MOLECULAR EPIDEMIOLOGY OF CIRCULATING HUMAN RHINOVIRUS/ENTEROVIRUS IN CHILDREN AND ADULTS WITH ACUTE RESPIRATORY INFECTIONS AT A TERTIARY HOSPITAL IN FREE STATE

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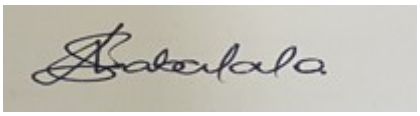
DECLARATION

I hereby declare that this project entitled *Molecular epidemiology of circulating human rhinovirus/enterovirus in children and adults with acute respiratory infections at a tertiary hospital in Free State* submitted to the Division of Virology, University of the Witwatersrand, is entirely my work and the information from the literature has been duly acknowledged in the text and a list of references provided.

The work contained in this dissertation has not been submitted to any university/ institution to award any degree or has not been published any time before.



30 November 2024

DEDICATION

I dedicate this paper to the Almighty God, who has given me the strength, knowledge, wisdom, and protection to complete this study.

I wish to express my deepest gratitude to my supervisor, Professor Zinhle Makatini, whose invaluable guidance and expertise have been critical to the successful completion of this research. Your thoughtful mentorship, continuous feedback, and unwavering dedication to my academic growth have not only helped shape this project but have also profoundly influenced my development as a researcher. I am truly fortunate to have had the opportunity to learn from your wisdom and experience, and I will carry on the lessons you have imparted with me throughout my career.

To my entire family, I extend my heartfelt thanks for their endless support and patience throughout this process.

A big shout out to my best friend, Ngiyabonga, Msezane, Raketsi Iami!

ACKNOWLEDGEMENTS

I would like to acknowledge the Poliomyelitis Research (PRF) Foundation and the National Research Foundation (NRF) for the bursary to support the MSc studies, the National Health Laboratory Service (NHLS) Universitas in Bloemfontein for allowing access to samples and use of the laboratory. In addition, I wish to acknowledge the Academic Affairs and Research Office (AARMS) for granting permission to access the data.

ABSTRACT

Background: The study focuses on the prevalence and molecular characterization of human rhinoviruses (HRV) and/enteroviruses (EV), describing circulating HRV genotypes in children and adults with acute respiratory infections managed in the Free State province public sector hospitals between January – October 2024.

Methods: This descriptive, cross-sectional study analysed nasopharyngeal samples from patients with acute respiratory infections (ARI). RNA from stored samples was extracted, reverse transcribed into cDNA and amplified using nested PCR targeting the VP4/VP2 region. Sequencing was performed on the ONT platform, followed by phylogenetic analysis using iqtree2. Data on patient demographics, clinical details, and Ct values were obtained from the NHLS database, and statistical analysis was conducted to identify significant findings ($p < 0.05$).

Results: The detection rate for circulating respiratory viruses in the Free State province over the study period (10 months) was 59.5% (329/553). Of the 329 samples, 82 (24.9%) were adults and 247 (75.1%) children - with a predominance of infants (n=137; 55.5%). The median age (interquartile range [IQR] of the adult study participants was 26.5 years [32 -58.5] and that of children 1.89 years [0.11 - 2 years]. Human rhinovirus was positive in 35.3% (116/329) of the samples, however only 30 samples were appropriate for genotyping. The majority of viruses occurred as single detections (n=79; 69.1%) however, 37(31.9%) positive for HRV were also positive for another pathogen. RSV was the most common pathogen (n=16; 43 %) found in viral co-detections. Twenty-one samples were successfully genotyped into HRV genotype A, B and C. The majority (n=15, 71.4%) yielded genotype C. HRV genotypes A and B were detected at a frequency of 5 (23.8%) and 1 (4.8%) respectively. Association of HRV genotype C with severity of disease was not undertaken.

Conclusion: All three genotypes of HRV were identified in our study cohort, although HRV-C was the commonest, followed by HRV-A and only one HRV-B. HRV genotype association with severity of ARI should be conducted in future studies.

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LIST OF ABBREVIATIONS/ACRONYMS

AdV: Adenovirus

AFM: Acute Flaccid Myelitis

ARDS: Acute Respiratory Distress Syndrome

ARI: Acute Respiratory Infections

BAL: Bronchoalveolar Lavage

CAP: Community-acquired pneumonia

CD: Cluster of differentiation

CDHR-3: cadherin related family member 3

COPD: Chronic obstructive pulmonary disease

Ct: Cycle threshold

CV: Coxsackievirus

EV: Enterovirus

HMPV: Human metapneumovirus

HRV: Human rhinovirus

ICAM-1: Intercellular Adhesion Molecule-1

ICTV: International Committee on Taxonomy of Viruses

IFN: Interferons

Influ A: Influenza A

Influ B: Influenza B

Kb: Kilobases

IRES: Internal Ribosome Entry Site

LDLR: Low-Density Lipoprotein Receptor

LRTI: Lower Respiratory Tract Infection

NK: Natural killer

nm: nanometers

ORF: Open Reading Frame

PRR: Pathogen recognition receptor

RIG-1: Retinoic acid inducible gene 1

RT-PCR: reverse transcription polymerase chain reaction

SOP: Standard operating procedure

ssRNA: Single-Stranded Ribonucleic Acid

TLR: Toll-Like Receptors

UTR: Untranslated region

URTI: Upper Respiratory Tract Infection

VP: Viral Protein

RSV: Respiratory syncytial virus

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Chapter 1: Literature Review

1.1 Introduction

Respiratory pathogens are recognised as a leading cause of acute respiratory infection in both children and adults (Lozano et al., 2012). Acute respiratory infections (ARI), in particular, constitute a major cause of morbidity and mortality worldwide in 11–22% of children aged < 5 years, globally (Tomczyk et al., 2019). Surveillance data from the severe acute respiratory infection (SARI) monitoring system in South Africa reports a high incidence of lower respiratory illnesses amongst children younger than five years (~2260/100,000), with an even higher incidence in those under one year (~8000–11,000/100,000 population) (Cohen et al., 2015).

Respiratory viruses, including Rhinoviruses (HRVs) and Enteroviruses (EVs), are some of the most common causes of ARI (Heikkinen et al., 2003). Human rhinoviruses alone are responsible for approximately 20%–50% of respiratory tract infections annually (Zlateva et al., 2020), particularly affecting children, the elderly, and individuals with preexisting respiratory conditions (Luka et al., 2020; Kenmoe et al., 2021). These viruses not only are responsible for significant morbidity, but also medical costs and absence from school as well as the workplace (Stobart et al., 2017).

1.2 Overview of Respiratory Pathogens

The most common viral respiratory pathogens causing respiratory tract infections include influenza (Influ) A, B, and C viruses, respiratory syncytial virus (RSV), parainfluenza virus (PIV), respiratory adenovirus (AdV), human metapneumovirus (hMPV), human coronavirus (SARS-CoV-2), and human bocavirus (HBoV). Whilst influenza, RSV, and PIV are often associated with pneumonia, especially in children under 5 years of age and commonly reported in those those living in developing countries, (Iwane et al., 2004; Lee et al., 2005; Nair et al., 2011; Nair et al., 2010; Yu et al., 2012), AdV, HCoV and HBoV are associated with a substantial proportion of RTI in infants and young children (Ahmed et al., 2012; Lu et al., 2012; Khamis et al., 2012; Song et al., 2010). In addition, RSV and PIV may also contribute substantially to mortality among the elderly and the annual outbreaks. The occurrence of recurrent

infections suggest that RSV and PIV may contribute to the burden of disease throughout life (Van Asten et al., 2012).

1.3 Commonly detected pathogens

1.3.1 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is a pleomorphic, enveloped, single-stranded RNA virus with a genome size between ~15-16 kilobases (kb). Based on antigenic heterogeneity, RSV is classified into two specific and distinct subgroups: type A and type B and these are further classified into genotypes based on genetic variation within the highly variable G-glycoprotein (Vandini et al., 2017).

Although the seasonal nature of RSV transmission varies globally, in tropical climates RSV cases are detected all year-round (Jha et al., 2016). Although RSV infections may occur across all age groups, it is commonly detected most frequently in the first two to three years of life, with re-infections regularly observed (Jagušić et al., 2017). Whilst there is a substantial burden of severe RSV disease amongst older adults, a higher incidence of severe clinical cases of RSV-associated ARI in children under six months of age is a recognised phenomenon (Zar et al., 2020).

Currently, there is no vaccine for RSV; however, passive immunoprophylaxis is available for infants at high-risk (Rocca et al., 2021).

1.3.2 Influenza Virus

Influenza belongs to the *Orthomyxoviridae* family and is divided into 3 main types - A, B, C and D. Influenza A viruses are further divided into subtypes based on two proteins on the surface of the virus, hemagglutinin (H) and neuraminidase (N). Eighteen different hemagglutinin subtypes and 11 different neuraminidase subtypes (H1 - H18 and N1 - N11, respectively) have been characterized.

Current subtypes of influenza A viruses that routinely circulate in humans include A(H1N1) and A(H3N2). Currently circulating influenza, A(H1N1) viruses are related to the pandemic and are known as A(H1N1) pdm09. This is a quadruple reassorting virus consisting of two swine-origin viruses, one avian-origin virus and one human-origin virus (Gatherer et al., 2009). Influenza B viruses are not divided into subtypes and are

instead further classified into two lineages: B/Yamagata and B/Victoria. Most of the epidemics and outbreaks of influenza are caused by influenza types A and B, with type C being generally responsible for sporadic mild upper respiratory symptoms (Mosnier et al., 2015; Poon et al., 2016).

The influenza season in South Africa started in April 2024, peaking in June and ending in October. The first peak of the season was predominated by A(H1N1)pdm09, with influenza transmission and impact reaching a high level. A second lower peak was observed in August 2024 and was predominated by B/Victoria (https://www.nicd.ac.za/centre for respiratory diseases sentinel surveillance 2024).

1.3.3 Human Parainfluenza Virus

Human parainfluenza viruses (HPIV) belong to the family Paramyxoviridae (CDC, 2024; Elboukariet al., 2023) and have four recognised genotypes, HPIV-1 to HIPV-4. HPIVs are common and an important cause of upper and lower RTIs, such as bronchitis, croup, and pneumonia (Branche et al., 2016; Elboukari et al.,2024). Primary HPIV infection typically occurs before the age of five, with nearly 100% seropositivity by that age and numerous reports of re-infection (Branche et al., 2016). Children are often infected with HPIV-1, 2, and 3, typically presenting as LRTI, whereas HPIV-4 infection is often seen as an URTIs in children and adults (Branche et al., 2016; Lau et al., 2005).

Currently, neither licensed anti-HPIV medications or vaccinations are available to prevent HPIV infections (Pawełczyk et al., 2017).

1.3.4 Human metapneumovirus

Human metapneumovirus (HMPV) belongs to the family Pneumoviridae and genus metapneumovirus (Jagušić et al., 2017) and is classified into two genotypes, HMPV-A and HMPV-B (Ballegeer et al., 2020; Carr et al., 2008; Uddin et al., 2024).

HMPV has been detected in 4 - 16% of patients with ARIs (Turner et al., 2013; Lu et al., 2013). The incidence of HMPV may vary from year to year in the same area (Maggi et al., 2003). HMPV causes disease primarily in children, but can infect adults,

immunocompromised and the elderly - who would be at risk of hospitalisation (Ali et al., 2013). The clinical features of the illness caused by HMPV infection range from a mild upper respiratory tract infection to life-threatening severe bronchiolitis and pneumonia.

1.3.5 Respiratory Adenovirus

Human adenoviruses (AdVs) are a group of double-stranded nonenveloped DNA viruses belonging to the genus Mastadenovirus of the *Adenoviridae* family (Kulanayake et al., 2021). Seven AdV species (A-G), containing more than 79 genotypes, have been identified and defined (Ismail et al., 2018; Kaján et al., 2018). The more common sites of infection for HAdV include the respiratory tract, corneal epithelia, and the intestinal tract. Infections due to AdV frequently present as respiratory illnesses, especially in children under five (Shieh, 2022). Human adenovirus may induce upper and lower respiratory tract infections, including pneumonia, bronchiolitis, pharyngitis, and croup (Shieh, 2022; van der Zalm et al., 2024). Most ADV epidemics occur in the winter or early spring, but infections occur throughout the year with no clear seasonality (Ison et al., 2006).

1.4 Respiratory Co-detection

Patients diagnosed with community acquired pneumonia (CAP) and who have bacterial or viral co-infection are likely to have increased disease severity and subsequent higher mortality rates (McCullers et al., 2006; Ruuskanen et al., 2011; Lim et al., 2019).

Respiratory viruses, particularly influenza and RSV, are recognized as an increasingly important cause of CAP in adults. HRV co-infection with other respiratory viruses, such as respiratory syncytial virus (RSV), adenovirus, coronavirus, and parainfluenza viruses, occurs frequently.

Viral co-infection appears to be more common in young children (Mandelia et al., 2021). Most instances of viral co-detection were found in children under the age of two, while no co-infections were identified in HRV-positive samples from individuals over 14 years old (Golke et al., 2021). HRV and RSV are both important contributors

to childhood respiratory illnesses and HRV is one of the most common viruses detected in RSV-positive samples (Comte et al., 2020).

1.5 Human Rhinovirus

1.5.1 Human rhinovirus/Enterovirus structure

Human rhinovirus/enteroviruses possess a positive-sense, single-stranded RNA (ssRNA) genome ranging from 7.2 to 7.5 kb and packaged within a stable icosahedral capsid of approximately 30 nanometres (nm) in diameter (Fig 1.1) There are 60 copies of each of the four capsid proteins, giving the virion an icosahedral structure (Jacobs et al., 2013). X-ray crystallography of the HRV icosahedral structure revealed a canyon in VP1 encircling each central plateau around the 5-fold axes of symmetry, with a canyon in VP1 serving as the site of attachment to cell surface receptors (Turner et al.,1999; Paul et al., 1998).

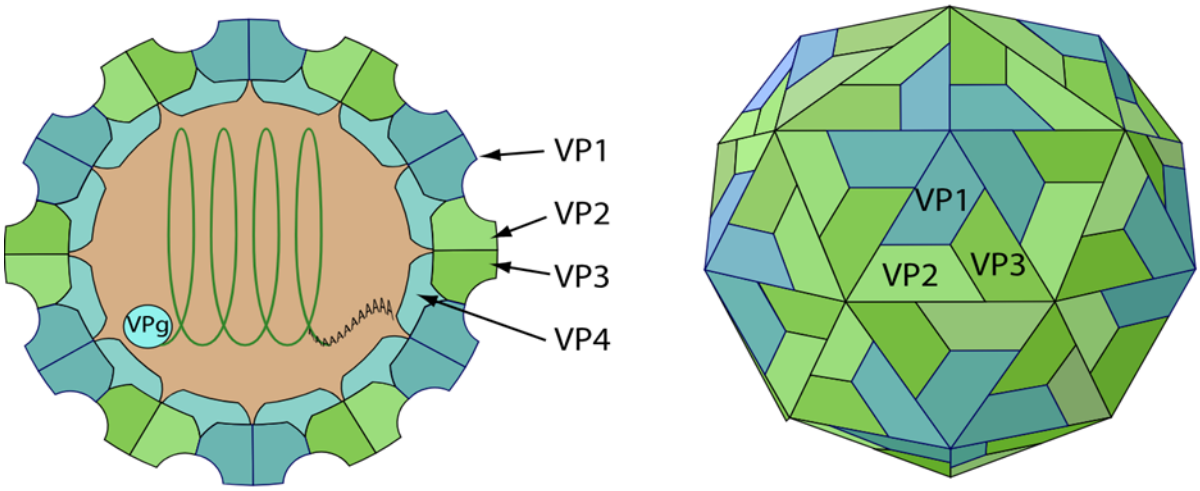


Figure 1.1: The genomic structure of Human Rhinovirus.

Adapted from: Viral Zone Website

The genome consists of a single gene encoding a polyprotein (Palmenberg et al., 2010) and the translated polyprotein is cleaved by viral proteases 2A and 3C to yield 11 mature proteins. Four of the structural proteins, VP1, VP2, VP3, and VP4, make up the viral capsid, while the remaining nonstructural proteins are involved in viral replication and assembly (Bizot et al., 2021). Whilst VP1, VP2, and VP3 proteins

contribute to the virus's antigenic diversity and are present on the cell surface, VP4 serves to anchor the RNA core to the capsid (Georgieva et al., 2023).

The HRV genotypes use three different cellular membrane glycoproteins expressed in the respiratory epithelium for virion attachment to the host cell surface. The majority of HRV-A and B genotypes use the intercellular adhesion molecule (ICAM-1), whilst 12 of HRV-A use the low-density lipoprotein receptor (LDRL) and HRV-A using the cadherin-related family member 3 (CDHR3) (Greve et al., 1989; Tomassini et al., 1989; Staunton et al., 1989). The binding of HRV-A, B and C to their respective ICAM-1, LDLR or CDHR3 cell receptor is thought to initiate the process of receptor-mediated endocytosis, which allows viral particle entry and the subsequent genome uncoating into the host cell (Fuchs et al., 2010).

1.5.1.1 Genome Organization

The human rhinovirus/enterovirus (HRV/EV) genome is composed of two untranslated regions (UTRs) - the 5′ UTR, which is typically 610 to 630 bases long and precedes the open reading frame, and the 3′ UTR, which consists of 40 to 45 bases upstream of the poly(A) tract.

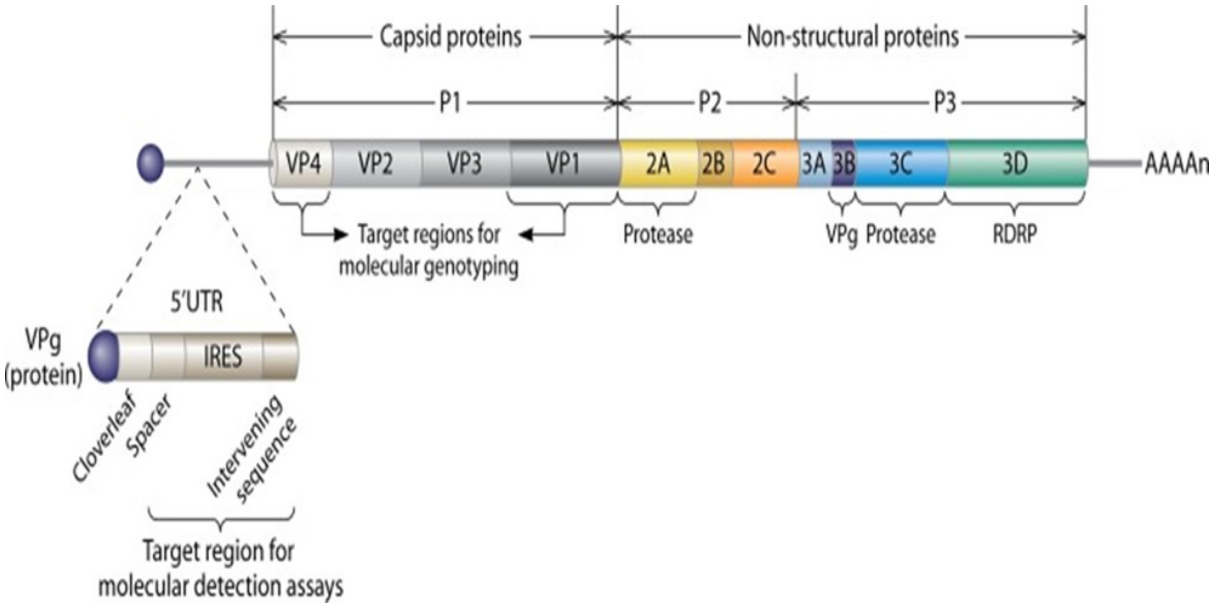


Figure 1.2: HRV genomic structure.

Adapted from: (Jacobs et al., 2013)

The ORF encodes a polyprotein, and the translated polyprotein is cleaved by viral proteases 2A and 3C to yield 11 mature proteins which includes four structural viral

proteins (VP) 1 to 4 and the non-structural proteins including 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Baillie et al., 2020; Zhao et al., 2018).

Based on sequence analyses of HRVs, there is a high genetic diversity, especially in the capsid-coding region as well as evidence for recombination events mapped mainly in the 5′ UTR and the protease 2A gene (Huang et al., 2009; McIntyre et al., 2010). The HRV genome is highly prone to random point mutations due to the absence of typical RNA proofreading mechanisms. This leads to extensive antigenic variation, allowing individuals to experience multiple seasonal HRV infections, even though each infection triggers a memory immune response. The vast antigenic diversity also poses a significant challenge to the development of vaccines and antiviral treatments, contributing to the difficulty in creating effective therapies (Coultas et al., 2021).

1.5.2 Human Rhinovirus/Enterovirus Classification

Classification of HRV/EVs is based on overt similarities in genome organization, capsid properties and primary sequence conservation (Palmenberg et al., 2010). Rhinoviruses are now genetically assigned to the species Rhinovirus A or Rhinovirus B on the basis of metrics of sequence divergence (Zell et al., 2017). A species letter prefix is used to indicate these species assignments (e.g. RV-B2, RV-A16). Rhinovirus C are also numbered consecutively from RV-C1 to RV-C57. Enteroviruses infecting humans are assigned to the species Enterovirus A – Enterovirus D and all newly identified enteroviruses are numbered sequentially e.g. EV-D68, EV-B69, EV-D70 and EV-A71.

The molecular cross-reactivity between HRVs and EVs has resulted in the two viruses reported together as HRVs and/or EVs and in the molecular diagnostic platform, this cross reactivity is reflected between PCR assays for HRV and EV, making differentiation difficult (Jacobs et al., 2013). EV D68 belongs to the species EV D and shares more physicochemical properties with HRVs (Blomqvist et al., 2002). The availability of molecular assays has improved our understanding of the genetic connections between rhinovirus and enterovirus, as well as among different rhinovirus species. For example, rhinovirus 87 and enterovirus D68 are closely related, and both are sensitive to acid. As a result, rhinovirus 87 is now classified as enterovirus D68.

EV-D68 infections affect all age groups and are reported to have no sex predilection (Rahamat-Langendoen et al., 2011; Meijer et al., 2012).

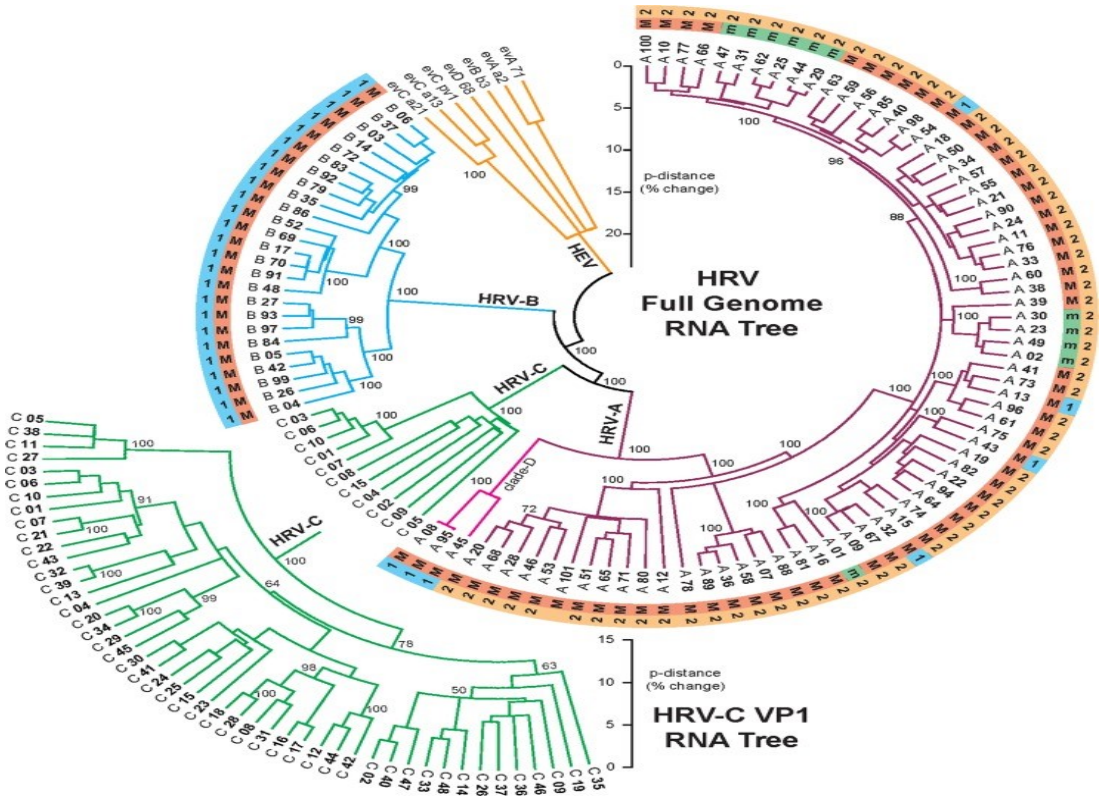


Figure 1.3: Phylogenetic tree. Shown are circle phylogram relationships for known genotypes of HRV-A, HRV-B, and HRV-C

Adapted from: (Jacobs et al., 2013)

1.5.3 HRV/EV Genotypes

HRVs are a diverse group of viruses with over 168 known genotypes (Kuroda et al., 2015) (Table 1.1), classified into three genetically distinct types: HRV-A, HRV-B, and HRV-C (Stobart et al., 2017). HRV/EV genotypes have been classified into different genotypes based on various parameters, including receptor specificity, antiviral susceptibility, and nucleotide sequence homology (Lau et al., 2007). HRV-C, -A and -B share 53-57% amino acid homology and in addition, HRV species use different host surface protein receptors for viral entry, and it is these receptor differences that are support distinct mechanisms of infection and replication for the three genotypes,

Genus	Species	Genotypes	Main clinical manifestations
`Enterovirus	Human rhinovirus A	1A, 1B, 2, 7, 9–13, 15, 16, 18–25, 28–34, 36, 38–41, 43, 44, 46, 47, 49–51, 53–68, 71, 73–78, 80–82, 85, 88–90, 94, 96, 98, 100, Hanks	Common cold
	Human rhinovirus B	HRV3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91-93, 97, 99	Acute lower respiratory tract infections
	Human rhinovirus C	HRV-C1 to HRV-C54	Common cold
	Human Enterovirus D	68 and 70	pneumonia and bronchiolitis, acute haemorrhagic conjunctivitis (EV70), meningoencephalitis (EV70

potentially leading to variations in pathogenicity among the three HRV species (Baillie et al., 2020).

Table 1.1: Picornavirus genera, HRV and EV species, and human genotypes.

Adapted from: (Rollinger & Schmidtke, 2011)

1.5.4 HRV/EV Genotype Clinical Manifestation

1.5.4.1 Human rhinovirus

The clinical characteristics of HRV can vary from being asymptomatic to causing infections in the upper respiratory tract or severe infections in the lower respiratory tract requiring hospitalisation (Comte et al., 2020). The vast diversity of HRV species is thought to account for the differences in clinical phenotypes between symptomatic and asymptomatic individuals (Baillie et al., 2020). The differences in symptom presentation are not regarded enough to differentiate between HRV-A, HRV-B, and HRV-C (Esneau et al., 2022).

The hallmark symptoms of HRV infection include nasal manifestations such as rhinorrhoea, nasal congestion, and frequent sneezing. Patients often report a sore or scratchy throat, which can vary in severity. Coughing is also common and may present as either a dry or productive cough. Additionally, systemic symptoms such as mild headaches, fatigue, and malaise are frequently reported. While fever can occur, it is typically low-grade and less common in rhinovirus infections compared to other respiratory viruses.

HRV-A is more commonly identified in patients with bronchiolitis and pneumonia; HRV-C is associated with acute wheezing, bronchiolitis, asthma exacerbation, and structural lung disease; and HRV-B is associated with cardiovascular disease, mild respiratory disease, or asymptomatic manifestations (Golke et al., 2021; Howard et al., 2016; Hung et al., 2019). HRV-B genotypes are typically associated with milder symptoms, mostly confined to the upper respiratory tract, such as rhinorrhoea (runny nose), sore throat, and cough. These infections are less likely to lead to severe complications but can still cause significant discomfort and missed school or work. While HRV-A and HRV-B cause primarily upper respiratory tract infections, HRV-C is more often linked to severe disease. Children infected with HRV-C are more likely to develop wheezing, bronchiolitis, and pneumonia, with an increased risk of hospitalization compared to those infected with HRV-A or HRV-B (Cox et al., 2013).

1.5.4.2 Enterovirus

Enteroviruses, particularly Enterovirus D68 (EV-D68), are another significant cause of respiratory illness (Larsson et al., 2022). EV-D68 is associated with more severe respiratory infections, especially in children. Additionally, EV-D68 is known to exacerbate asthma, often leading to increased hospitalizations in children with pre-existing asthma. Unlike other enteroviruses, EV-D68 is also notable for its potential to cause neurological symptoms, such as acute flaccid myelitis (AFM), a rare but serious condition that causes muscle weakness and paralysis. Although EV-D68 is known to cause neurological symptoms, it is regarded primarily as a respiratory pathogen (Messacar et al., 2018).

Other EVs, including coxsackieviruses and echoviruses, can also cause respiratory infections, but they are generally associated with milder upper respiratory symptoms, similar to HRVs. Additionally, coxsackieviruses and echoviruses can exacerbate respiratory symptoms in individuals with asthma or other chronic respiratory conditions (Royston & Tapparel, 2016).

1.6 Laboratory Diagnosis

1.6.1 Respiratory RT- Multiplex PCR Panel Pathogen Detect

Molecular methods for viral genome detection and sequencing directly in clinical samples are essential for RV diagnosis. The typical workflow for PCR-based RV diagnosis includes RNA extraction from clinical samples followed by reverse transcription (RT) and multiplex PCR (Coiras et al., 2004; Lamson et al., 2006; Lee et al., 2007) and partial sequencing to determine virus type.

RV-specific diagnostic RT-PCR assays can target the 5′-untranslated region (UTR)/viral protein (VP) gene 4 and the VP4/VP2 region. Nested RT-PCR targeting of the 5′-UTR was employed for HRV screening, and of the VP4–VP2 regions for genotyping. Primers that anneal to highly conserved motifs in the 5′ UTR provide the most sensitive assays for detection of both prototypic strains (Gama et al., 1989; Ireland et al., 1993) and novel RV variants (Lee et al., 2007, Kiang et al, 2008; Lu et al. 2008, Tapparel et al. 2009).

Sequencing: The PCR products are sequenced directly using an automatic DNA analyser.

1.6.2 Cycle Threshold

Whilst respiratory viruses can be detected in less than 2% of asymptomatic patients, (Britain-Long et al., 2010), detection of a specific virus in a respiratory sample using multiplex RT- PCR of a patient with ARI, is considered to be a definitive aetiological diagnosis (Nair et al., 2010; Taubenberger et al., 2017).

In Virology diagnostic laboratories where commercial multiplex RT-PCR assays are used to establish a viral diagnosis, result outcomes are qualitative, indicating the presence or absence of a respiratory pathogen with the Ct (cycle threshold) value presented in the final result. The Ct value, ≤40, expresses the cycle in which the detection system considers that the sample is positive for a certain virus. Samples with a high viral load of a respiratory pathogen, have a low Ct value and in turn, those with a low viral load have a higher Ct value (Do et al, 2012).

Whilst acute respiratory infections may result in mild infections, severe infections leading to hospitalisation do occur. Whether real-time (rt)-PCR cycle threshold (Ct) values can be used to guide clinical decisions or not has remained unclear. This is exemplified by a number of studies evaluating an association between the detected Ct value of a given respiratory pathogen and disease severity, ICU admission as well as length of stay in hospital (Feikin et al., 2017; Hasegawa et al., 2015; Wishaupt et al., 2017). On the contrary, the amount of virus and therefore Ct value in association with disease severity has been questioned with the argument that host factors or genetic factors with predispose an individual to more severe disease instead (Wang et al., 1995; Brand et al., 2015; Mejias et al., 2013).

1.6.3 Limitations of Assay

Multiplex RT PCR assays due to their high sensitivity and specificity, are used in clinical laboratories for detection of respiratory viruses (Lee et al., 2019). A pitfall associated with the multiplex testing approach includes limits to the number of targets or targeting of conserved regions of the viral sequence or virus genotype – a limitation that may result in an atypical or novel viruses evading detection due to only a few fluorophores being available (Mackay et al., 2002).The introduction of next-generation sequencing technologies in clinical settings therefore overcome these limitations and supplement conventional molecular methods to provide more valuable clinical information (Jerome et al., 2019; Li et al., 2019).

1.7 Sequencing

1.7.1 Next generation sequencing

Next-generation sequencing (NGS) technologies not only allow for simultaneous sequencing of a large number of samples per run, but it also has the added advantage of characterization of multiple agents in a single sample. NGS will potentially detect viral within a clinical specimen without actively targeting them, while simultaneously analysing the genetic sequence. The use of NGS eliminates the need for prior knowledge of virus genomic sequences.

1.7.2 Oxford Nanopore sequencing

Nanopore-based sequencing, in comparison to the commonly used Illumina sequencing, is not synthesis-based but instead has nucleotides passing through a polymer membrane via a nanoscale protein pore (Nanopore) and are typed through the disruption of an electric current across the membrane. The entire sequencing reaction is integrated on a single flow cell, which can contain thousands of Nanopores. There are different ONT flow cells which vary in throughput and sequencing accuracy (Mosbruger et al., 2020) and the small size of the flow cells as well as the development of compact sequencing instruments, provide portable sequencing options (Wang et al., 2021) thus allowing for real-time monitoring (Hu et al., 2021).

1.7.3 Phylogenetic analysis

The amplified gene sequences are compared to the reference HRV genotype sequence deposited in GenBank. A phylogenetic tree is constructed using the generated sequences and the reference sequences of each HRV species.

1.8 Study Rationale

From a virus initially thought to only cause a common cold, there has been growing evidence indicating that HRV is one of the most common respiratory pathogens identified in hospitalized patients with pneumonia as well as its role in exacerbation of underlying chronic lung diseases. Whilst in children, HRV typically causes bronchiolitis, the very young, the immunocompromised patients and the elderly are more likely to progress to more severe disease on infection with HRV.

HRV is classified into HRV-A, HRV-B and HRV-C and 168 genotypes are recognised, with HRV-A, HRV-B and HRV-C having 80, 32 and 56 genotypes respectively. Due to the HRV genetic diversity as well as the wide clinical spectrum resulting from infection with HRV, managing all HRV genotypes as one biological and clinical entity is no longer supported. Whilst a number of studies have indicated that RV-C was associated with more severe illness, findings from other studies reports no solid links between HRV genotypes and specific clinical respiratory pathologies.

Use of molecular methods to test respiratory clinical samples is the key to guarantee the accuracy of HRV diagnosis. HRV/EV capsid proteins exhibit a high degree of heterogeneity and as such are routinely screened for by an RT-PCR method targeting a 5'UTR and genotyped by sequencing the VP4-VP2 region of the genome.

This molecular approach has allowed rapid diagnosis of rhinovirus infection in everyday clinical practice. There is an increasing awareness from clinicians of ruling out HRV/EV in critically ill patients with pneumonia and co-pathogens. However, such diagnostic tests which can provide rapid virological confirmation are only available in large academic laboratories.

There is paucity of data on the genotypic diversity and the epidemiology of HRVs in children and adults with acute respiratory infections (ARI). We therefore set out to evaluate the predominant HRV genotypes and their clinical characteristics in children and adults managed with ARI in the public sector hospitals across the Free State, South Africa.

1.9 Study Aims and Objectives

1.9.1 Study Aim

This study aims to characterise the molecular epidemiology of rhinoviruses (RV) and/enteroviruses (EV) as well as corresponding circulating genotypes in children and adults with acute respiratory infections at Universitas Academic Hospital between January 2024 – October 2024.

1.9.2 Objectives

The study will focus on the following objectives:

- To determine the circulating molecular RV/EV genotypes in respiratory specimens collected from children and adults over an 8-month period;
- To describe circulating RV/EV genotypes associated with mild, moderate to severe respiratory infections;
- To describe the proportion of children and adults with co-detection of RV/EV with other respiratory viruses from NPS submitted for respiratory panel testing.

Chapter 2: Materials and Methods

2.1 Study design

The study is a descriptive, cross-sectional study design conducted on paediatric and adult patient samples who had presented with acute respiratory infections (ARI) at public health sector sites from across the Free State and whose nasopharyngeal samples were submitted to the NHLS/Universitas Virology laboratory for respiratory viral panel RT-PCR testing between January 2024 - October 2024.

2.2 Study Setting and Study Population

Universitas Academic Hospital is a private and provincial hospital in Bloemfontein. The hospital offers specialist services including intensive and high-care facilities. The diagnostic laboratory service is supported by the NHLS.

Respiratory panel testing was conducted at the NHLS/Universitas laboratory. Nasopharyngeal samples were referred to the NHLS/Universitas Virology laboratory from 3 districts: Mangaung Metro, Fezile Dabi District and Lejweleputswa District. The Mangaung Metro includes Universitas Academic Hospital, Botshabelo District Hospital, Pelonomi Hospital and the National District Hospital. Respiratory viral panel samples from the Fezile District are from Boitumelo Regional Hospital and Toko District Hospital. Bongani District Hospital, situated in the Lejweleputswa District, also referred its samples to the Virology Laboratory.

NHLS/Universitas Virology laboratory conducts Respiratory viral panel RT-PCR testing for 7 respiratory pathogens (respiratory syncytial virus, human rhinovirus, Parainfluenza types 1-4, Influenza A, Influenza B, Human metapneumovirus, respiratory adenovirus) using Allplex 2019-nCoV (Allplex; Seegene, Seoul, Korea). According to the Virology Laboratory standard operating procedure (SOP), residual nasopharyngeal swab specimens that tested positive for any respiratory pathogen/s were stored in the departmental repository at -80 ℃. For the study purposes samples that tested positive for HRV were selected for study purposes, following ethical

clearance and departmental and NHLS approval and according to a study specific eligibility criterion.

2.3 Eligibility Criterion

2.3.1 Inclusion criteria

a) Nasopharyngeal swabs submitted for respiratory virus panel testing during April 2024 to October 2024 were included in the study;

b) Nasopharyngeal samples that tested positive for HRV/EV with a Ct value ≤ 40 in keeping with the laboratory's cut-off threshold for a positive respiratory panel RT-PCR outcome.

2.3.2 Exclusion criteria

a) Nasopharyngeal samples with inadequate residual volumes following initial routine diagnostic respiratory RT PCR testing;

b) Nasopharyngeal samples with missing data values.

2.4 Study Definitions

At the NHLS/Universitas laboratory, a run is valid if the internal control is positive for both the sample and control. An internal control that is negative for a sample is suggestive of PCR inhibition and the sample should, according to the Virology SOP, be repeated.

A cycle threshold (Ct) value of ≤ 40 is interpreted as a positive for a given respiratory pathogen. A Ct value ≥40 is interpreted as negative for detection of any of the 7 respiratory pathogens. If the Ct value of an internal control is >40, then the test is repeated.

2.5 Laboratory Methods

Human rhinovirus positive residual samples initially stored at -80 ℃, were retrieved for analytical processing.

2.5.1 Total Nucleic Extraction

The total nucleic acid extraction was performed from 500 µL of each known HRV positive residual sample. The extraction was carried out using the NucliSENS® easyMag® system (bioMérieux, USA), a widely used automated platform for isolating high-quality nucleic acids from biological samples. Following the manufacturer's instructions, samples were processed, and nucleic acids were eluted into 50 µL of the elution buffer. This method is known for its efficiency in yielding RNA suitable for sensitive downstream applications, including reverse transcription PCR (RT-PCR) and other molecular assays.

2.5.2 Nested PCR

2.5.2.1 Complementary DNA (cDNA) synthesis

The extracted RNA was reverse transcribed into cDNA using the LunaScript® RT SuperMix (NEB #E3010) (New England Biolabs, USA), a highly efficient and reliable enzyme mix specifically designed for optimal cDNA synthesis from RNA templates. The reverse transcription process was initiated by combining 8 µL of the extracted RNA with 2 µL of LunaScript® RT SuperMix, following the manufacturer's detailed protocol. The reaction was subjected to a specific thermal cycling profile to ensure accurate and efficient conversion of RNA to cDNA. Initially, the reaction mix was held at 25°C for 2 minutes to facilitate primer annealing, ensuring optimal binding of primers to the RNA template, a step that is crucial for preparing the template for reverse transcription.

The reaction was then heated to 55°C for 20 minutes, during which the reverse transcriptase enzyme actively synthesized the complementary DNA strand from the RNA template. Following the synthesis phase, the reaction was raised to 95°C for 1 minute to inactivate the reverse transcriptase enzyme, halting the reverse transcription process. Finally, the reaction was held at 4°C to preserve the integrity of the newly

synthesized cDNA before amplification. This carefully controlled thermal profile ensures high-quality cDNA synthesis, critical for downstream applications such as quantitative PCR and further molecular analysis.

2.5.2.2 RT-PCR Testing

The VP4/VP2 region was amplified and sequenced using nested PCR and primers that were synthesized from the published primer sequences (Table 1) (Wisdom et al., 2009). The first amplification was done using the Q5® Hot Start High-Fidelity DNA Polymerase (NEB#M0493L) and Deoxynucleotide Solution Mix (NEB#N0447S) from New England Biolabs (NEB) (New England Biolabs, USA) according to the manufacturer's instructions with primers HRV-EV-OS OS458 (CCGGCCCCTGAATGYGGCTAA) and HRV-EV-OAS OAS1125 (ACATRTTYTSNCCAAANAYDCCCAT) (Wisdom et al. 2009).

The reaction mixture contained 14.25 µL of RNAse-free water, 5 µL of 5x Q5® buffer, 0.5 µL of 10 mM dNTPs, 0.25 µL of Q5® Hot Start High-Fidelity DNA Polymerase, 1.25 µL of 10 µM forward primers, 1.25 µL of 10 µM reverse primers and 2.5 µL of cDNA. The thermal profile entailed a hot-start of one cycle at 98°C to activate the enzyme followed by PCR activation for 35 cycles at 95 °C for 10 seconds to achieve DNA separation, then 35 cycles at 56°C for 20 seconds to anneal the primers. Thereafter, it would undergo 35 cycles at 72 °C for 60 minutes before finishing with 1 cycle at 72 °C for 2 min.

The second, nested, amplification was done using the same Q5® Hot Start High-Fidelity DNA Polymerase (NEB#M0493L) and Deoxynucleotide Solution Mix (NEB#N0447S) from New England Biolabs (New England Biolabs, USA) according to the manufacturer's instructions, this time with the inner sense and antisense primers HRV-EV-IS IS547 (ACCRACTACTTTGGGTGTCCGTG) and HRV-EV-IAS IAS1087 (TCWGGHARYTTCCAMCACCANCC) (Wisdom et al. 2009).

The reaction mixture used the same components and contained similar concentrations from the first amplification (14.25 µL of RNAse-free water, 5 µL of 5x Q5® buffer, 0.5 µL of 10 mM dNTPs, 0.25 µL of Q5® Hot Start High-Fidelity DNA Polymerase) this time using the inner primers (1.25 µL of 10 µM forward primers and 1.25 µL of 10 µM

reverse primers) and 2.5 µL the DNA template. The thermal profile was the same as in the first amplification (a hot-start of one cycle at 98°C to activate the enzyme followed by PCR activation for 35 cycles at 95 °C for 10 seconds to achieve DNA separation) differing at the annealing step, with 35 cycles at 58°C for 20 seconds to anneal the primers. Thereafter, it would undergo 35 cycles at 72 °C for 60 minutes before finishing with 1 cycle at 72 °C for 2 min.

The PCR products were purified from agarose gel using the gel extraction kit (QIAGEN, USA) according to the manufacturer's instructions in preparation for sequencing using BigDye Terminator 1.1® kit (Thermo Fisher Scientific) on an ABI 310 sequencer according to the manufacturer's recommendations.

Table 2.1: Primers used for amplification of HRV VP4-VP2 regions

Target	**Sequence**	**Region**	**Position**	**Orientation**
HRV and EV	CCGGCCCCTGAATGYGGCTAA	VP4-VP2	458	OS
HRV and EV	ACCRACTACTTTGGGTGTCCGTG		547	IS
HRV and EV	TCWGGHARYTTCCAMCACCANCC		1087	IAS
HRV and EV	ACATRTTYTSNCCAAANAYDCCCAT		1125	OAS

OS= Outer sense IS= Inner sense OAS= Outer antisense IAS= Inner antisense

Adapted from: Wisdom et al., 2009

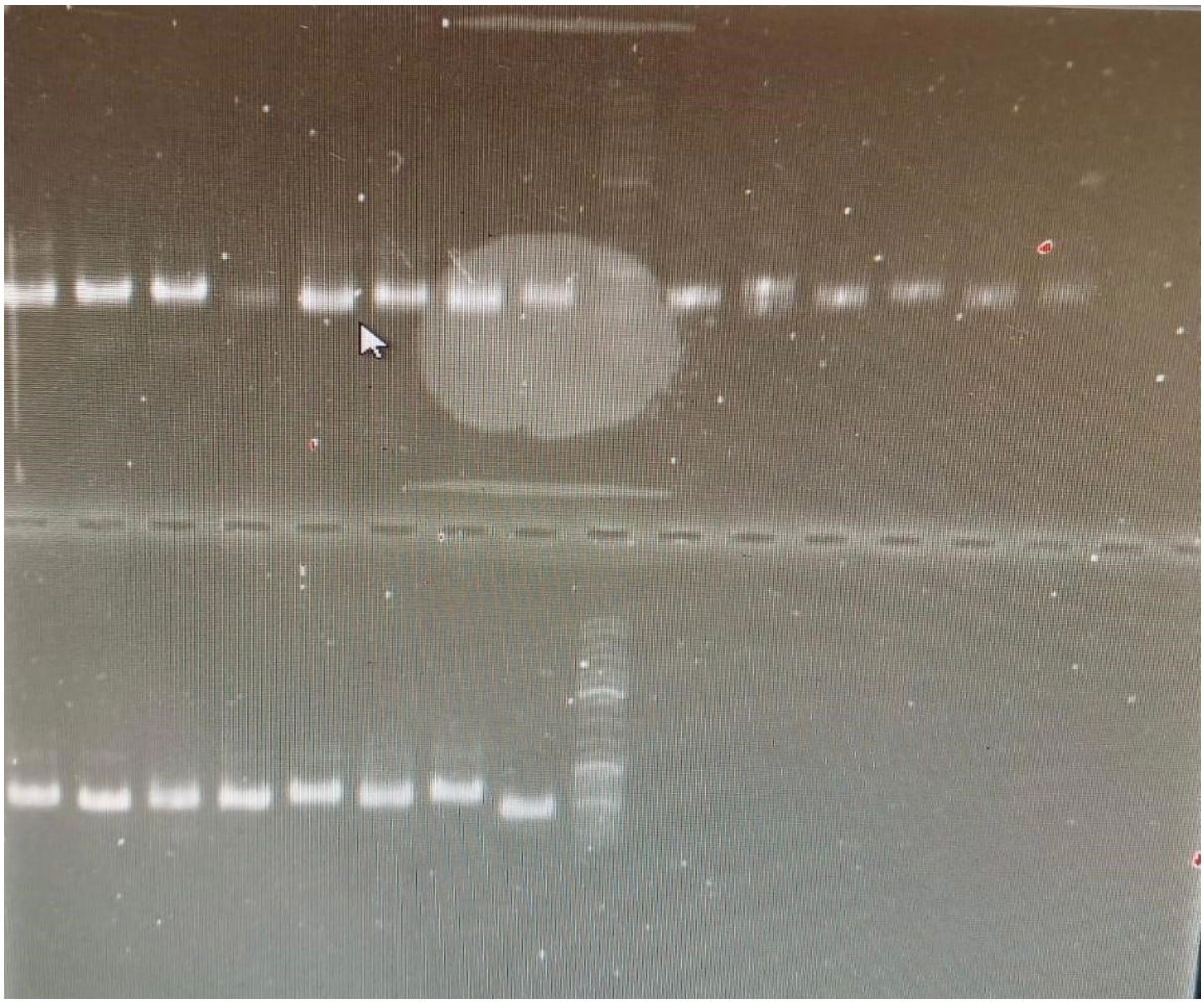


Figure 2.1: The amplification of the unique HRV 5'-UTR region produced a sharp 397-bp band in 21 of 24

HRV RT-PCR. Products were visualized by agarose gel electrophoresis. Top left to right: Lanes 1-3, 5-8, 10-21 show 397-bp bands and belong to HRV positive cases. A 100-bp DNA marker (Roche Applied Science, Mannheim, Germany) was used to designate the band's size (lane L). C-, negative control; and C+, positive control.

2.6 Sequencing

2.6.1 Library Preparation

The next step involved constructing the sequencing library, which included end repair, barcode ligation, and adaptor ligation. For the end preparation, the reaction mixture was assembled using 1.2 µL of buffer and 0.5 µL of enzyme mix from the NEBNext® Ultra™ II End Repair/dA-Tailing Module (NEB#E7546L, New England Biolabs, USA)

following the manufacturer's protocol. To this, 5 µL of RNAse-free water and 3.3 µL of the amplicon sample were added, completing the end repair and dA-tailing process. This step ensured that the DNA ends are properly repaired and tailed for efficient ligation of adaptors.

For barcode ligation, the Blunt/TA Ligase Master Mix (NEB#M0367L, New England Biolabs, USA) was used as per the manufacturer's instructions (Table 2). The ligation reaction included 5 µL of the Native Adaptor Kit (Oxford Nanopore Technologies, UK), 35 µL of the Blunt/TA Ligase Master Mix, and 30 µL of the barcoded amplicon pool. This step was essential for attaching unique barcodes to each sample, enabling multiplexed sequencing. The reaction was carried out following the recommended conditions from the manufacturer to ensure efficient ligation of the adaptors to the prepared DNA ends, completing the library construction for subsequent sequencing analysis.

The assembled library, consisting of 24 barcoded samples, was then sequenced using an Oxford Nanopore Technologies' (ONT) Flongle Flow Cell R10.4.1 9 (FLO-FLG114) (Oxford Nanopore Technologies, UK) over 12 hours according to the manufacturer's recommendations.

Table 2.2: Preparation of Reaction Mix for Library Preparation

Number of Samples	**24**
End Preparation Sample	1.5 µL
Barcode	1.5 µL
Blunt/TA Ligase Master Mix	5 µL
H2O	2 µL
	Total: 10 µL
Mastermix	7 µL
Sample	1.5 µL
Barcode	1.5 µL
	Total: 10 µL per sample

2.7 Sequence Assembly and Phylogenetic Analysis

For the base calling step, Dorado (version 0.6.1 + 79b5da5) a toolkit developed by Oxford Nanopore Technologies (Oxford Nanopore Technologies, Oxford, UK) that incorporates their base calling algorithms, was used. This tool converts the raw sequencing output from .pod5 format to .bam files, which are more suitable for downstream analyses. Once the conversion was complete, the resulting .bam files were demultiplexed, separating the reads based on their unique barcodes to ensure each sample could be analysed individually. To generate preliminary sequences, an Amplicon sorter was applied (Vierstraete et al., 2022, ONT), which processed the data to yield initial amplicon sequences. These sequences were then subjected to a blasting analysis using the NCBI database to identify the closest matching sequences. The blast results were filtered and sorted by bitscore, retaining only the top-scoring alignment for each query sequence, a crucial step in identifying suitable reference sequences for the viral samples.

Taxonomic classification was performed using Taxonkit (Shen et al., 2021), a tool designed to assign taxonomic labels based on the NCBI taxonomy database. This helped categorize the viral sequences accurately, providing insight into the classification and ranking of each sample. To refine the analysis, the best matching reference sequences from the blasting results were selected for mapping. A combination of minimap2 was used, samtools-sort, and samtools-index to align the barcoded FASTQ files to these reference sequences. Minimap2 provided fast and accurate alignment, while samtools-sort and samtools-index facilitated sorting and indexing of the resulting .bam files for structured data analysis.

Samtools-consensus was then used to generate consensus sequences for each sample from the aligned reads, representing the most likely nucleotide sequence for each viral genome. These consensus sequences underwent further alignment using MAFFT to ensure correct alignment across all samples. UNIPRO Ugene was used (version 50.0) to identify and remove nested primer sequences from the alignment. This step was crucial for eliminating artifacts introduced during PCR amplification, ensuring the integrity of the sequences for the next phase of analysis.

Finally, the processed consensus sequences were combined with manually curated HRV sequences for phylogenetic analysis. The iqtree which uses Model Finder to determine the best fit model based in criteria such as AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) was used. GTR (General Time Reversible) DNA substitution model + I + G were identified as the best model. Letter I indicate invariable site with G indicating the use of a gamma model to account for use for rate of heterogeneity across the sites. Ultrafast bootstraps were used with 1000 replicates and also a 1000 SH-like approximate likehood rations test replicates.

2. 8 Sample Size Determination and Data Management

2.8.1 Sample Size

Sample size determination was based on the prevalence rate of each genotype in the South African paediatric population as outlined (Baillie et al., 2020).

The sample size was calculated using the equation $n = Z^2_{1-a/2} * P (1-P) / E^2$

Where:

n is the minimum sample size

P is the estimated prevalence of indicator.

a is the level of significance which is 5 % (0.05)

Za is the z-score corresponding to the degree of confidence. Z= 1.96 for a confidence level of 95%.

E is the desired precision. E= 5%.

Rhinovirus Genotype A:

P is the estimated prevalence of indicator. p= 45% (0, 45)

*Therefore, n = 1,962 * 0, 45(1-0,45)/0,052*

n = 386 samples for HRV-A

Rhinovirus Genotype B:

P is the estimated prevalence of indicator. p= 8%(0,08)

*Therefore, n = 1,962 * 0, 08(1-0,08)/0,052*

n=111 samples for HRV-B

Rhinovirus Genotype C:

P is the estimated prevalence of indicator. p= 40% (0,4)

Therefore, n = 1,962 * 0, 40(1-0,40)/0,052

n=369 samples for RV-C

2.9 Data Collection

Selected variables for the HRV positive samples that met the inclusion criteria were requested from the National Health Laboratory Services Central Data Warehouse (CDW). This included the patient's episode number, age, gender, clinical ward, dates of testing, cycle threshold values (Ct) and respiratory co-infections – all captured on TrakCare laboratory Information System.

2.10 Statistical Analysis

Descriptive statistics are presented as frequencies and percentages for categorical data and for continuous measures, determined using the median and interquartile ranges (IQR). Where appropriate, Chi-square, paired t-test, Pearson's correlation coefficient and the Wilcoxon signed-rank test was used to evaluate statistical differences. Variables with *p values* < 0.05 are considered statistically significant. All data was analyzed using Microsoft Excel and GraphPad Software (GraphPad Inc., USA).

2.11 Ethical Approval

Ethical approval for this study was granted by the Human Research Ethics Committee at the University of the Witwatersrand (Protocol no: M240845). Permission to collect data was obtained from the NHLS Academic Affairs and Research Management System (AARMS) reference no PR22455037. The patient's samples were assigned

episode numbers by the Virology Laboratory. These were used and hence there was no personal identifying information by which samples could be traced back to patients.

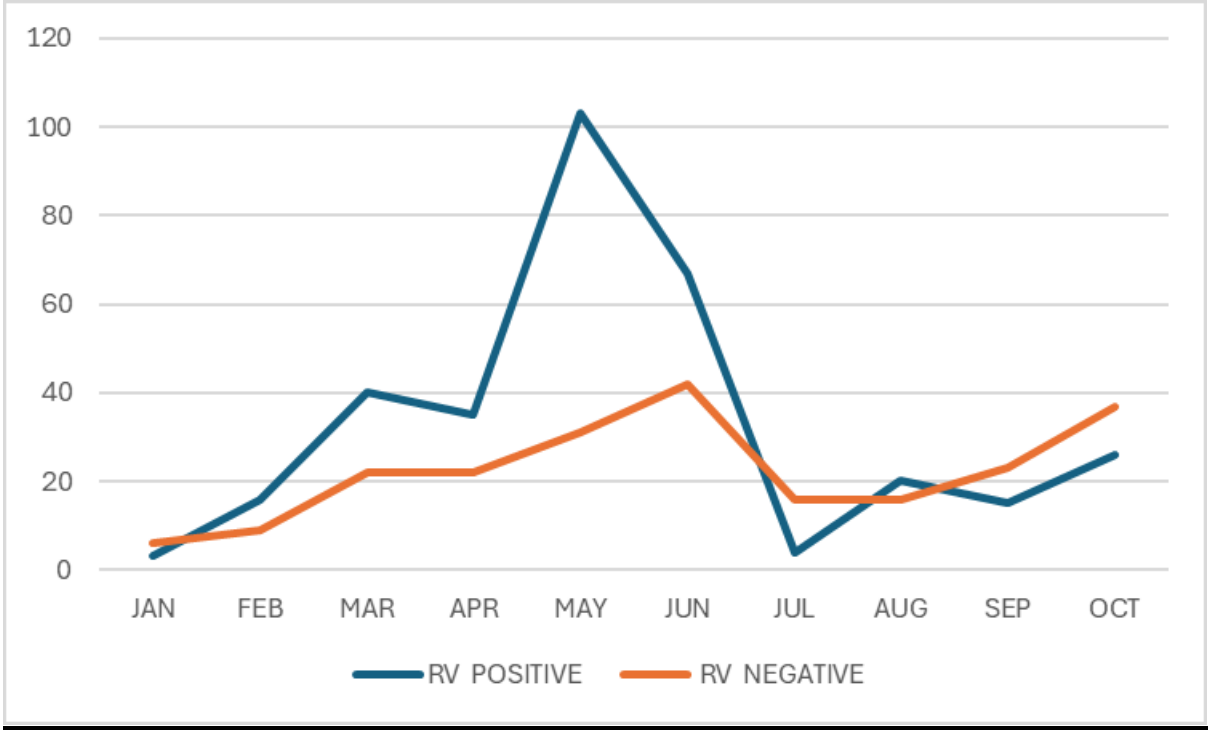
Chapter 3: Results

3.1 Overall respiratory panel cohort

Over a 10-month period from January to October 2024, a total of 585 nasopharyngeal swab samples collected from children and adult patients were tested at the National Laboratory Health Service NHLS/Universitas for a comprehensive respiratory panel pathogen screening. The Allplex™ Respiratory multi-essential panel 7 used by the Laboratory, facilitates the detection of the following clinically relevant respiratory pathogens - RSV, HMPV, AdV, Influ viruses A and B, as well as PIV types 1 to 4. The samples were from patients that attended various public sector clinics and hospitals across various districts in the Free State. The majority (82.4%) of patients whose nasopharyngeal samples were submitted for respiratory panel testing, had been referred from a hospital compared to a local clinic.

Of a total of 585 samples received and tested in the laboratory for respiratory panel testing, 32 samples (5.5%) were invalid on testing and thus excluded. The final study cohort thus consisted of 553 samples (94.5%), eligible for inclusion in the analysis.

On stratifying both respiratory virus (RV) positive and negative samples by month covering the study period, we observed that as the number of samples testing positive for RV increased, RV negative samples submitted to our laboratory, displayed a corresponding increase (Fig 3.1).



*RV positive=329 positive respiratory viruses (RV); RV negative= 224 respiratory pathogens testing negative

Figure 3.1: Repartition of positive swabs for respiratory viruses (RV) tested according to the timing of sampling in months.

3.2 Baseline Characteristics

From the overall 553 patient samples, 329 (59.5%) tested positive for the respiratory pathogens, with the remaining 224 (40.5%) with no respiratory pathogen detected.

Of the 329 samples who were tested initially by the NHLS Virology laboratory using the respiratory panel multiplex PCR, 82 (24.9%) were adults and 247 (75.1%) children - with a predominance of infants (n=137; 55.5%). The median age (interquartile range [IQR] of the adult study participants was 26.5 years [32 -58.5] and that of children 1.89 years [0.11 - 2 years]. There was an approximately equal distribution of male and females, with 51.1% males.

3.2.1 Respiratory Pathogens Detection Rate

Whilst the detection rate of respiratory viruses was 54.5% (n=329), a total of 284 (86.32%) patient's swabs tested positive for one respiratory pathogen. Two distinct viral respiratory pathogens were detected from 40 patient samples (12.2%), with the remaining 5 samples (1.5%) revealing the presence of three respiratory pathogens from a single nasopharyngeal swab. This resulted in a total of 379 pathogen detections across the 329 swab samples.

Human rhinovirus (n=116; 35.3%) was the most frequently detected respiratory virus in this cohort, followed by respiratory syncytial virus (n=95; 25.1%), Influenza A (n=72; 19%) Influenza B (n=54; 4.2%), parainfluenza virus 1-4 (17; 4.5%), adenovirus (n=13, 3.4%) and human metapneumovirus (n=12; 3.2%).

3.2.2 Distribution by gender and age

3.2.2.1 Age

The proportion of patient samples testing positive for respiratory pathogens reporting was highest among those in the 0-14 years age group (55.3%) and lowest among those in the 15-25 years age group (2.4%) (Table 3.1).

Table 3.1: Demographic and baseline characteristics of patients that tested for a respiratory virus pathogen/s

Characteristic	Group	Overall (n=329;%)	HRV-positive (n=116;%)	coefficient	p-value
Neonates (age in months)	0-1	45(13.7)	11(9.5)	-0.12516	0.77
Infants (age in months)	2-11	137(41.6)	46(39.7)	1.321084	0.30
Children (age in years)	1-2	21(6.4)	21(18.1)	1.282016	0.00
	3-5	25(7.6)	10(8.6)		
	5.1 - 14	19(5.6)	6(5.2)		
Adults (Age in yrs)	15-25	8(2.4)	3(2.6)	-1.033	0.00
	26-40	30(9.1)	13(11.2)		
	41-60	26(7.9)	4(3.4)		
	>60	18(5.5)	2(1.7)		
Gender	Male	168(51.1)	66(56.9)	0.3956	0.15
	Female	161(48.9)	50(43.1)		
Type of Facility	Hospital	271(82.4)	102(87.9)	0.3956	0.15
	Clinic	58(17.6)	14(12.01)		

In terms of distribution of detected respiratory pathogens across the study cohort, in children < 1 year of age, the most commonly detected respiratory pathogen at a frequency of 76,6% and 50.9% was RSV and HRV respectively. HRV and RSV infection was higher in children <1 years compared to the adult population.

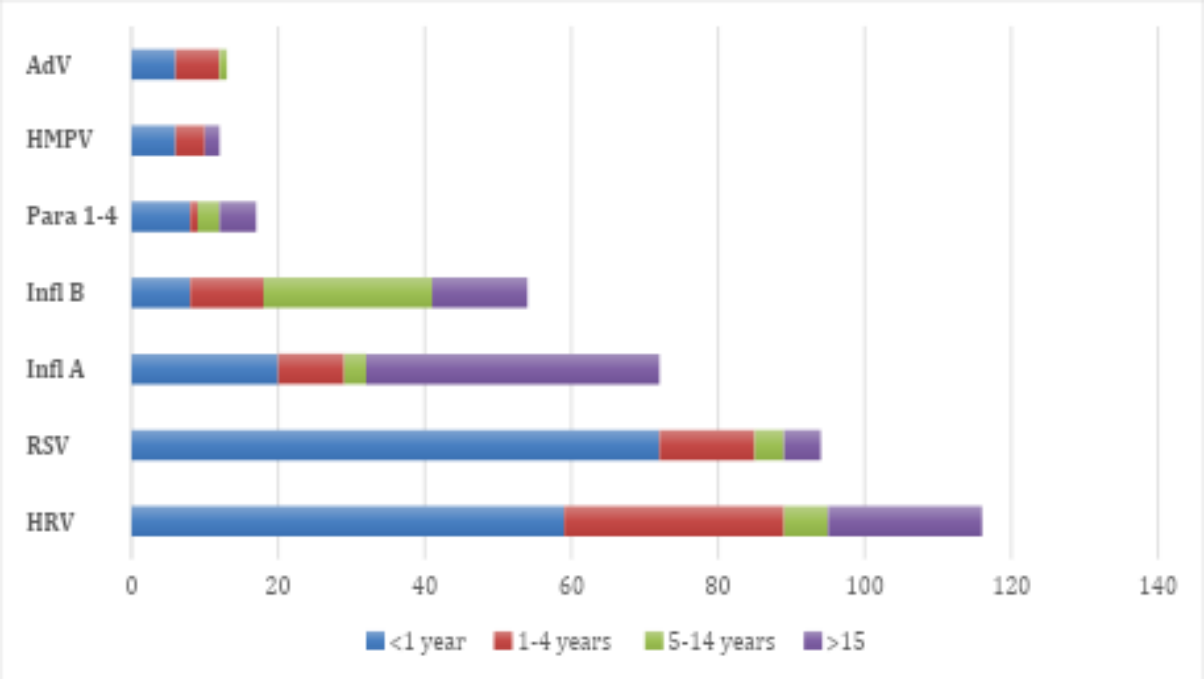


Figure 3.2: Infection rates of respiratory pathogens among different age groups.

Influenza A infection was significantly lower within infants (<1 year; 27.8%), compared to older age groups (>15; 55.6%). On the contrary, infants (<1 year) demonstrated the highest proportion of RSV (76.6%), HRV (50.9%) followed by children aged 1–4 (Figure 3.2).

3.2.2.2 Gender

We found a higher proportion of detected respiratory viruses in men (n=168; 51.1%) than in women (n=161; 48.9%). In general, HRV and RSV, followed by human metapneumovirus showed higher circulation among males (Fig. 3.3).

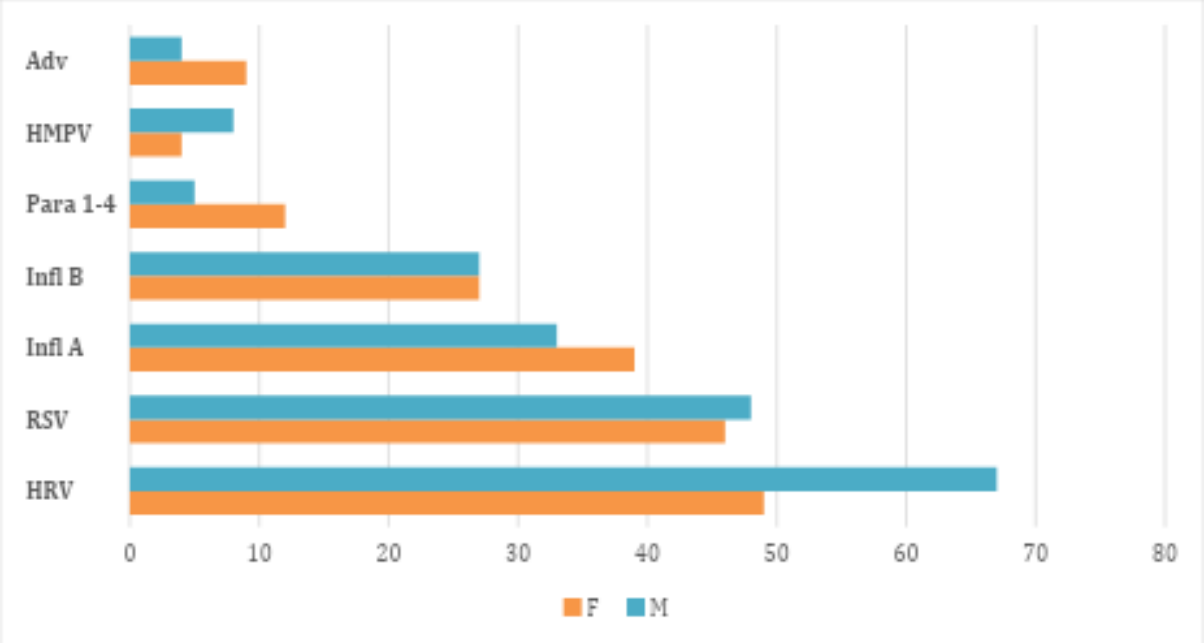


Figure 3.3: Infection rates respiratory pathogens among males and females

ARIs were most frequent in May, followed by June. The rate of ARIs steadily decreased in the subsequent months (Figure 3.4).

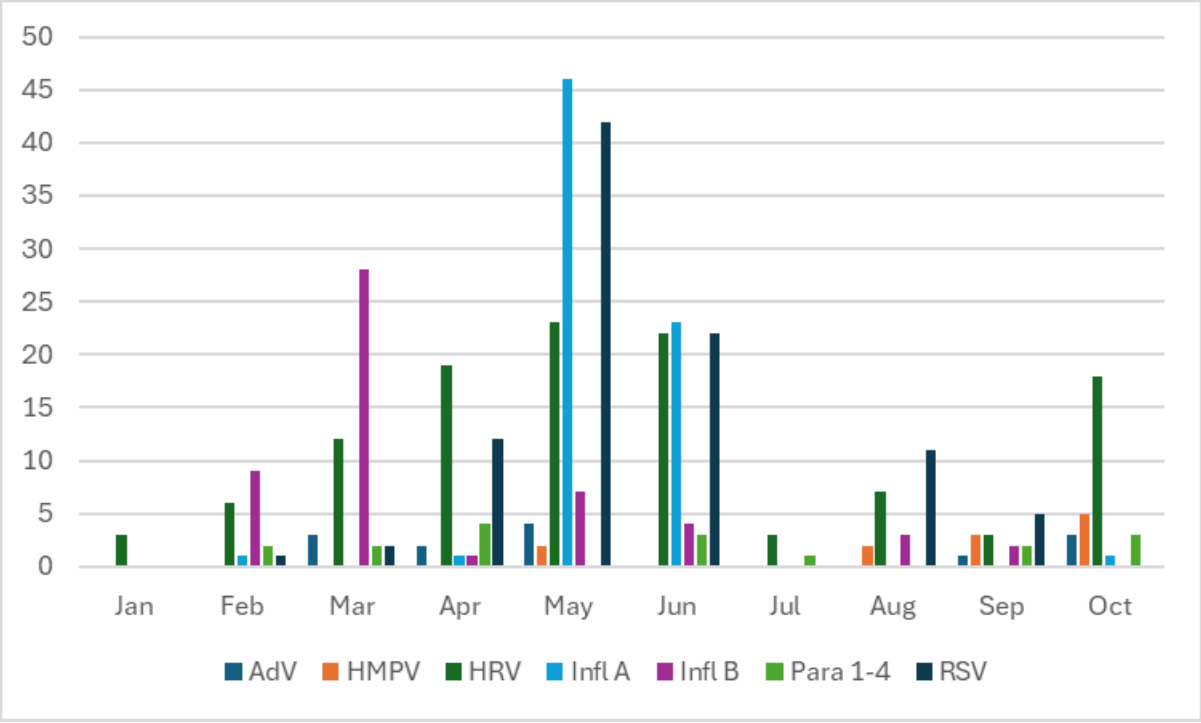


Figure 3.4: Distribution of respiratory viruses on a 10-month period

From the detection of only 3 (1.0%) positive samples in January, there was a steady increase in samples testing positive, with the first small peak in March (12.1%), followed by a major peak in positivity in May (31.3%) and a subsequent drop thereafter to 1.22% in July.

The highest number of positive respiratory viruses (RV) was recorded between May and June and corresponds to the autumn and early winter months in the Southern Hemisphere, a period typically characterized by cooler temperatures and lower humidity conditions that are conducive to the increased transmission of respiratory viruses.

3.2.1 Seasonality

It is evident from Fig 3.4, that viral respiratory infections were circulating throughout the year, with monthly peaks of activity. Rhinovirus, RSV and Influenza A demonstrated pronounced seasonality, with peak infection rate.

Circulating RSV in the Free State was evident in February, peaking in May and ending in September. Influenza A virus, typically detected in winter months, was detected early - with the first few cases in February and a sharp increase culminating in a peak

in May and ending in October. Nationally, the influenza season was dominated by the Influenza A A(H1N1)pdm09,as per NICD surveillance reports. We are not however able to report on the influenza typing as typing of strains is not routinely performed in the NHLS diagnostic laboratories. Human rhinovirus was circulating throughout the 10-month period, with peak infection rate occurring in April, May and June.

3.3 Respiratory Co-detection

From the 329 positive samples tested, a total of 284 (86.3%) patient's nasopharyngeal swabs tested positive for at least one respiratory pathogen. Two distinct viral respiratory pathogens were detected from 40 samples (12.2%), with the remaining 5 samples (1.5%) revealing the presence of three respiratory pathogens from a single nasopharyngeal swab. This resulted in a total of 379 pathogen detections across the 329 swab samples (Table 3.2).

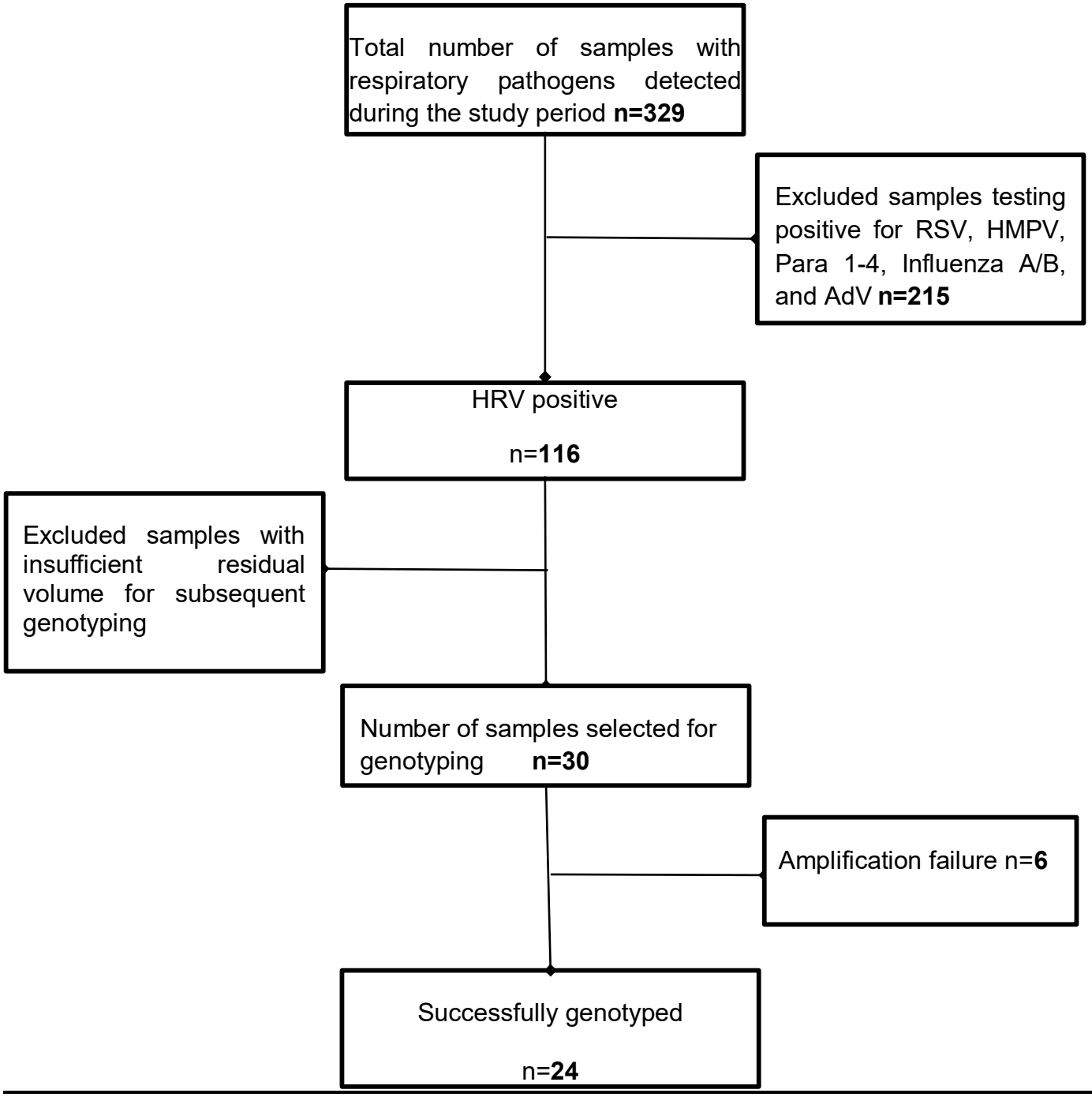
Table 3.2: Detected viruses during acute respiratory infections

Detected Virus	**Acute Respiratory Infections,(n=379) No.(%)**
Human rhinovirus (HRV)	116 (30.6%)
Respiratory syncytial virus (RSV)	95 (25.1%)
Influenza A (Influ A)	72 (19.0%)
Influenza B (Influ B)	54 (14.2%)
Parainfluenza 1-4 (PIV 1-4)	17 (4.5%)
Adenovirus virus (AdV)	13 (3.4%)
Human metapneumovirus (HMPV)	12 (3.2%)

3.4 Selection of Human Rhinovirus samples for genotyping

Of the 329 respiratory virus positive samples that had been tested and stored by the Virology laboratory, 116 samples were positive for human rhinovirus. There was an insufficient residual volume in 84 of the human rhinovirus positive samples stored for HRV genotyping and these samples had to be excluded, leaving 30 samples that were appropriate for genotyping analysis. PCR amplification failure of 3 samples resulted in only 21 samples being included in the final study cohort (Figure 3.4).

Figure 3.5: Flow diagram for the selection of NHLS/Universitas Bloemfontein for Human rhinovirus genotyping



3.5 Co-detection of Human Rhinovirus positive samples

The table provides data on the number of respiratory viral co-detections observed in individuals, where two or more viruses are detected simultaneously in a sample. The data is split into two categories: 2 viral pairs and 3 viral pairs.

Of the 116 human rhinovirus positive samples, the majority of viruses occurred as single detections (n=79; 69.1%) of all viruses. However, 37(31.9%) positive for HRV were also positive for another pathogen (6 with dual infection and 2 with triple viral infection). In addition, two HRV positive samples were observed to be positive for not only HRV but 2 other respiratory pathogens. No sample had more than 3 viruses detected, and RSV was the most common pathogen (n=16; 43 %) found in viral co-detections.

Most HRV detections occurred as a single detection (n=79; 69.10%) of RVs, most often in autumn (when RSV detection also peaked) and spring (when MPV, PIV detections peaked.

Table 3.3: overall incidence of co-detected viral pairs.

Number of respiratory viral co-detection			
Dual Infection		3 viral pairs	
HRV-AdV	7	HRV-RSV-AdV	3
HRV-Para1-4	3	HRV-RSV-Influenza A	3
HRV-RSV	16		
HRV-Influenza A	2		
HRV-Influenza B	2		
HRV-HMPV	2		

3.6 Genotypes

Twenty-one samples were successfully genotyped into HRV genotype A, B and C. The majority (n=15, 71.4%) of the known HRV positive samples yielded genotype C. HRV genotypes A and B were detected at a frequency of 5 (23.8%) and 1 (4.8%) respectively.

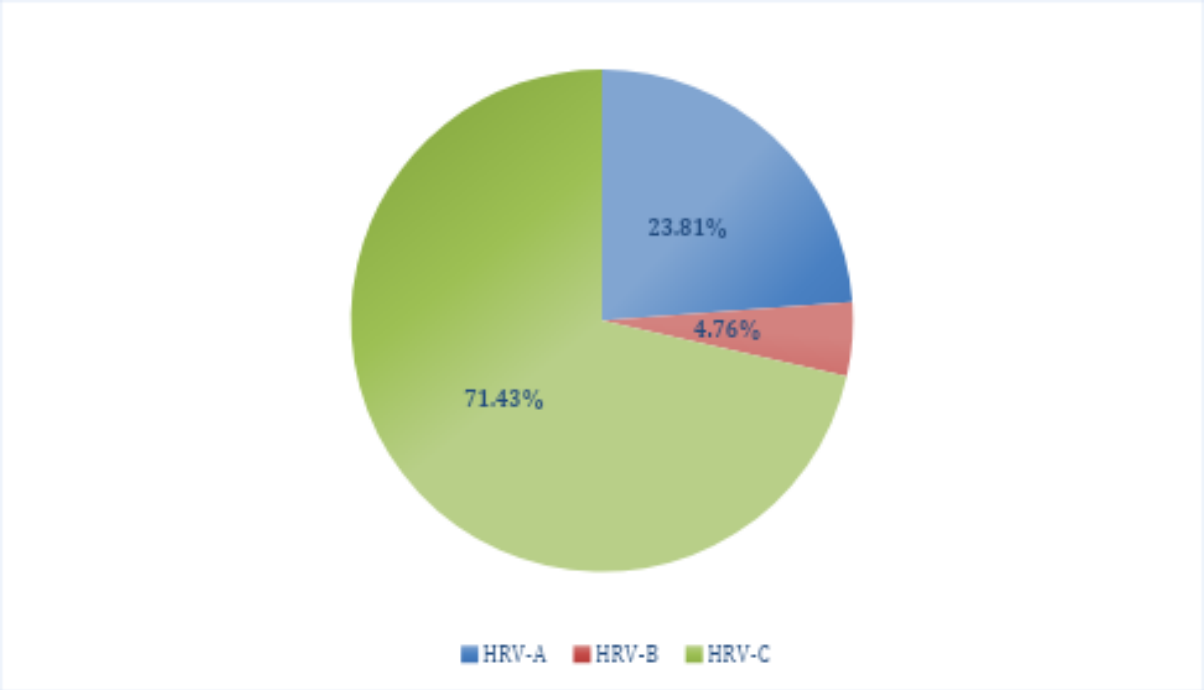


Figure 3.6: Distribution of HRV species

The Table 2 below provides baseline characteristics of the nasopharyngeal samples of 21 patients.

In the under 1 year age group, the distribution of HRV species differed by age group, with HRV-C (n=6;40%) being the most prevalent followed by HRV-A (n=3; 60%). Of note is that HRV-C (60%) was more prevalent among > 18 years.

Table 3.4: Demographic Characteristics of HRV-Positive Patients stratified by HRV species.

Characteristic	HRV-A (n = 5) No. (%)	HRV-B (n = 1) No. (%)	HRV-C (n = 15) No. (%)	Total (n =21) No. (%)	p-value
Age					
<1 year	3(60%)	0(0,0%)	6(40,0%)	9(42,9%)	**0.04**
1-5 years	1(20%)	0(0,0%)	2(13.3%)	3(14,3%)	0.20
6-18 years	0(0,0%)	1(100%)	1(6,7%)	2(9,5%)	0.14
>18 years	1(20%)	0(0,0%)	6(40,0%)	7(33,3%)	0.29
Gender					
Female	1(20%)	1(100%)	9(60%)	11(53,4%)	0.91
Male	4(80%)	0(0,0%)	6(40%)	10(47,6%)	0.92
ICU	1(20,0%)	0(0,0%)	3(20,0%)	4(19,0%)	0.88
Non-ICU	4(80,0%)	1(100%)	12(80%)	17(81,0%)	0.64

We did not undertake clinical file review, but documented patients admitted to the Intensive Care Unit (ICU) with ARI as a proxy for severity of disease from detection of HRV-C. Patients with ARI were managed either in the ICU or non-ICU setting in the hospital. Of the four admitted to ICU, three (n=3; 2%) had HRV-C detected compared to twelve (n=12; 8%) with HRV-C detected but managed in the non-ICU setting.

3.6.1 Sequence Diversity

Sequence analysis based on the VP4-VP2 yielded 4 HRV-A: A2, A56, A58, A98, 1 untyped, followed by 1 HRV-B: B27. Within each HRV genotype, except for genotype B, there was a broad range of genotypes identified. There were 12 HRV-C, most frequently detected: C1, C3, C8, C11, C43, C47 and 2 untyped. Within each HRV species, there was a broad range of genotypes identified (Table 3.5).

Table 3.5: Distribution of HRV species and genotypes in 21 HRV-infected patients

HRV Species	**Genotypes**
A	A56, A2, A58, A94, Untyped
B	B27
C	C1, C1, C1, C3, C8, C8, C8, C11, C43, C43, C47, C48, Untyped, Untyped, Untyped

In the Virology laboratory, the threshold for positivity is a Ct value 40. As Ct value is inversely proportional to viral load, we sought to determine the relationship between the viral load of different HRV genotypes and the severity of the disease.

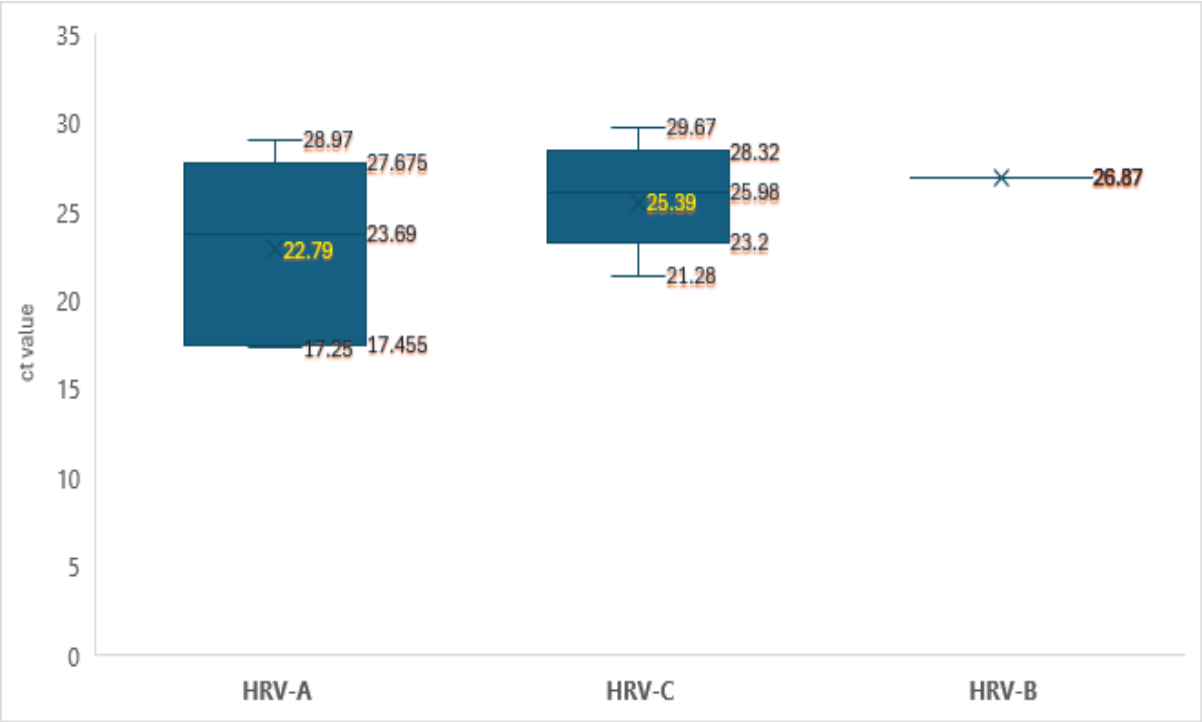


Figure 3.7: Box and whisker plots showing Ct values of the HRV species.

The boxes represent the interquartile range (difference between first and third quartile, IQR), and the lower and upper limits of the whiskers represent the maximal and minimal value. The number within each box is the mean Ct value per group.

We describe Ct values for each HRV species. As shown in Fig. 7, the Ct value of HRV-A ranged from 17.45 to 27.67 and HRV-C ranged from 23.2 to 28.32. The viral load of HRV-A was higher than that of HRV-C.

Heatmap showing the distribution of frequent genotypes across the HRV positive samples sequenced. The intensity of the colour correlates to the frequency of samples.

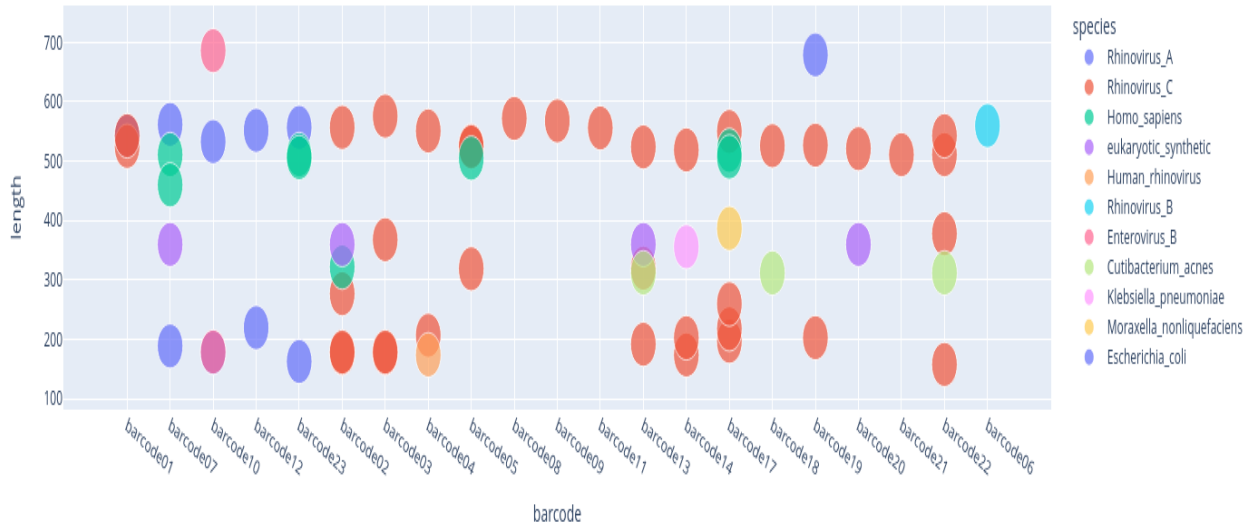


Figure 3.8: Scatter plot of Distribution of genotypes.

Reference sequences are presented by GenBank accession number and strain. Isolates from clinical samples are indicated by a sample barcode. Barcodes 15 and 16 were not included in the tree because they had non-HRV sequences

One virus that did not cluster with HRVs was identified as Echovirus 25.

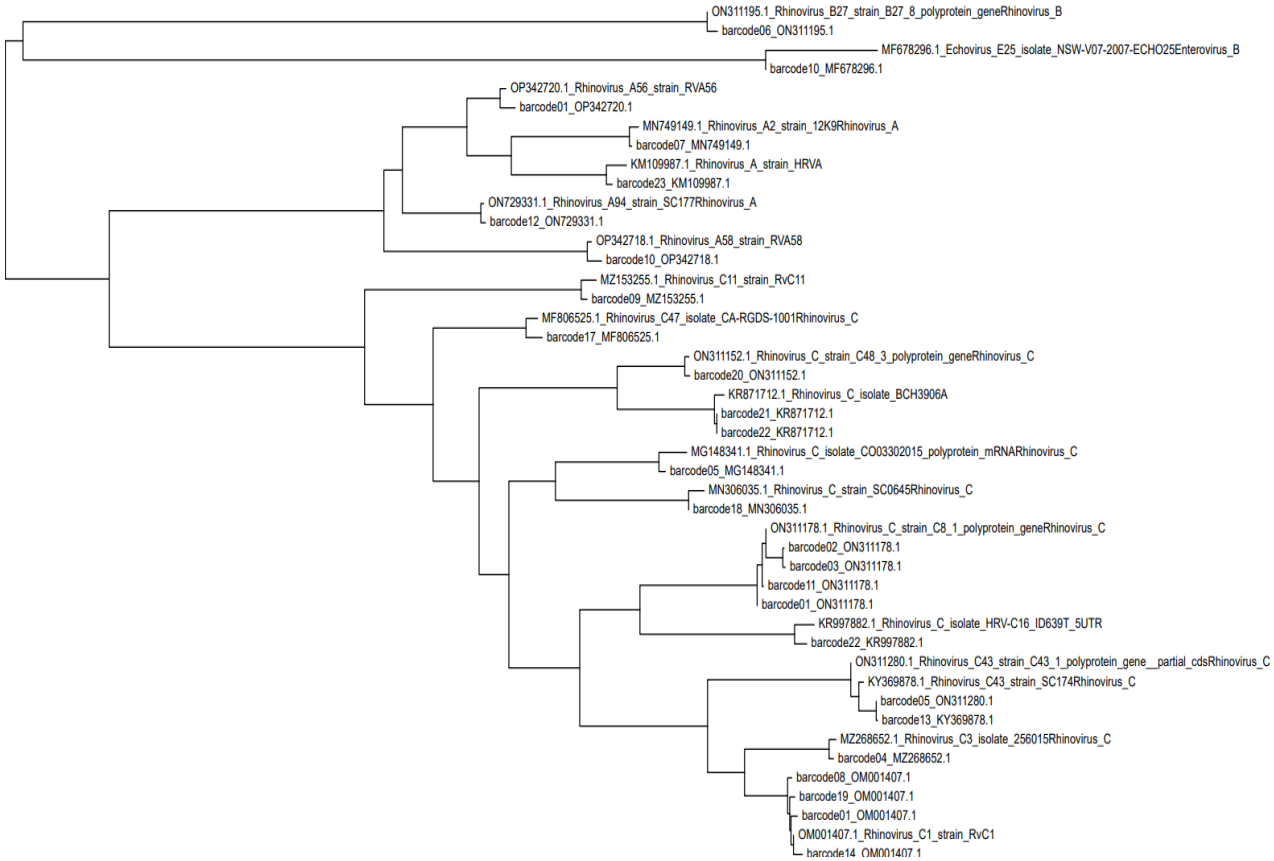


Figure 3.9: Phylogenetic tree on HRV based on VP4/VP2 coding region.

Chapter 4: Discussion

Human rhinoviruses are highly prevalent and continue to constitute a significant public health burden. There is a large body of epidemiologic evidence supporting high rates of HRV detection in children and adults with common colds, wheezing illnesses, and pneumonia, and even in asymptomatic individuals. In addition, the advent of increasingly sensitive molecular techniques for viral respiratory pathogen diagnosis has led to an increased appreciation of a wide range of clinical illness, a result of infection by HRVs.

The present study is the first to determine the different genotypes of HRVs circulating in the Free State among children and adults with acute respiratory infection between January 2024 - October 2024.

4.1 HRV prevalence

Our study describes respiratory viruses (RV) circulating among patients from all age groups with acute respiratory tract infections (ARI) managed in public sector clinics and hospitals across the Free State Province in South Africa. During the 10-month period, in 329 (59.50%) samples positive for respiratory viruses, 116 (35.3%) were human rhinovirus positive. Rhinovirus was the most frequently detected virus, followed by RSV, Influenza A Influenza B and PIV whilst HAdV and HMPV were also detected but at <3% of cases.

Our data on 35.2% HRV detection rates is consistent with prior studies conducted in the Free State, reporting HRV prevalence rates of 30% in hospitalised children (Ogunbayo et al., 2019). Smuts et al, reported higher HRV prevalence rates of 58.2% in children managed at the Red Cross Hospital in Cape Town. In addition, in a study of sudden unexpected death in infancy (SUDI), HRV was detected in 50% and 10% from use of flocculated swabs in sampling the tracheal and lower lobes of both lungs respectively (Vanmali et al, 2024).

Lower HRV prevalence has however been reported from a number of studies from 2010 to 2020, reporting HRV prevalence rates of 21- 25% (Baillie et al.,2019; Helfeeresce et al., 2019, Pretorius et al., 2014).

4.2 Co-detection

Advances in laboratory diagnostic techniques have resulted in an increasing incidence of virus to virus codetection, creating a diagnostic challenge as a number of these pathogens have an uncertain pathogenicity.

We reported a co-detection rate of 30%, with HRV-RSV combination being the most common (n=16; 43%). A number of studies have reported an incidence of virus-virus codetection between 15% and 45% - although this would be influenced by age and testing methods (Aberle et al., 2005; Peng et al., 2009; Kouni et al., 2013).

A number of reports have argued that co-detection is merely a reflection of viruses in circulation at the time (Xiang et al., 2010; van der Linden et al., 2016; Blaschke et al., 2018). Given the high rates of RSV and rhinovirus infections in our cohort, we think it acceptable that they should be the most commonly identified viruses in co-infected patients. This occurrence has been demonstrated in multiple studies (Cebey-López Miriam et al., 2015; Goka et al., 2015; Goka et al., 2014). Although virus-virus codetection is thought to have a limited impact on clinical severity among children with ARI, infection with specific pathogen pairs might be associated with more severe outcomes (Lim et al., 2016).

4.3 RV genotype

Our study is one of a few studies to describe the epidemiology of HRV genotypes among children and adults managed in the public sector in the Free State. All three HRV genotypes were shown to circulate with more than half (57.1%) of the HRV strains belonging to HRV-C, and 19% of the typed HRV samples belonging to HRV-A, while only one (4.8%) were HRV-B. The finding is consistent to several studies of children hospitalized with respiratory illness where HRV-C was the most frequently identified species (Linsuwanon et al., 2009; Bizzintino et al., 2011; Miller et al., 2009; Huang et al., 2009; Lau et al., 2009). A study by Smut et al in Cape Town also reported the predominance of HRV-C genotypes in hospitalised children. (Smuts et al., 2011). The predominance of HRV-A and C reported in adult and paediatric patients in other South African studies, was not seen in our cohort. In addition, we were only able to report on one case of HRV-B in our cohort and our findings is consistent with reports of HRV-B being often only found at very low levels (0–3%) or being observed

sporadically (Saraya et al., 2017; Yan et al., 2017; Howard et al., 2016; Bruning et al., 2015).

It is acknowledged that most of the HRV genotypes circulating in a given population may change over time resulting in variation in the prevalent HRV genotype at any given time (van der Linden et al., 2016). In our study, we identified 47 HRV strains in 21 patients. In the Free State cohort, 17 HRV genotypes were detected and predominant HRV-A genotypes were A-56, A-2, A-58, and A-94; those of HRV-B were B-27, and the predominant HRV-C genotypes were C-1 (3), C-3, C-8 (3), and C-43 (2), C-11, C-47, C-48 and 3 untyped.

Four samples from our cohort were untyped, 3 from HRV-C, with one from HRV-A genotypes. (Saraya et al., 2017; Yan et al., 2017; Howard et al., 2016; Bruning et al., 2015)

4.4 Severity of Disease

An association between HRV genotypes and the severity of the disease is widely recognised (Fawkner- Corbett et al., 2016; Marcone et al., 2014 ; Zhao et al., 2018. In addition, HRV-C has been identified as a major contributor to HRV disease.

Although we did not review clinic files to assess for disease severity, a study involving HRV-positive specimens identified through severe acute respiratory illness (SARI) surveillance in four provinces from 2009 to 2010 in South Africa, did not identify any difference in disease severity due to different HRV type (Pretorius et al in 2014).

Conclusion

The ARI incidence as well as pathogen detection rates, were higher during the months of May through June 2024 in the Free State. HRV and RSV were the predominant viral causative agents in under-five ARIs. Our findings showed that there was a high diversity in the sequences of the HRV strains that circulated in hospitalized South African patients with respiratory disease. We were not able to undertake review of patient files and determine the association between symptoms and HRV genotypes. Further studies are necessary to determine severity of HRV genotype C and if other factors play a role in RV-associated disease.

Limitations

The study is limited to a single tertiary institution which is in the Free State, so the findings may not generalize all the South African population. The study also provides a snapshot of circulating genotypes circulating in a 8 month-period, which will make it impossible to determine how prevalence changes over several years or the emergence of new genotypes. The study lacks detailed clinical information about the outcome of infection (mild, moderate or severe) of children making it challenging to associate genotype and clinical outcome. Four HRV genotypes were untypable (Table 3.5). All the 4 samples that failed to be typed had high Ct values and thus low HRV viral loads. It was therefore speculated that the low viral titres, based on the high Ct values were responsible for the failure to sequence. Another notable limitation of this study is the relatively small sample size that will be utilized due to budgetary constraints.

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Annexure

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Ms L Shabalala

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M240845

NAME: (Principal Investigators)	Ms L Shabalala	**DEGREE:** MSc
SCHOOL:	School of Pathology Department of Virology Faculty of Health Sciences	
PROJECT TITLE:	*Molecular epidemiology of circulating Human Rhinovirus/ Enterovirus in children and adults with acute respiratory infections at a tertiary hospital in Free State* Change of study title noted on 2024/11/12	
DATE CONSIDERED:	Ad hoc	
DECISION:	Approved unconditionally	
CONDITIONS:	Laboratory study	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>	
SUPERVISOR:	Drs Z Makatini, N Nxele and M Moloi; Ms H Mthinyane	
APPROVED BY:	Professor P Ruff, Chairperson, HREC (Medical)	
DATE OF APPROVAL:	2024/08/19	**EXPIRY DATE:** 2029/08/18

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report** in the format available at https://wits.ac.za/research/researcher-support/research-ethics/ethics-committees/. This report is due annually, for the duration of the Clearance Certificate, beginning on the first anniversary of Date of Approval above. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Shabalala
Signature of Principal Investigator

30/11/2024
Date