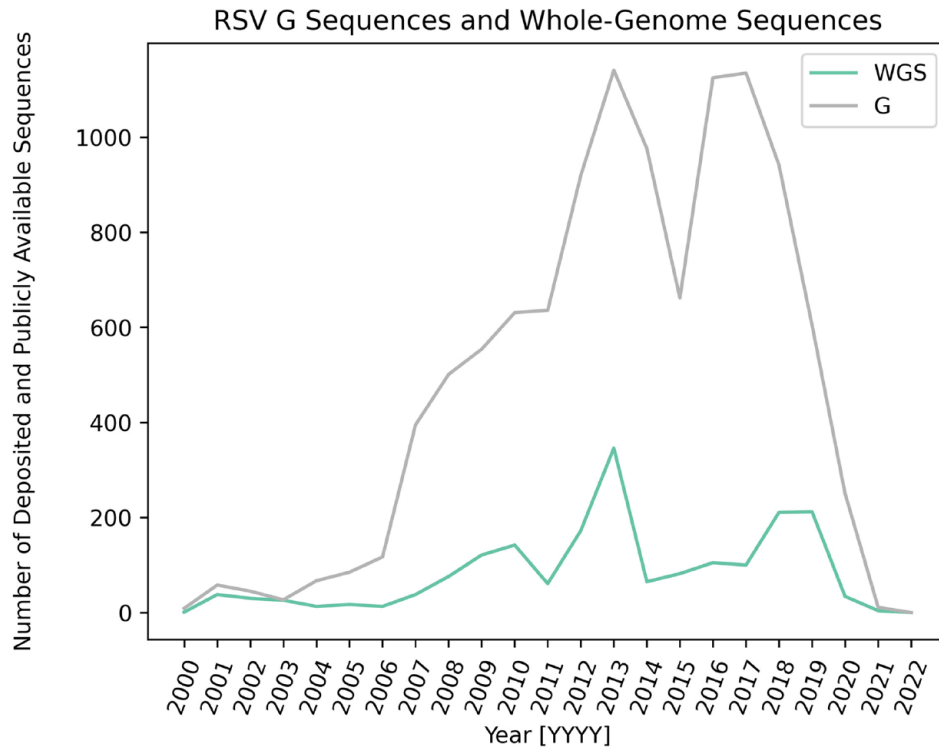


Figure 3. RSV sequences deposited in National Institute of Health (NIH) sequence database GenBank over time. Plot of the number of publicly available sequences deposited in GenBank from 2000 to 2022. RSV-G sequences are depicted in gray and WGS data are depicted in green.

Although RSV cases typically follow a seasonal pattern with the peak of infections occurring from late December to late February in the Northern Hemisphere, RSV seasonality is variable in tropical regions⁴⁹ and near the equator.⁵⁰ Environmental factors such as humidity and temperature were not found to be causal of RSV seasonality, and subtropical climates displayed continuous RSV cases throughout the year.⁵¹ Although there can be a heterogeneous population of genotypes co-circulating in populations during one RSV season, it has been observed that a single genotype dominates a geographic area with subtype switching occurring year to year (RSV-A followed by RSV-B and back). For example, RSV epidemics in Kilifi, Kenya, displayed sequential replacement of local circulating genotypes over 11 recurrent epidemics.⁵² Similar phenomena had occurred with GA2, GA5, and GA7 genotypes (RSV-A) being outcompeted by NA1, NA2, and ON1 genotypes (RSV-A), which contain a 72 nucleotide duplication in the C-terminal region of the G gene, in Malaysia,⁵³ Canada,⁵⁴ India,⁵⁵

South Korea,⁵⁶ and China.⁵⁷ While efforts are underway to explore these trends through increased genomic surveillance, the lack of a standardized nomenclature for genotypes and limited whole-genome sequencing (WGS) data have complicated this effort.

Currently, there are approximately 13 different documented methods³⁸ to define RSV genotypes,⁴³ illustrating a broad lack of agreement on how to classify viral variants. This remains a major problem in the field as the adoption of standard nomenclature for genotype designations is crucial for effective surveillance, clinical risk analysis, and public health communication. For example, the rapid adoption of standard nomenclature for describing SARS-CoV-2 variants assisted scientists and epidemiologists in tracking the virus and in communicating risks with the public.⁵⁸ To address this issue in the RSV field, Goya *et al.* worked to better define RSV clades *via* G-ectodomains. The authors proposed a classification system based on average genetic distance

among and within RSV genotypes with a cut-off value based on the genetic distance of previously defined genotypes. Resolution of phylogenetic trees is increased when using whole-genome sequencing, but the G-ectodomain was found to be sufficient to calculate genotype designations. This method resulted in a reduction of recognized genotypes by almost threefold while additionally allowing for sub-genotypes and lineage designation.⁴³ Likewise, efforts to standardize nomenclature when reporting RSV sequences to improve data accessibility and analysis have been proposed, akin to the standards used for influenza virus sequencing and reporting.⁵⁹

While phylogenetic analysis has traditionally relied on G, several WGS strategies have since been developed to understand viral diversity in other ORFs. These same approaches are currently being explored to refine genotype and clade designation.^{60,61} There are other genetic ‘hotspots’ spanning the genome in addition to G that justify the need for whole-genome surveillance, including in the 5′ untranslated region (UTR) of SH, in M2, and in NS1. Both RSV-A and RSV-B have high nucleotide diversity in the 5′UTR of SH, potentially signaling selection for SH expression that may influence RSV pathogenesis.^{13,62} In a cohort of RSV+ infants in Vietnam, M2-2 was found to be under positive selection, with an increased ratio of nonsynonymous mutations to synonymous mutations in both RSV-A and RSV-B isolates.⁶³ Some mutational hotspots have also been found to be subtype specific, including sites in NS1 and N in RSV-A with still uncharacterized functionality.⁶³ Understanding the rate of viral evolution specific to these components of RSV is crucial, as some of these ORFs (i.e. M2-2, NS2, and N) contribute to RSV pathogenicity and are the primary target of some vaccine candidates and monoclonal antibodies in phase 1 clinical trials.⁶⁴

Nevertheless, WGS approaches to catalog RSV genomic diversity remain the exception. Approximately 2200 RSV whole-genome sequences have been publicly deposited in GenBank since 1956 (Figure 3), as opposed to over 9.6 million whole-genome sequences of SARS-CoV-2 since 2020. Scientific studies and public health investigations have leveraged these sequences to define the convergent evolution of G gene duplication in RSV genomes and to carry out healthcare worker outbreak investigations.^{65–70} All but one of these studies focus on

infants less than 1 year of age, with a large subset focusing on infants with severe respiratory illness, highlighting the dearth of knowledge available on whole-genome RSV diversity and evolution in adult populations. Overall, advances in WGS creates an optimal opportunity to address ongoing questions surrounding RSV viral evolution and knowledge gaps surrounding under-served populations or under-sequenced RSV genomic regions. However, there are certain technical challenges to conduct molecular surveillance. For example, polymerase chain reaction (PCR) primer mismatches due to the increased diversity in regions of the RSV genome presents another challenge, resulting in unsuccessful amplification of certain ORF fragments of the RSV genome from one season to the next.⁷¹ Consequently, publicly available RSV sequences become critical to then aid in the design of primer schematics as a solution to primer mismatching with the goal of whole-genome coverage.⁷²

RSV global surveillance efforts

Despite its large global disease burden, less is understood about the transmission, seasonality, and pathogenicity of RSV when compared with other pathogenic respiratory viruses. This is in part due to the lack of a widespread surveillance system to track RSV epidemiological information, in contrast to the more developed systems in place for influenza viruses and SARS-CoV-2. To resolve these issues of limited reporting, the Bill & Melinda Gates Foundation provided dedicated funding for RSV surveillance in several low- and middle-income countries (LMICs) to track mortality in infants aged < 6 months through PCR-based diagnostics between 2011 and 2013.⁷³ The resulting data uncovered a staggering RSV disease burden and mortality in these countries, eliciting a broader call for action in Argentina, India, Pakistan, and Zambia.⁷³ Subsequently, several countries began embedding RSV surveillance efforts into pre-existing influenza surveillance systems.⁷⁴ Since then, there have been four major initiatives for increased global surveillance of RSV that have enhanced our understanding of seasonality, trends, and genomic evolution: (1) Global Influenza Surveillance and Response System (GISRS), (2) Global Epidemiology of RSV initiative (GERi), (3) Respiratory Syncytial Virus Consortium in Europe (RESCEU), and (4) International Network for Optimal Resistance Monitoring (INFORM).

The World Health Organization (WHO) adapted the framework of the GISRS into a pilot program for RSV surveillance from 2017 to 2019.⁷⁵ GISRS tracked RSV cases in 14 countries (Argentina, Australia, Brazil, Canada, Chile, Cote d'Ivoire, Egypt, India, Mongolia, Mozambique, Russian Federation, South Africa, Thailand, and the United Kingdom) with the primary goal to establish a better understanding of RSV disease burden, seasonality, and high-risk groups. By using real-time PCR (RT-PCR) molecular diagnostic testing to detect genotype RSV, the study concluded that the seasonality of RSV was found to be dissimilar among participating surveillance locations depending on temperate and tropical climates, although there were regional overlaps with seasonal influenza virus cases. In addition, the GERi – modeled off the Global Influenza B Study (GIBS) – was founded in 2016 with the goal to determine genotype distribution, demographic factors, and environmental factors that influence RSV epidemics using national surveillance systems that provide information on RSV-subtype and clinical data.^{76,77} Additional regional specific efforts have emerged since that time, including RESCEU, which focuses on incidence data from a global prospective study of 10,000 infants to inform the RSV attributable economic burden and establish a national framework and biobank to support future vaccine development.⁷⁸ While these efforts have focused on improved RSV surveillance to better understand disease burden, seasonality, and virological behavior, less emphasis has been placed on genomic surveillance systems and their potential long-term benefits for therapeutic design. To begin to address that gap, the INFORM-RSV study – led by the Respiratory Syncytial Virus Network (ReSViNET) Foundation – has proposed to sequence more than 4000 whole genomes from clinical isolates across 17 countries from 2017 to 2021. These data will be used to track the emergence of new genotypes and polymorphisms in RSV antigenic sites of the F protein that could impede therapeutic design.⁷⁹

While traditional and molecular-based surveillance approaches are critical to improve our understanding of RSV incidence and diversity, much less is understood as to how this diversity influences clinical outcomes. These studies tend to be more regionally constrained due to the need to aggregate and compare in-depth medical

record data. As one example of these efforts, the Infant Susceptibility to Pulmonary Infections and Asthma Following RSV Exposure (INSPIRE) longitudinal birth cohort of healthy infants implemented sequencing efforts in Tennessee from 2014 to 2016 to establish correlation between viral diversity and recurrent wheezing and asthma in healthy infants through WGS and phylogenetic analysis.^{13,80,81} Overall, these initiatives utilized similar infrastructural frameworks to increase RSV surveillance with the goal to address distinct phylogenetic, clinical, or molecular gaps in knowledge. However, all of these studies faced several challenges that highlight critical areas for improvement of RSV surveillance. For example, non-standardized platforms for testing, limited genome sequencing technologies/capabilities, and linkage to influenza surveillance systems that operate in constrained time frames limited some of the takeaways from these studies.

Efforts to perform and understand RSV surveillance were complicated even further by the COVID-19 pandemic. Widespread implementation of public health measures against respiratory viruses in 2020 brought about a stark reduction in RSV cases, ranging from a decrease of 57%⁸² to 85%⁸³ in different regions of the world. This significant decrease in cases parallels that of several other respiratory viruses, such as influenza viruses, rhinoviruses, and metapneumoviruses.^{84–87} As public health recommendations and restrictions such as indoor masking and remote learning eased, communities around the globe began to experience a resurgence in RSV cases. Reemergence of a delayed RSV season was observed in Japan⁸⁸ and Argentina⁸⁹ with higher than usual cases and introductions from other countries in 2021. Likewise, in March 2021, the Centers for Disease Control and Prevention (CDC) reported increases in RSV infections through the National Respiratory and Enteric Virus Surveillance System, resulting in the issuance of a health advisory for interseasonal activity in the southern United States.⁹⁰ Predictive analyses propose that the combination of implemented public health mitigation strategies and decreased exposure to RSV will increase the likelihood and intensity of an RSV epidemic reemergence with an atypical broader age range.^{14,15}

Taken together, global surveillance efforts have substantially improved our understanding of RSV

epidemiology. One major conclusion drawn from these surveillance efforts is that RSV prevalence and mortality are grossly underestimated with as many as three additional RSV-associated deaths occurring in the community for every one RSV-associated death reported in the hospital.⁴ These efforts additionally highlighted the absence of significant RSV surveillance in adult and older populations. In addition, the association of global RSV-subtype distributions among different age groups remain unclear.⁷⁶ Finally, these global surveillance efforts have facilitated characterization of the spread of persistent global RSV subtypes, such as RSV-A ON1.⁹¹ Continued surveillance will be critical to filling in these knowledge gaps and assessing how RSV molecular epidemiology has or has not changed following the COVID-19 pandemic.

Impacts of genetic diversity on disease severity and clinical outcome

Changes in viral genotype can result in changes in viral load, cell tropism, and immune evasion, and subsequently impact disease presentation, severity, and clinical outcome. Different subtypes of influenza virus and different variants of SARS-CoV-2 have both been associated with exacerbated severe or mild disease with a similar pattern observed in patient outcomes.^{92,93} Several studies have examined the relationship between RSV genotype and clinical severity, although these have failed to reach consensus with studies coming to disparate conclusions.¹⁰ The reason for the contradicting conclusions is likely multifaceted and attributable to differences in inclusion criteria, cohort demographics, prior immunity, and sample size.¹⁰ These studies have largely relied on G sequencing to elucidate genotype with the assessment of clinical severity through available medical health records and severity metrics, such as proprietary severity scores, intubation, and length of hospitalization. For example, RSV-A genotype ON1 was suggested to worsen bronchiolitis clinical severity in hospitalized infants, particularly in the 2016–2017 and 2017–2018 RSV seasons.⁹⁴ Likewise, another study that coupled transcriptome analysis, genotyping, and clinical metadata in a multicenter prospective study in the United States found that genotype GA5 (RSV-A) was associated with increased severity and a unique host immune response in five consecutive RSV seasons.⁶⁶ Other studies, however, have found no associations between

either subtype RSV-A or RSV-B and any clinical outcomes in hospitalized patients or patients with severe bronchiolitis.^{69,95,96} Viral load has been similarly analyzed in association to clinical severity with similarly disparate conclusions.^{97,98} Overall, these findings (summarized in Table 1) illustrate the complexity of measuring how circulating RSV genotypes may impact disease severity and subsequent clinical outcomes in infants, the elderly, and the immunocompromised.^{65–70,99–102} Furthermore, it must be reiterated that RSV genomic data used in these studies is overwhelmingly based on G sequencing; how mutations in other parts of the genome may influence these phenotypes is even less well understood. Emphasis to characterize glycoproteins and their role in viral infection has similarly been observed in other viruses, such as influenza, SARS-CoV-2, and dengue.¹⁰³ However, while changes in the SARS-CoV-2 glycoprotein, Spike, have been thought to largely drive the observed changes in COVID-19 disease severity, changes outside of the influenza virus glycoproteins have been associated with more severe disease, including changes in the innate immune regulatory protein NS1.^{104,105}

Ultimately, there are several challenges associated with elucidating the link between RSV genotype and clinical severity. Future studies should strive to include full genome sequence data for RSV, particularly across the NS1, M2, and SH ORFs, to determine if the increased diversity across these genes correlates with any particular outcome. Furthermore, the populations included in these studies should be expanded to include other at-risk groups besides infants, such as the immunocompromised and elderly. Finally, standardization of genotype and clade designation on a global scale is crucial and needs to be achieved for conclusions to be readily shared and translated for an international audience.

Impacts of genetic diversity on therapeutic efficacy

Beyond changes in disease severity, changes in viral genotype can have massive consequences on the efficacy of antiviral therapeutics, monoclonal antibody treatments, and vaccines. The evolution of antiviral resistance has been well documented in human immunodeficiency virus type 1 (HIV-1) and influenza viruses, requiring patient-specific drug selection and the careful employment of antiviral stewardship strategies.^{106–108} Monoclonal

Table 1. Representative table of studies linking RSV subtype and genotype to clinical severity and outcome.

Location	Year	Population	Results	Reference
United States	2004–2009, 2010–2011	Infants hospitalized over 5 nonconsecutive respiratory seasons	RSV-A (GA5) was associated with increased disease severity	66
Italy	2005–2017	Infants hospitalized with bronchiolitis	RSV NA1 was associated with more severe bronchiolitis as opposed to ON1 or BA genotypes	68
Vietnam	2010–2011	Infants hospitalized over 5 nonconsecutive respiratory seasons	RSV-A virus was associated with higher clinical severity score	65
Cyprus	2010–2013	<12-year-old hospitalized children with acute respiratory tract infections	RSV-B BA infections was correlated with a higher risk in requiring oxygen. RSV-A ON1 was associated with a milder respiratory tract infection	100
Germany	2011–2017	Children with acute respiratory tract infections (ARTs)	RSV-A ON1 from children in the pediatric intensive care unit (PICU) had worsened clinical severity as opposed to non-ON1 genotypes	70
South Africa	2012–2015	Hospitalized patients (all ages)	No reported difference in clinical outcome between RSV-B and RSV-A. More <i>streptococcus pneumoniae</i> co-infections were associated with RSV-A infections	69
Japan	2012–2015	<5-year-old infants visiting pediatric outpatient clinics with respiratory symptoms over 3 consecutive respiratory seasons	RSV-A ON1 had a 6.9-fold increase in hospitalization risk as compared with NA1 genotype	99
Chile	2013–2014	Hospitalized infants with lower respiratory tract infections	No correlation in clinical outcome or severity with specific RSV-B and RSV-A genotypes	101
China	2013–2015	Hospitalized children 0–14 years old	RSV-A virus was associated with higher clinical severity score	67
Netherlands	2016–2018	Hospitalized adult patients	RSV-A strain with 8 amino acid changes unique to the region had higher clinical severity as measured by severity scores (CURB-65)	102

Location: the location in which the study took place and where the samples were collected; Year: the time frame of sample collection date; Population: a description of the study population and any comorbidities or clinical symptoms of interest; Results: the conclusions of association between specified clinical severity parameters/outcomes and RSV genetic diversity.

antibody therapeutics are likewise highly susceptible to antigenic shift. For example, the emergence of each new variant of SARS-CoV-2 has required reassessment of monoclonal antibody efficacy.¹⁰⁹ Finally, vaccine efficacy is strongly linked to its compatibility to the circulating variant. The ongoing antigenic drift and shift of influenza viruses have required constant surveillance and annual reformulation/administration of the preventive vaccine.¹¹⁰ Likewise, the emergence of the highly

divergent Omicron variant of SARS-CoV-2 has resulted in an increase in breakthrough infections.¹¹¹ All these represent critical tools to prevent transmission and lower disease burden, so understanding the impact of viral variation on therapeutics is essential for effective clinical care and public health response.

There are currently only two Food and Drug Administration (FDA)–approved treatments for

RSV: ribavirin and palivizumab. Ribavirin is a nucleoside inhibitor with broad antiviral efficacy against RSV, hepatitis C virus, Lassa virus, and other viruses.¹¹² Palivizumab is a monoclonal antibody that targets the F protein, though it is reserved for use as a prophylactic in high-risk infant populations.¹¹³ While there are currently limited options, there are over 14 different antiviral and immune-based therapeutics in various stages of clinical trial for the prevention and treatment of RSV infection.¹¹⁴ These include a number of vaccine candidates, most of which rely on either delivery of live attenuated virus or virus-like particles.¹¹⁵ While most of these are being formulated for use in infants, additional trials are underway for the protection of other at-risk groups, including the elderly and immunocompromised. For example, Pfizer recently commenced a phase 3 clinical trial for a RSV prefusion F vaccine candidate for individuals over the age of 60.¹¹⁶

RSV genetic diversity has proven to be a major challenge in the development of next-generation therapies. For example, phase 2b trials of the RSV fusion inhibitor, presatovir, employed partial fusion protein sequencing pre- and post-treatment to identify mutations associated with fusion inhibitor resistance. Of the 233 subjects administered therapy, 18 developed resistance.¹¹⁷ Discouragingly, certain resistance-associated mutations in F, such as V127A, were already present in a subset of subjects and had previously been documented to be circulating in South Africa,¹¹⁸ Korea,¹¹⁹ and Buenos Aires^{12,120} at varying frequencies. Likewise, the proposed monoclonal antibody therapeutic sputavumab failed a randomized, double-blind, placebo-controlled phase 3 trial due to the emergence of RSV-B strains with L172Q and S173 L mutations in F that decreased the binding affinity of the antibody.¹²¹ As a result, the trial failed to achieve its secondary endpoint of decreased RSV-confirmed hospitalizations or outpatient visits among enrolled patients. These mutations in F were found in the 88.6% of RSV-B genotypes in sequenced clinical samples in 2015–2016,¹²² demonstrating a strong need for genomic surveillance to inform therapeutic design and efficacy. Fortunately, resistance to the current monoclonal antibody therapy palivizumab has been sparse,^{123,124} with only a few reports emerging in Japan,¹²⁵ Lebanon,¹²⁶ and in a multinational cohort of patients deemed to be at high risk for

respiratory virus-related illness.¹²⁷ An early 2010 study identified N276S as a potential palivizumab resistance mutation in RSV-A,¹²⁷ but this mutation was present in approximately 44% of RSV strains in 2008–2009 and 100% of RSV strains in 2009–2010 in Canada with no reported decrease in therapeutic efficacy.¹²⁵ Overall, consideration of RSV viral evolution should be taken into account when developing next-generation therapies to minimize the potential for decreased efficacy from RSV therapeutic resistant mutations in circulating genotypes.

Improved RSV surveillance, especially longitudinal surveillance in the context of drug trials, will be critical to assessing the risk of therapeutic resistance going forward. Given the already limited options for antiviral treatment, continued surveillance for resistance to current treatment regimens will be further required to best inform clinical use and stewardship. The emphasis on therapeutic resistance has been heavily weighted toward infant populations; additional assessment of RSV evolution in response to therapeutic treatments in elderly or immunocompromised populations will be required to fully understand the risk of population-based antiviral resistance. In sum, improved molecular surveillance of RSV is sorely needed to not only inform how genetic variability impacts epidemiology and pathogenesis, but also the efficacy of current and future therapeutics.

Toward a molecular understanding of RSV biology

Understanding the mechanism by which changes in the RSV genome impact viral spread, disease pathogenesis, and therapeutic efficacy depends on an understanding of the molecular virology and selective pressures that underlie RSV replication and evolution. In general, mutations that improve a virus' ability to replicate in a given environment (i.e. improve viral fitness) will be selected for over time and vice versa.¹²⁸ For example, mutations that improve transmission, enhance immune evasion, or provide resistance to an antiviral therapeutic may enhance viral fitness in some cellular environments, providing an advantage to the viruses that carry them.¹²⁹ While genomic surveillance can be remarkably effective in identifying mutations that are under selection, interpreting why they are selected requires lab-based inquiry.

The RSV glycoprotein G and fusion protein F are among the most heavily studied viral proteins given their critical role in the viral entry and their importance as therapeutic targets. F and G are expressed on the outer surface of virions and mediate binding to the target cell surface through interactions with host attachment factors and entry receptors.¹³⁰ An array of entry receptors and attachment factors have been described, including CX3CR1 and heparan sulfate proteoglycans (HSPGs) that interact with G as well as nucleolin, epidermal growth factor receptor (EGFR), IGF1R, and ICAM-1 that interact with F.¹³¹ Receptor binding ultimately enables F to initiate membrane fusion and deposit the viral genetic material into the cell.²⁵ Beyond their role in viral entry, F and G also serve critical roles in immune evasion as prominent targets for the humoral immune response, including neutralizing antibodies that block receptor binding.¹³² To help circumvent this response, G is heavily glycosylated to mask potential protein epitopes and is shed in a soluble form to neutralize and effectively lower antibody titers.^{133,134} As a result, G experiences strong selective pressure and is among the most heavily diversified ORFs in RSV. F, on the contrary, is more evolutionarily constrained and as such serves as a common therapeutic target.²³ High selective pressure in the presence of fusion inhibitors, however, can lead to the emergence of resistance mutations. Understanding the impact of G and F mutations on viral entry, viral transmission, and therapeutic efficacy is therefore of critical importance.

To determine the functional impact of mutations in the RSV viral glycoproteins, a number of biochemical and cellular tools have been developed.^{135–137} For example, different variants of F and G can be used to pseudotype non-infectious reporter viruses *in vitro* to test the effect of mutations on cell entry and antibody neutralization.¹³⁸ Similar systems have been developed to understand the ongoing evolution of the SARS-CoV-2 Spike protein, which faces similar selective pressures.¹³⁹ Using these assays, several mutations in RSV G and F have been reported to influence their interaction with their respective host factors, influencing the efficiency of cellular attachment and membrane fusion.¹⁴⁰ Similarly, several escape mutations reported in F following *in vitro* selection for fusion inhibitor resistance have been

found to have secondary impacts on cell entry, resulting in a decrease in viral fitness.¹⁴¹ Beyond influencing viral entry and therapeutic efficacy, these mutations may also influence pathogenicity and host response. For example, mutations in the F protein of RSV-A strains 2-20 and A2 have been found to alter engagement with EGFR, which has downstream consequences on airway mucin production.¹⁴² In sum, implementation of *in vitro* strategies to elucidate the consequences of mutations on RSV replication will be crucial for informing the development of novel therapeutic and preventive treatments targeting these proteins.

While much is known about the function and selective pressures acting on F and G, much more needs to be done to fully understand the replicative lifecycle of RSV and the critical interactions that might drive viral evolution. Indeed, a series of genetic and biochemical studies in recent years have identified several new RSV host factors that have unique proviral or antiviral functions. For example, the role of nucleolin as a primary cellular receptor *in vivo* was only recently identified through transcriptomics analyses followed by siRNA knockdown and quantitative RSV plaque assays.¹⁴³ Both genome-wide loss-of-function^{144,145} and gain-of-function³⁸ screens in lung epithelial models have likewise identified a number of new RSV host factors. For example, a recent study identified chemokine (C-X-X Motif) Ligand 4 as a novel antiviral host factor that inhibited RSV entry and was correlated with disease severity in RSV-infected patients.¹⁴⁶ Proteomic approaches have likewise been used to identify host proteins critical for viral replication or the immune response to infection. For instance, a study using immunoprecipitation mass spectrometry demonstrated that, in addition to perturbing immune responses, NS1 binds to mediator complexes of RNA polymerase II to regulate antiviral gene expression.¹⁴⁷ Similar high-throughput approaches have been used to identify molecular correlates of disease that may serve as future biomarkers. For example, differentially expressed proteins involved in glycolysis (BPGM, TPI1, PRDX2, and CFL1) in nasopharyngeal secretions of children in the acute or convalescent phase of RSV infection were upregulated and identified using isobaric tags for relative and absolute quantitation (iTRAQ) analysis.¹⁴⁸

While these studies have contributed toward our understanding of the molecular virology of RSV, most of this work fails to account for the genetic makeup of most currently circulating strains, instead relying on the use of a handful of lab-adapted strains that grow well in laboratory cultures and models. Five out of the six most commonly used strains (RSV Long, A2, Line19, Line19F, and CH-18537) were isolated before 1970 and do not recapitulate the immune response of more recent isolates.¹⁴⁹ A more recent strain (Memphis 37) was isolated from nasal aspirates in 2001, but likewise does not contain the aforementioned duplication event in G or accurately represent currently circulating variants. Furthermore, these strains display differential disease phenotypes and severity in murine models¹⁵⁰ and different cytopathology in primary pediatric bronchial epithelial cells when compared with clinical isolates.¹⁵¹ Analysis of viral gene expression between lab-adapted strains and current circulating genotypes (GA1, ON, GB1, and BA) revealed clear differences in viral lifecycle regulation between these strains that may underlie different host responses.¹⁵²

Much more work needs to be done to understand what molecular determinants underlie RSV replication, how these determinants drive viral evolution, and ultimately how the resultant variants differ in their replicative lifecycle and interaction with the host. Implementation of molecular biology and systems biology strategies in tandem with molecular surveillance will be required to identify the factors driving differences in viral replication and pathogenesis *in vivo*. Meanwhile, the inclusion of more relevant lab models and relevant RSV isolates will be critical to refining our understanding of the proviral and antiviral host factors underlying RSV replication in different microenvironments.

Strain-specific differences in the immune response

While limited, some studies have emphasized how RSV genotype can influence the innate and cellular immune response to infection. Much of this work has been done through comparison of different viral strains in well-characterized murine models of infection. For example, examination of strain-specific differences in the pathogenesis of RSV in BALB/c mice revealed differential levels of neutrophils and IL-13 expression in CD4⁺ T cells attributable to strain variation in the F

protein and its fusion activity in epithelial cells.¹⁵³ Similarly, when measuring phenotypic parameters in mice infected with laboratory strain A2 (RSV-A) and clinical isolate strain Line 19 (RSV-A), production of cytokines IL-10 and IL-13 was found to be higher and lower, respectively.¹⁵⁴ Outside of murine models, some work has been done with different clinical isolates in standard cell line models to compare effects in culture. For example, a point mutation in the transcriptional termination signal of G found in 98% of circulating genotypes between 2009 and 2017 was speculated to decrease F expression relative to RSV strain A2 as a way to evade the humoral response.¹⁵⁵

Studies of immune responses in human cohorts are more difficult given that diversity in both the hosts and the pathogen could drive meaningful changes that are hard to assign to pathogen diversity alone. For example, when assessing correlates of the host response to bronchiolitis severity, One study found that induced ISGs differed between patients infected with NA1 (RSV-A), ON1 (RSV-A), or BA (RSV-B) and that these patterns were associated with severity in bronchiolitis clinical manifestations.¹⁵⁶ Likewise, using microarrays and high-throughput sequencing, microRNAs responsible for regulating inflammatory mediators produced by leukocytes were identified to be dysregulated in RSV-infected infants and contributes to airway hyperreactivity.¹⁵⁷ When evaluating inflammation in these patients, elevated levels of neutrophils were identified in the bronchoalveolar lavage in children with RSV-induced bronchiolitis.¹⁵⁸ Using paired transcriptomic analysis, specifically low-input RNA sequencing of neutrophils in sputum and blood from infants admitted in the ICU, this same study identified upregulation of neutrophil activation genes, IL-6, and NF- κ B signaling.¹⁵⁹ Although the role of neutrophilia in RSV pathophysiology is not fully understood, excessive neutrophilia has been identified in severe tissue damage and inflammation in RSV bronchiolitis.¹⁶⁰ While these findings relate back to studies in murine models suggesting strain-specific differences in inflammation and neutrophil levels, it is still unclear if RSV genotype was a significant driver of the differential immune responses and infant outcomes. Overall, *in vitro* work, animal models, and measurements of immune-related gene expression patterns in clinical cohorts support the notion that RSV genetic diversity is involved in altered immune responses,

though much more work needs to be done to determine how these interactions drive viral evolution.

Bridging molecular epidemiology and global systems biology

Molecular epidemiology seeks to relate the molecular mechanism of disease to environmental and genetic factors on a population-based scale to inform and predict pathogenic spread for the advancement of public health.¹⁶¹ The collection and dissemination of molecular surveillance data potentiates a positive feedback loop in tracking emerging variants, implementing successful public health interventions, and identifying promising molecular targets for next-generation therapeutics. Systems biology approaches seek to provide an unbiased molecular network of interactions on a genomic, metabolomic, transcriptomic, or proteomic scale to shed light on the underlying biology of a disease.¹⁶² The integration of these techniques, known as systems epidemiology, has been previously implemented in cancer biology to identify networks of host factors that contribute to translatable outcome metrics, such as tumor growth and risk of disease.¹⁶³ Likewise, this multidisciplinary field has been identified as a particularly beneficial approach to rapid responses for viruses such as Zika through integration of molecular surveillance, analysis of the host response, and linkage to clinical data.¹⁶⁴ Overall, systems epidemiology provides etiological insights and information on prevention strategies on a global scale.¹⁶⁵ While the field of infectious disease has a long way to go before systems epidemiology can be effectively employed, the COVID-19 pandemic exemplified how molecular surveillance and systems biology approaches could be used in tandem to quickly gain insight into an evolving pathogen in near real-time. Hopefully, these tools can be effectively leveraged in the future to better inform our understanding of RSV and how viral diversity drives differences in viral replication, disease pathogenesis, patient outcome, and therapeutic efficacy.

Conclusion

The ongoing viral evolution and high global disease burden of RSV necessitate concerted efforts in molecular surveillance to understand the impact of circulating genotypes on clinical manifestations and therapeutic efficacy. Current global

surveillance efforts have broadened our understanding of disease burden, seasonality, and evolution; identification of new genotypes [ON1 (RSV-A) and BA (RSV-B)] and patterns of convergent evolution have resulted from these global efforts. Continued efforts to expand these surveillance systems, standardize best practices, and implement whole-genome sequencing approaches will help advance future efforts in characterizing RSV diversity. These efforts will enable a more holistic understanding of RSV pathogenesis and pathophysiology beyond our current glycoprotein-centric understanding. The need for these efforts is especially acute in the context of viral evolution in response to existing and emerging therapeutic strategies. While enhanced surveillance will help us identify critical associations with viral genotype, improved understanding of RSV molecular virology will be critical for establishing causation and identifying the drivers of viral evolution. Expanded use of high-throughput, systems-based biochemical, cellular, and immunological assays promise rapid advancement in our understanding of underlying RSV biology. Ultimately, biomedical research and public health surveillance efforts should work to integrate molecular biology approaches within an epidemiological framework. Integration and collaboration will propel the field forward toward improved understanding of how RSV molecular diversity impacts viral replication, disease pathogenesis, patient outcome, and therapeutic efficacy.

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Not applicable

Consent for publication

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Author contributions

Estefany Rios Guzman: Conceptualization; Visualization; Writing – original draft; Writing – review & editing.

Judd F. Hultquist: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Visualization; Writing – original draft; Writing – review & editing.

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