CLINICAL SEVERITY OF RHINOVIRUS/ENTEROVIRUS COMPARED TO OTHER RESPIRATORY VIRUSES IN CHILDREN

By

Sandra A. Asner

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AUTHOR:	Sandra A. Asner MD (University of Bern, Switzerland)
SUPERVISOR:	Professor Marek Smieja, MD, PhD
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ABBREVIATIONS

ADV	Adenovirus
ARIs	Acute respiratory infections
CAP	Community-acquired pneumonia
FLU A/B	Influenza A/B
HBoV	Human bocavirus
HSCT	Hematopoietic stem cell transplant
hMPV	Human metapneumovirus
HRV/ENT	Human rhinovirus/enterovirus
LRTI	Lower respiratory tract infections
PIV 1-4	Parainfluenza virus types 1-4
RSV A/B	Respiratory syncytial virus A and B
SOT	Solid-organ transplant
URTI	Upper respiratory tract infections

ABSTRACT

Recent evidence suggests that human rhinovirus/enterovirus (HRV/ENT) infections are commonly identified in children with acute respiratory infections (ARIs), such as upper respiratory tract infection, acute asthma exacerbations, bronchiolitis or community-acquired pneumonia. However, data on the clinical disease severity of HRV/ENT infections remains limited. Observational studies conducted amongst hospitalized children with single respiratory viral infections have reported varying results of clinical disease severity of HRV/ENT infections. Whether HRV/ENT acute respiratory infections are associated with severe clinical disease remains unclear. Assessing the association of HRV/ENT infections with severe disease as compared to other respiratory viruses is of particular interest given its prevalence, morbidity and economic burden.

We conducted a retrospective cohort study of children under 18 years of age at the Hospital for Sick Children, a tertiary care paediatric hospital in Toronto, Ontario, Canada, to compare the clinical severity and the rates of community-acquired pneumonia (CAP) in children with HRV/ENT, versus those with respiratory syncytial virus (RSV), influenza A/B (FLU A/B), or other common respiratory viruses.

A total of 742 children with any respiratory illness were evaluated, of whom 462 (62.3%) were tested positive for one or more respiratory viruses by two or more of the following molecular assays: ResPlex II v2.0, Seeplex RV15 kit, xTAG- RVP and xTAG-RVP Fast. Among these, 381 (82.5%) tested positive for a single respiratory infection: 116

HRV/ENT, 102 RSV, 99 FLU A/B and 64 other common respiratory viruses were identified. The remaining 81 (17.5%) had two or more respiratory viruses and were excluded from this analysis.

Children with single HRV/ENT infections presented with significantly higher rates of underlying immunosuppressive conditions compared to those with RSV (37.9% vs 13.6%; p<0.001), FLU A/B (37.9% vs 22%; p=0.018) or any other single viral infection (37.9% vs 22.5%; p=0.024). In multivariable analysis adjusted for underlying conditions and age, children with HRV/ENT infections had increased odds of hospitalization compared to children with RSV infections (OR 2.6; 95% CI : (1.4, 4.8); p<0.003) or with FLU A/B infections (OR 3.0; 95% CI: (1.6, 5.8); <0.001), and increased odds of severe clinical disease among inpatients (OR 3.0; 95% CI: (1.6, 5.6); p=0.001) when compared to those with FLU A/B infections.

In conclusion, children with HRV/ENT had significantly higher rates of underlying comorbidities and a more severe clinical course than those with RSV and FLUA/B infections. These findings emphasize the importance of considering HRV/ENT infection in children presenting with severe acute respiratory tract infections.

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Part I. INTRODUCTION

Human rhinovirus/enterovirus

Classification

Human rhinovirus/enterovirus (HRV/ENT) belongs to the picornaviridae family, which consists of 12 genera and 28 species (Knowles 2010). HRV/ENT belongs to the Enterovirus genus, which consists of 10 species. Until 2009, rhinoviruses and enteroviruses were separate genera, but have been recently reclassified together as genus Enterovirus to correct phylogenetic inconsistencies, and were further classified into Enterovirus species A-D and Rhinovirus (HRV) Species A to C. The picornaviruses are single-stranded positive sense RNA viruses of 22-30 nm, icosahedral in structure and containing no lipid envelope. The capsid contains four viral proteins (VP1, VP2, VP3 and VP4) of which VP1-VP3 constitute the major part of the capsid. They have a limited host range (human and animal) and are transmitted to humans horizontally by contact or droplets (Bella & Rossman, 2000; Gwaltney, 2002). Enteroviruses can infect humans and other mammals including pigs, sheep, buffalo and cows. Human enterovirus A (ENT-A) and B (ENT-B) affect humans and animals (monkeys for ENT-A and monkeys and pigs for ENT-B) whereas human enterovirus C (ENT-C) and human enterovirus D (ENT-D) affect humans only. Other species strictly infect the animal reservoirs such as Simian enterovirus A, Bovine enterovirus and Porcine enterovirus B. Until recently, HRVs were

classified into two species HRV-A and HRV-B, based on phylogenetic sequence criteria. The development of highly sensitive molecular techniques for the identification of HRV in clinical specimens led to the identification and designation of a novel species, HRV-C, by the International Committee on Taxonomy of viruses in 2009 (Jacobs et al, 2013). HRV-C strains do not grow in standard cell culture, likely postponing their discovery. Therefore, a genetically based classification system was developed. HRV-C strains have a genomic organization similar to that of HRV-A and HRV-B. However, there are several distinct characteristics that support their classification as a new species. To date, at least 50 different types of HRV-C have been identified (Jacobs et al, 2013).

Pathogenesis

The incubation period of HRV/ENT consists of 8-12 hours, whereas their period of communicability is 3-11 days, but can be extended up to 4 weeks in immunocompromised subjects (Cate et al, 1964; van Elden et al, 2008). The majority (90%) of HRV A and B genotypes bind to intracellular adhesion molecule-1 (ICAM-1) as their cell receptor (Bella & Rossman, 2000; Bella & Rossman, 1999), while a minority (10%) bind to the low density lipoprotein (LDL) receptor (Jacobs et al, 2013). A different receptor seemed to be used by HRV-C although it remains as yet unidentified
(Bochkov et al, 2012). The attachment to the ciliated epithelial cells in the upper respiratory tract, results in uncoating of the capsid and further endocytosis of the viral RNA (Kennedy et al, 2012). The capsid is recognized by toll-like receptor (TLR)-2 on the

epithelium and HRV nucleic acids are detected by TLR3, TLR7, TLR8, melanoma differentiation associated gene-5 (MDA-5) and retinoic acid inducible gene-I (RIG-I) (Triantafilou et al, 2011; Slater et al, 2010). Activation of these receptors triggers the innate immune system to release type I and type III interferons to inhibit virus replication. In contrast to other respiratory viruses, HRV/ENT does not cause cytopathology but rather disrupts tight junctions in the epithelial barrier resulting in increased vascular leakage and mucus secretions (Kennedy et al, 2012). Viral titers begin to decline approximately 72 hours post-infection.

Epidemiology

Although HRV/ENT infections are isolated in all months of the year, the peak occurs at the beginning of the school year, presumably because contact among children increases (Monto et al, 1994; Arruda et al, 1997). The incidence of HRV/ENT infections in children under 2 years of life is 0.7-0.8 infections per person per year and up to 6 infections per person per year in school-aged children (Blomqvist et al, 2002; van Benten et al, 2003).

Diagnostic methods

Improved detection methods have increased the detection rates of asymptomatic HRV/ENT infections and resulted in ambiguity in the interpretation of positive test results (Jansen et al, 2011). The prevalence of asymptomatic HRV infections ranges from 5-40% as a result of different study-populations, definitions of asymptomatic events, and detection methods used (Peltola et al, 2008; Greenberg, 2011; Brownlee & Turner, 2007). HRV/ENT is commonly detected from respiratory samples, although case reports are increasingly identifying HRV/ENT in pericardial fluid, stool, plasma, and cases of HRV/ENT infections have been reported in urine in neonates. Conventional diagnostic methods include viral culture in primary pulmonary fibroblasts cells and acid lability testing (Santti et al, 1999). However, these diagnostic techniques are limited in their sensitivity as a result of numerous HRV/ENT strains, and are labor intensive, as they required separate equipment such as an incubator set at 33˚C (Lu et al, 2008) and prolonged culture of up to two weeks. Furthermore, serologic testing is not a diagnostic method of choice as a consequence of numerous HRV/ENT serotypes. This being said, direct fluorescent antibody (DFA) testing for multiple serotypes is also not a practical diagnostic option for HRV/ENT. Given these limitations, molecular assays such as reverse transcription-PCR (RT-PCR), are the diagnostic methods recommended for HRV/ENT testing (Santti et al,1999). Since 1988, numerous molecular assays, primarily targeting the 5’ untranslated region (5’-UTR) (Lu et al, 2008; Savolainen et al, 2003; Schibler et al, 2012; Do et al, 2010; Gambarino et al, 2009; Mahony, 2008; Santti et al, 1999), have been

reported for HRV/ENT detection. These assays are limited in that they do not distinguish between ENT and HRV without sequencing the amplicon, hybridization with an internal HRV-specific probes, or use of nested PCR (Mahony, 2008; Savolainen et al, 2003). The capsid proteins VP2/4 and VP1 have also been used for species classification. Their major limitation is their variablility in utilization for explicit species, which impairs the development of universal primers (Bochkov & Gern, 2012). Commercial diagnostic assays such as ResPlex II v2.0 (Qiagen, Mississauga, ON, Canada), Seeplex RV15 kit (Seegene Inc., Seoul, Korea), and xTAG-RVP /xTAG-RVP Fast, (Luminex, Austin TX) have been recently developed with different levels of analytical sensitivity for HRV/ENT detection. While xTAG-RVP and xTAG-RVP Fast are more sensitive than ResPlex II v2.0 and Seeplex RV15 kit for HRV/ENT detection, they do not distinguish HRV from ENT. Furthermore, these assays are limited in that they cannot detect all known HRV genotypes and cannot quantify viral load, which is poorly correlated to clinical outcomes (Utokaparch et al, 2011; Franz et al, 2010). Other commercial assays such as MassTag PCR (detection of final product using mass spectrometry, Centre for Infection and Immunity, Columbia University, NY) and Respiratory MultiCode-Plx Assay (detection of final product using flow cytometry e.g EraGen Bisosciences Inc.,Madison, WI) contain both 5'UTR and capsid gene primers for rapid, sensitive detection and species typing (Bochkov & Gern, 2012). MassTag PCR has been utilized to identify novel HRV C genotypes (Xiang et al, 2010; Miller et al, 2009; Fuji et al, 2011; Iwane et al, 2011).
The advent of molecular assays has increased the detection of HRV/ENT by 85% (van Der Zalm et al, 2009). Furthermore, there are limited data on the correlation between viral

loads and clinical significance or severity. In the few studies that have investigated HRV viral loads, only hospitalized children were included (Utokaparch et al, 2011; Franz et al, 2010). These findings may suggest that a minimum amount of HRV viral loads may contribute to symptomatic illness and hospitalizations in children. Future studies, conducted in different patient-population (asthmatics, children with CAP or bronchiolitis), including important factors such as timing of sampling, sample quality and patient's underlying conditions, may clarify the evidence for this association (Jansen et al, 2011). The development of assays including measurement of viral loads may also encourage the development of HRV/ENT antivirals, as their efficacy could be monitored through the course of an infection.

Treatment

There are no specific treatments for HRV/ENT that are currently available. Given the large number of HRV/ENT serotypes, vaccines against HRV/ENT are not an option. Other prevention strategies such as vitamin D, ICAM blockers or Zinc have not been solely assessed for HRV/ENT. The identification of the crystal structure of HRV 14 in the late 1980s triggered the search for HRV/ENT inhibitors targeting the protein capsid (Rossmann et al, 1985; Thibaut et al, 2012). The first compounds developed, known as "WIN" compounds, were found to bind to the hydrophobic pocket of the capsid increasing the rigidity of the virion and decreasing its ability to bind to the receptor (Thibaut et al, 2012). The WIN compound pleconaril was tested in clinical trials in 1996 and showed

good tolerance and significant reduction in the duration and severity of colds but was rejected by the US. Food and Drugs Administration (FDA) in 2002 due to safety concerns as it was suspected in reducing the effectiveness of oral contraceptives through the production of CYP3A4, which likely led to intermenstrual bleeding in woman taking pleconaril and estrogen-based oral contraceptives (Hershenson, 2010). Another VP1 capsid inhibitor vapendavir or BTA 798 which interferes with the connection to ICAM-1 receptor has been tested in Phase I (2007) and Phase IIa clinical trials (2009) and achieved proof-of-concept by demonstrating reduction in incidence of viral shedding and symptomatic infection in volunteers infected experimentally. Alternatives to the capsid binding agents are proteolytic enzyme targets such as proteases 2A, 3C, and 3CD which are essential for viral replication (Thibaut et al, 2012). The most potent inhibitor developed was rupintivir, but it did not decrease the clinical severity or viral load in natural infections in a RCT/observational study (Thibaut et al, 2012). Another protease inhibitor is LVLQTM, targeting the 2A protease which has been shown to inhibit HRV 2 and 14 replication in A549 cells 500- and 150-fold respectively (Falah et al, 2012). Recent modeling of the capsid structure of HRV C showed that it had a shorter VP1 protein with a more restricted hydrophobic pocket than either HRV A or B thus suggesting that novel capsid-binding agents will be needed for HRV-C genotypes.

In summary the advent of molecular assays has enabled the detection of HRV/ENT from patients with various respiratory conditions although the spectrum of illness associated with these infections is not fully defined, as HRV/ENT testing is not done

routinely in most hospitals.

Clinical presentation

Human rhinovirus/enterovirus is the leading pathogen detected in children with symptoms of a common cold, but has been increasingly documented in hospitalized children with serious lower respiratory tract infections (LRTIs) (Iwane et al, 2011). The impact of HRV/ENT infections in LRTIs has been previously underestimated as many studies used conventional techniques for viral detection and were conducted outside high HRV/ENT prevalence periods (early fall and late spring months in the northern hemisphere) or for short periods of observation. Consequently, studies focusing on the winter months did not reflect the relative contribution of HRV/ENT to illness (Gwaltney et al, 2000). The advent of molecular assays has resulted in increased detection of HRV/ENT among children with acute asthma exacerbations, bronchiolitis and viral community-acquired pneumonia (CAP) (Hayden, 2004; Calvo et al, 2010; Miller et al, 2009; Messacar et al, 2013, Ruuskanen et al, 2011; Malcolm et al, 2001). Direct evidence for HRV/ENT invasion of the pulmonary parenchyma has been found in individual paediatric patients with pneumonia by virus isolation from bronchoalveolar lavage (BAL) and by immunohistochemistry of lung tissue in one case (Imakita et al, 2000). Furthermore, bronchiolitis, asthma exacerbations and pneumonia are the most common clinical entities described in children under 5 years of age admitted to hospital with HRV/ENT infection. (Imakita et al, 2000)

A review of seven studies reported the clinical profile of 643 HRV/ENT infections in children admitted to hospital has been reported (Ruuskanen, 2011) and 11-53% had a CAP. A study by Xiang et al, 2010 reported that 17.9% of CAP was due to HRV/ENT among a cohort of 528 children with community-acquired pneumonia. An older retrospective study over a six year period (Kim et al, 1998) reported that 95% of the 93 HRV/ENT culture-positive paediatric patients presented with LRTI and 14% with fever and suspected sepsis. Among these, almost 70% of the children presented with underlying conditions including prematurity, reactive airways and congenital cardiac disease, thus suggesting that patients with underlying co-morbidities were particularly at risk for HRV/ENT LRTI. According to a recent review of 431 virus-positive BAL or bronchial biopsy cultures from immunocompromised subjects aged 2.5-86 years and hospitalized with an acute respiratory illness, 7% were documented HRV/ENT positive, representing the third most common virus detected after CMV (64% of positive samples) and HSV (21%) (Malcolm et al, 2001). Detection of HRV/ENT was associated with a poor prognosis as 60% of the patients were admitted to the intensive care unit (ICU) and 25% died during hospitalization thus suggesting that HRV/ENT infections could be associated with severe lower respiratory tract disease in transplant recipients. A prospective study over an eight year period in bone marrow and stem cell transplant patients identified 31 patients with HRV/ENT infections, representing one quarter of community respiratory viral infections (Bowden, 1997). A recent retrospective analysis of 77 bone marrow-transplant adult and paediatric patients (Ison et al, 2003) with pneumonia during one respiratory season identified eight BAL samples (6% of 122 tested) in six patients (8%)

that were positive for HRV RNA by RT-PCR. The fatality rate in HRV-infected patients was 83%, but all of them had significant co-infections. This available evidence indicates that HRV/ENT may be associated with LRT disease in highly immunocompromised marrow transplants recipients, frequent co-infections and often poor prognosis. However the contribution of HRV/ENT to overall pathogenesis remains uncertain with regard to causing direct viral damage or predisposition to secondary invaders (bacterial or fungal) common in this patient-population. The timing of infection in relation to transplantation and type of transplant may be an important variable in regard to severity of disease, and more data are needed from both hematopoietic stem cell transplant (HSCT) and solid organ, particularly lung transplant recipients.

Recent evidence from the literature (Midulla et al, 2012; Papadopooulos et al, 2002) suggest that HRV/ENT is the second most common etiology of bronchiolitis after RSV in infants and possibly the most common cause of bronchiolitis in older children. A study by Midulla et al (Midulla et al, 2010) reported that HRV/ENT was identified in 9% of infants admitted with bronchiolitis whereas another study by Papadopoulos and colleagues (Papadopooulos et al, 2002), which focused on older children, reported more than 30% of HRV/ENT bronchiolitis thus suggesting the importance of this pathogen in older children. Human rhinovirus/enterovirus is also the leading respiratory virus documented in asthma exacerbations in children above 12 months of age (Johnston et al, 1996; Busse et al, 2010; Tan, 2005). The above mentioned study by Johnston et al, reported that 80-85% of asthma exacerbations were associated with viral infections in

children 9-11 years. Among these, HRV/ENT accounted for almost 70% of the detected viruses. Furthermore, the seasonality of asthma exacerbations is associated with the seasonality of HRV infections. A study by Johnston et al, 1996, suggested a correlation ($r=0.72$, $p<0.0001$) between the peaks of HRV/ENT infections usually more common in the fall and spring when children return to school from vacation and admission for asthma exacerbations. Recent evidence from the literature (Jackson et al 2008) suggests that early infections with HRV/ENT were a potential trigger of asthma exacerbation with increased odds for asthma exacerbations (OR = 31.7) compared to RSV (OR = 13.6). Although HRV/ENT has been well documented in asthma exacerbations in children, the role of HRV/ENT bronchiolitis occurring early in life in the development of asthma remains unclear. Only a small number of infants with HRV/ENT associated bronchiolitis will develop wheezing or asthma later in life, suggesting the importance of other factors such as recurrent viral infections, atopic background, which may all contribute to the development of asthma. (Kieninger & Regamey, 2012; Midulla et al, 2012).

While recent studies identified HRV/ENT as the leading pathogen in acute asthma exacerbations, bronchiolitis and viral pneumonia in older children, the clinical severity of respiratory illnesses attributed to HRV/ENT remains uncertain (Calvo et al, 2010; Miller et al, 2009; Papadopoulos et al, 2002; Ruuskanen et al, 2011; Malcolm et al, 2011).

Clinical disease severity

Clinical severity attributable to HRV/ENT LRTI remains controversial. A recent study (Iwane et al, 2011) reported equivalent disease severity between respiratory illnesses caused by HRV/ENT and those caused by other common respiratory viruses in hospitalized children above 5 years of age. In contrast, others studies (Midulla et al, 2012) conducted amongst infants admitted with bronchiolitis reported that HRV/ENT bronchiolitis resulted in a less severe disease compared to RSV bronchiolitis. Conversely, a study by Papadopulos et al. (2002) suggested that HRV/ENT was a significant predictor for severe bronchiolitis compared to other respiratory viruses. Explanations for the discrepancies observed amongst these studies (Johnston et al, 1995; Busse et al, 2010; Tan et al, 2005) include the use of different clinical criteria to assess clinical severity (clinical severity scores versus clinical outcomes) and the focus on different patient-populations and age groups (inpatients and young children).

One potential explanation for the discrepancies in clinical severity documented among HRV/ENT infected children and across studies can be explained by the pathogenicitiy of different HRV/ENT strains: HRV/ENT genotyping suggests that HRV species C results in more severe lower respiratory tract infections in young children compared to HRV- A or -B (Bochkov & Gern, 2012; Lau et al, 2007; Tapparel et al, 2009; Khetsuriani et al, 2008; Arden & Mackay, 2010). Other studies of ARI cases seen in emergency or urgent care settings reported significantly higher detection of HRV-A

(Khetsuriani et al, 2008; Piotrowska et al, 2009) and HRV-C (Wisdom et al, 2009) compared with asymptomatic controls thus suggesting that HRV-A and C were mainly associated with disease whereas HRV-B may be more commonly detected among asymptomatic subjects. While HRV-C has been associated with more severe outcomes, a recent genetic analysis of 144 HRV clinical samples from lung transplant patients and hospital patients (children and adults) with upper and lower respiratory tract infections showed no correlation between a given species and ability to invade the lower respiratory tract (Arakawa et al, 2012). In conclusion, only a limited number of studies addressed the question of clinical severity and included genotyping of HRV/ENT strains, therefore findings from published studies remain unclear.

Limitation of Previous Research

Previous studies have several key limitations. Most of the studies did not report underlying co-morbidities, thus the contribution of HRV/ENT infection versus host-related factors such as underlying immunosuppression to the severity of the disease was not reported in most studies (Iwane et al, 2011; Khetsuriani et al, 2008; Piotrowska et al, 2009). Also, most of these studies did not provide information on bacterial co-infections or antibiotic prescription, which are important confounding variables to adjust for in multivariable analyses when addressing the question of clinical severity. Furthermore, most of published studies did not include a control group of asymptomatic subjects in their analyses. This is an important point as HRV/ENT can be detected in asymptomatic

subjects in up to 40% of cases. Furthermore results from different observational studies addressing the question of disease severity of HRV/ENT infections are conflicting as a result of different patient-population and age groups studied (inpatients vs young children) and different clinical criteria to assess clinical severity (clinical severity scores versus clinical outcomes). Also a selection bias may have occurred in these observational studies as these studies did not enroll consecutive patients. Finally, different diagnostic molecular assays and specimens (nasal swabs vs. mid-turbinate vs. throat swabs) were used by these studies which may in turn affect the sensitivity for viral detection as available molecular assays have different sensitivities for the detection of HRV/ENT infections.

Rationale

There is growing evidence that HRV/ENT is the most commonly identified virus in both adults and children with respiratory symptoms, and thus needs to be regarded as a pathogen responsible for much more than just the common cold. Human rhinovirus/enterovirus (HRV/ENT) infections may result in significant morbidity and cost. Available literature has provided controversial conclusions about the severity of HRV/ENT infections. To date, the most recent study by Iwane et al, 2011 evaluating the severity of HRV/ENT, only included in-patients and compared HRV/ENT to any other single respiratory viral infection instead of comparing HRV/ENT to FLU or RSV individually. This is an important limitation as FLU or RSV infections may result in more

severe outcomes compared to HBoV or coronaviruses, thus limiting the conclusions that can be drawn.

A study, which would include inpatients and outpatients and would allow individual comparison of HRV/ENT with FLU, RSV and any other respiratory infections would provide better insight into that question. By documenting that HRV/ENT, RSV, FLU infections may result in equivalent severity of disease, our study would reinforce the need for routine screening in hospital settings as HRV/ENT infections may cause more severe disease than assumed. Also, at this point, preventive strategies and antivirals are available for RSV or FLU but not for HRV/ENT. By demonstrating that HRV/ENT is associated with significant morbidities may enhance the need for developing preventing and treatment strategies. This may in turn, reinforce the need to focus on better HRV/ENT antiviral therapies as current options are limited.

PART II: OBJECTIVES, RESEARCH QUESTIONS AND METHODS

Objectives of the Project

This project had two main objectives. The first objective was to compare the clinical disease severity of HRV/ENT to respiratory syncytial virus (RSV), influenza A/B (FLU) and other common respiratory virus in children with ARIs, and to determine whether clinical disease severity was associated with viral status (HRV/ENT vs FLU, RSV or any other single respiratory infection) while controlling for host factors such as age or underlying co-morbidities.

The second aim was to determine the proportion of children presenting with upper respiratory tract infection (URTI) and CAP and to assess how these proportions of URTI and CAP compare to the ones identified in children with other traditional viruses.

Research Questions

Primary question

Amongst children under 18 years of age with ARTIs, evaluated at a tertiary care center (as inpatients or outpatients in the emergency room (ER)), is HRV/ENT associated with equivalent disease severity as compared to traditional viruses?

Secondary questions

Amongst children under 18 years of age presenting at a tertiary care center with HRV/ENT disease:

1) What is the proportion of children presenting with upper respiratory tract infection (URTI) and CAP?

2) How do these proportions of URTI and pneumonia compare to the proportions identified in children with traditional viruses?

Methods

The Dataset

Our dataset consists of data from the microbiology laboratory at the Hospital for Sick Children, Toronto, Ontario, Canada. The goal of the original study was to compare the sensitivities of four different multiplex assays: ResPlex II v2.0 (Qiagen, Mississauga, ON, Canada); Seeplex RV15 kit (Seegene Inc., Seoul, Korea); xTAG- RVP and xTAG-RVP Fast, (Luminex, Austin TX) (Gharabaghi et al, 2011). Details and results of this study have been previously published (Gharabaghi et al, 2011). The dataset was augmented through retrospective chart review, which provided information on patient-characteristics, clinical outcomes, laboratory data and antibiotic prescription.

Population and Definitions

Children under 18 years of age presenting with an acute respiratory illness and any single viral infection documented by molecular assays from mid-turbinate swabs to the Hospital for Sick Children, Toronto, Ontario, Canada were included in the study. Specimens were collected from November 2007 to April 2008 and January to March 2009, the times when multiplex PCR testing was utilized in consecutive randomly selected patients under 18 years of age presenting with ARTI with either an URTI or LRTI. Children presenting with ARI are routinely tested for respiratory viruses by DFA at the Hospital for Sick Children. Laboratory staff randomly selected the first 25 patients per week from a list of children with ARI in whom midturbinate swabs were sent for viral detection. Acute respiratory tract infections (ARTIs) included the common cold, pharyngitis, laryngitis, tracheitis, bronchitis, bronchiolitis and community-acquired pneumonia (CAP). URTI was defined as the detection of any single respiratory viral infection together with symptoms involving the upper respiratory tract (nose and pharynx) (Khanna et al, 2008). LRTI was defined as any patient with cough, tachypnea and or any respiratory distress or wheezing. Community-acquired pneumonia (CAP) was defined as any of the above LRTI symptoms with pulmonary infiltrates diagnosed on a chest-x-ray by a radiologist. (Khanna et al, 2008) Severe clinical disease was defined by hospital admission for outpatients, and by a composite end-point including intensive care (ICU) admission, hospitalization > 5 days, oxygen requirements or death in patients admitted to

the hospital. Ethics approval was obtained from the Research Ethics Board at The Hospital for Sick Children.

In addition to symptom data, information on age, gender and relevant baseline characteristics such as underlying co-morbidities and outcomes were all extracted from health records. Relevant underlying co-morbidities were grouped into 3 mutually exclusive categories: cardio-respiratory, prematurity and any immunosuppressive/metabolic conditions. One or more of the following immunodeficiency states were included in the latter group: Hematopoietic stem cell transplant (HSCT) (allogeneic and autologous transplants) and solid-organ transplant (SOT) recipients, recipients of cancer chemotherapy or long-term immunosuppression for any chronic disease, and congenital immunodeficiency states. Metabolic conditions included any inherited metabolic diseases such as cystinuria, phenylketonuria (PKU), gout and thyroid disease. In case of multiple co-morbidities, patients were referred to the group considered as the most significant co-morbidity: an underlying immunocompromised/metabolic condition was considered the most significant comorbidity, followed by a cardio-respiratory comorbidity. Outcomes consisted of hospital admission, duration of hospitalization, admission length > 5 days, intensive care (ICU) admission, any supplemental oxygen requirements, and presence of URTI, CAP and all-cause mortality.

Virology studies

From November 2007 to April 2008 and January 2009 to March 2009, 25 mid-turbinate nasal flocked swabs (FLOQSwabs, Copan Italia, Brescia, Italy) were randomly selected each week amongst all specimens collected from children with acute respiratory tract infections (ARTIs) and submitted to the clinical laboratory for routine virology testing including molecular assays. Neither laboratory staff nor clinicians were aware in advance which patient would be selected for viral detection by molecular assays, which minimized selection bias. Swabs were assayed by four different nucleic acid amplification-based assays: ResPlex II v2.0 (Qiagen, Mississauga, ON, Canada); Seeplex RV15 kit (Seegene Inc., Seoul, Korea); xTAG- RVP and xTAG-RVP Fast, (Luminex, Austin TX). These assays detect up to18 different respiratory viruses (RSV [A, B], coronaviruses [OC43, 229E, NL63, HKU1], rhinovirus/enterovirus (HRV/ENT), PIV [1, 2, 3, 4], FLU-A, FLU-B, bocavirus [HBoV], ADV [A,B,C,D,F]. and hMPV). ResPlex II v2.0, and Seeplex RV15 assays distinguished HRV from ENT whereas xTAG-RVP and xTAG-RVP fast assays reported a combined result of HRV/ENT. For simplicity, HRV/ENT were combined in this study. All specimens were also examined by direct fluorescent antigen assay (DFA) for 8 respiratory viruses (respiratory syncytial virus (RSV), influenza virus [A,B] (FLUA/FLUB), parainfluenza [1-3] (PIV), adenovirus (ADV) (SimulFluor®, Millipore, Temecula, CA) and human metapneumovirus (hMPV) (Diagnostic HYBRIDS, Athens, OH)) and/or viral culture. We defined a viral result as a true positive if positive by viral culture regardless of other tests; if positive by DFA and at

least one molecular test; or if positive by two different molecular tests for viruses not detectable by DFA. For those only detectable by molecular assays (HRV/ENT, coronaviruses, HBoV and PIV 4), a true positive result was defined as two or more positive test results from amongst the four molecular assays (Gharabaghi et al, 2011).

Study Design and Statistical Analysis

The first objective of the study was to compare the severity of illness in children presenting with HRV/ENT positive mid-turbinate swabs with RSV A/B, FLU A/B and other respiratory viruses, namely PIV 1-4, hMPV, ADV, HboV, HKU1,OC43, NL63 and 229E detected by molecular assays. The following sections outline the study design and the statistical analysis used.

Study design

Predictor of interest was viral status, where HRV/ENT was used as the reference and compared individually to RSV A/B, FLU A/B and other respiratory viruses (including PIV 1-4, hMPV, ADV, HboV, HKU1,OC43, NL63 and 229E). Covariables of interest included age, gender and presence of any of the three underlying conditions outlined above. Age was a continuous variable whereas any of the three underlying conditions were considered as categorical variables. Severe clinical disease was defined by hospital admission (primary outcome) for outpatients. For patients admitted to the hospital, a

composite end-point, which included admission to the intensive care unit (ICU), hospitalization >5 days, oxygen requirements or death was used as a proxy of clinical severity (secondary outcome). Both outcomes were dichotomous. In addition, length of admission in hospital, a continuous outcome, was measured among inpatients.

Statistical analysis on the primary objective

Standard descriptive and comparative statistical analysis were performed on data categorized by viral pathogen, where HRV/ENT was used as the reference group and compared to RSV, FLU, and to a category consisting of all other common viruses including PIV 1-4, hMPV, HBoV, ADV and coronaviruses. Standard descriptive and comparative statistical analysis were used to examine the study sample baseline characteristics such as age, gender and underlying conditions and clinical outcomes which included URTI, CAP, hospital admission, admission in the intensive care unit (ICU), oxygen requirements, length of hospital admission and mortality. The χ^2 test or Fisher's exact test was used to compare categorical variables between groups as appropriate. Continuous variables were reported using the median and interquartile range (IQR) for non-normally distributed data. Number and percentage were reported for dichotomous outcomes.

Logistic regression was used to examine viral status as a predictor of dichotomous outcomes (hospital admission and composite end-point). All relevant co-variables (viral

status, age, gender, presence of any of the three mutually exclusive underlying comorbidities) were first evaluated using univariable logistic regression. Variables with a P-value < 0.1 were considered for inclusion in the multivariable model and the final model was determined using a step-wise backwards elimination method.

Our *a priori* hypothesis was that subjects with underlying immunosuppressive conditions were at higher risk for HRV/ENT infections but were also more likely to be admitted to hospital. The association of immunosuppression with both the outcome (hospital admission) and viral status suggested confounding. We created thus another model, which included an interaction term measuring the combination of HRV/ENT positive status and underlying immunosuppressive conditions in addition to all relevant co-variables. Co-variables were analyzed and included in the final logistic regression model as outlined above. The model including the interaction variable was only tested for the primary outcome variable.

Linear regression was used to examine the relationship between co-variables of interest and length of admission in hospital. This continuous outcome was right skewed and was therefore found to violate the assumption of normality required in linear regression. A natural logarithmic transformation was performed and linear regression was fitted using the transformed variable as an outcome. Variables with a P-value < 0.1 were considered for inclusion in the multivariable model and the final model was determined using a step-wise backwards elimination method.

The geometric mean was derived by back transforming the arithmetic mean of the log- transformed length of stay for each co-variable. The back transformation of the mean difference between two 'viral status 'groups for the ln transformed data was equivalent to the ratio between these two means. The geometric means were on the same scale and with the same units as the original outcome measure (number of days of admission in hospital) (Sedgwick, 2012). Co-variables were analyzed and included in the final model similar to the logistic regression model as outlined above.

All estimates are presented with 95% confidence intervals. A P- value < 0.05 was considered significant. SPSS statistical software (version 20.0, SPSS Inc, Chicago, IL, USA) was used to conduct the analyses.

The second objective was to compare the proportion of common respiratory illness (URTI, pneumonia) between children presenting with HRV/ENT positive mid-turbinate swabs and the ones with RSV A/B, FLU A/B and other respiratory viruses (see above) positive mid-turbinate swabs detected by molecular assays.

The study design used to address this objective was the same as outlined above. Predictors of interest include viral status and all other co-variables outlined in the primary question. The two binary outcomes of interest were URTI and community-acquired pneumonia.

Statistical Analysis on the secondary objective:

Standard descriptive and comparative statistics were performed on data categorized by viral pathogen, where HRV/ENT was used as the reference group as outlined in the previous subsection above. These were used to examine the clinical outcomes. The χ^2 test or Fisher's exact test was used to compare theses categorical outcome variables between groups as appropriate.

Univariable and multivariable logistic regression models were used to examine the relationship between co-variables and URTI and CAP. Co-variables were analyzed and included in the final logistic regression model as outlined above.

All estimates are presented with 95% confidence intervals. A P-value < 0.05 was considered significant. SPSS statistical software (version 20.0, SPSS Inc, Chicago, IL, USA) was used to conduct the analyses.

PART III: RESULTS

A. Patient characteristics

A total of 742 children under 18 years of age, evaluated for any respiratory illness, were screened for respiratory viruses from mid-turbinate swabs by molecular assays. Of these, 462 (62.3%) children were detected positive for any respiratory virus. A total of 81 (17.5%) had two or more respiratory viruses and were excluded from analyses. The remaining 381 (82.5%), which tested positive for a single respiratory virus, were included in the study. Of these, 116 (30.4%) tested positive for HRV/ENT, 102 (26.8%) for RSV, 99 (26.0 %) for FLU and the remaining 64 (16.8%) for other respiratory viruses (Figure 1). Among the 462 patients included in this study, 205 (53.8%) were admitted in hospital and among these inpatients, 143 (37.5%) presented with severe disease.

Subject characteristics are presented in Table III-1. The median age was 1.2 years (interquartile range (IQR): (0.4-3.9); 62.2% were male. Immunosuppressive underlying conditions were present in 24.7% of the subjects (n=94); cardiorespiratory conditions in 18.4% subjects (n=70) and 7.6% (n=29) were children who were born under 37 weeks of gestational age. Children with HRV/ENT infections were significantly older compared to those with RSV infections (median age 1.2 years vs 0.5 years, $p<0.001$) but significantly younger than those with FLU infections (median age 1.2 years vs 3.7 years, $p=0.001$). Children with HRV/ENT infections presented with a significantly higher rate of

underlying cardio-respiratory co-morbidities compared to children with RSV (31.9% vs 14.6%; OR = 2.5; 95% CI: (1.4, 5.0); p=0.003), FLU (31.9% vs. 9.8%; OR = 5.0; 95% CI: (2,10); p=0.001) or any other single viral infection (31.9% vs. 12.5%; OR = 3.3; 95%CI: (1.4, 7.1); p=0.002). Likewise, children with HRV/ENT infections presented with significantly higher proportions of underlying immunosuppressive/metabolic conditions compared to those with RSV (37.9% vs. 13.6%; OR = 3.3; 95% CI: (2,10); p<0.001), FLU (37.9% vs. 22%; OR = 2.2; 95% CI: (1.1, 4.1); p=0.018) or any other single viral infection (37.9% vs. 22.5%; OR = 2.0; 95% CI: (1.1, 3.3); p=0.024). There were no significant differences in proportion of prematurity between children with HRV/ENT, RSV, FLU or any other single viral infection.

B. Clinical disease severity

1. Predictors of hospitalization

Predictors of hospitalization are outlined in Table III-2. In univariable analyses, viral status, age, underlying immunosuppressive/metabolic conditions and cardiorespiratory co-morbidities were associated with hospital admission, which was defined as a categorical variable. Children with HRV/ENT infections were significantly more likely to be hospitalized compared to those with RSV infections (OR = 4.3; 95% CI: (2.4, 7.5); p<0.001) or FLU infections (OR = 3.2; 95% CI: (1.8,5.6); p<0.001). Age (OR = 1.1 per year, 95% CI: (1.1,1.2); p<0.001), underlying immunocompromised/ metabolic

(OR = 8.2; 95% CI: (4.4,15.4); p<0.001) and cardiorespiratory conditions (OR = 2.0; 95% CI: (1.1, 3.4); p=0.014) were significant predictors of hospital admission. There were no interactions between underlying immunosuppressive/metabolic conditions and HRV/ENT infections (OR = 0.8; 95% CI: (0.2,3.0); p=0.702) as documented in a model which included an interaction variable between HRV/ENT and immunosuppression/metabolic underlying condition in addition to HRV/ENT positive status, age, underlying cardiorespiratory, immunosuppressive/metabolic conditions and prematurity. (Table III-4)

In multivariable analysis adjusted for age and co-morbidities, children with HRV/ENT infections were significantly more likely to be hospitalized compared to those with RSV infections (OR = 2.6; 95% CI: (1.4, 4.8); p<0.003) or FLU infections (OR = 3.0; 95% CI: (1.6-5.8); <0.001) (Table 2). Furthermore, age (OR = 1.1 per year; 95% CI: (1.0, 1.2); p=0.009) and underlying immunosuppressive/metabolic conditions (OR = 6.5; 95% CI: (3.4, 12.5); p<0.001) were significant predictors of hospital admission. The inclusion of variable representing an interaction between HRV/ENT positive status and underlying immunocompromised condition was not significant, and its inclusion did not affect the risk estimates above (Table III-3).

Assumption Testing and Model fit

Given the dichotomous dependent variable (hospital admission) used for this analysis, a logistic regression approach to modeling was chosen. Two Goodness of fit (GOF) measures were used to assess how effectively our multivariable model fits our data: the Hosmer-Lemeshow (HL) and the likelihood ratio test. The HL test, referred to as the gold standard GOF measure, provided information on the degree of superiority in fit of the current model to the null model without covariates. The likelihood ratio test was used to compare the deviance of two hierarchical non-saturated models. From our analysis, the final model with four predictors represented a good fit with a HL χ^2 (df 8) of 15.1, (p=0.057) under the null hypothesis of good fit. When comparing two multivariable models, we observed a lower deviance from the model with more predictors (439.9 vs. 441.4) indicating it presented a better fit (Table III-4). The model which included an interaction variable between HRV/ENT positive status and underlying immunosuppressive co-morbidities did not represent a better fit with a HL χ^2 (df 8) of 27.7, (p=0.001). (Table III-4)

2. Predictors of clinical disease severity amongst in-patients

In univariable analyses, viral status, age, underlying immunosuppressive and metabolic conditions and cardiorespiratory co-morbidities were associated with severe disease among inpatients (Table III-5). Children with HRV/ENT infections were

significantly more likely to present with severe clinical disease compared to those with RSV infections (OR = 2.9; 95% CI: (1.7-5.1); p<0.001), FLU infections (OR = 3.6; 95% CI: (2.0-6.4); p<0.001) or any other respiratory viral infection (OR = 2.6; 95% CI: (1.4-4.9); p=0.003). Age (OR = 1.1 per year, 95% CI: (1.0-1.1); p=0.005), underlying immunocompromised/metabolic (OR = 5.3; 95% CI: (3.2-8.7); p<0.001) and cardiorespiratory conditions (OR = 2.7; 95% CI: (1.6-4.6); p<0.001) were significant predictors of disease severity among inpatients.

In multivariable analyses, adjusted for age and underlying co-morbidities, HRV/ENT infected children were significantly more likely to present with severe clinical disease compared to children admitted to hospital with FLU infections (OR = 3.0; 95% CI: (1.6, 5.6); p=0.001) (Table III-5). Furthermore, underlying immunosuppressive/metabolic conditions (OR = 4.7; 95% CI: (2.8, 8.0); p<0.001) and underlying cardio-respiratory co-morbidities (OR = 2.2; 95% CI: (1.2, 4.0); p=0.007) were significant predictors of disease severity among inpatients.

Assumption Testing and Model fit

Given the dichotomous dependent variable (composite end-point) used for this analysis, a logistic regression approach to modeling was chosen. From our analysis, our final model with all four predictors did not represent a good fit under the null hypothesis of good fit (HL χ^2 (df 8) of 19.6, (p=0.012). Another model, which excluded age

represented a good fit with a HL χ^2 (df 6) of 1.6, (p=0.953). When comparing both multivariable models (Table III-6), we observed a lower deviance (432.6 vs. 434.4) from the model with four predictors (viral status, age, underlying immunosuppressive/metabolic and cardio-respiratory conditions) indicating it presented a better fit. Based on evidence from the literature, it was decided to include *a priori* all four predictors in multivariable analysis.

2. Admission length amongst in-patients

In univariable analyses, viral status and underlying immunosuppressive/metabolic conditions were significant predictors of admission length. Children admitted with HRV/ENT infections had an 80% increase in the length of stay compared to those admitted with FLU infections (β coefficient = 0.6, 95% CI: (0.1, 1.0); p=0.009). The β coefficient also known as the regression coefficient specifies the linear relationship between a predictor variable x and the outcome variable y (length of admission in hospital). In multiple linear regression it estimates the adjusted effect of one predictor variable x_1 on an outcome when other predictor variables are held constant. Children with underlying immunosuppressive or metabolic states had a 100% increase in the length of admission compared to those without underlying immunosuppressive or metabolic conditions (β coefficient = 0.6; 95% CI: (0.3, 0.9); p=0.001). Multivariable analysis adjusted for underlying co-morbidities led to the same results (Table III-7)

Assumption Testing and Model fit

Given the continuous dependent variable (length of admission in hospital) used for this analysis, a linear regression approach to modeling was chosen. Linear regression has several assumptions, one of which requires normal distribution of the outcome variable. The outcome variable was right skewed and found to violate the assumption of normality required in linear regression. Several approaches can be used to overcome this violation of normality including transformations using natural logarithmic (ln) or square root. In our study, the outcome variable was transformed using the natural logarithm function (referred simply as « ln transformed »), which involved obtaining the natural logarithm of each observation. After transformation, the data appeared to be normally distributed as illustrated in Figure 2(a), reflecting normally distributed residuals. The second assumption is equal variances (homoscedasticty), which is usually achieved after ln transformation (Sedgwick, 2012) as illustrated in Figure 2(b), where unstandardized residuals were plotted against the predicted value of the ln transformed outcome. As there were multiple independent variables, the predicted value of the outcome instead of the independent variable was used. The scatter plot suggested an equal number of data points above and below the zero reference line which suggested that the assumption of homoscedasticity was not violated. The assumption that Y observations were conditionally independent or in other words that independence between patients was not violated in this model as each Y observations resulted from uncorrelated and independent hospitalized children. Finally, the assumption that the predictor variables (age, gender, underlying comorbidities) were

known and error free was likely not violated.

Data points, which are a long distance away from the rest of the data can exercise undue influence on the regression line. Leverage values are numerical measures of extreme values. Leverage aimed at identifying those observations that are far away from corresponding average predictor values with the assumption that data points with high leverage have the potential of moving the regression line up or down as the case may be. In other words leverage h, measures distance of x_1 from the average predictor value x bar with values between 0 and 1 where 1 indicates that the regression is forced to fit y_i exactly at that point. Thus, any high leverage points could make our estimation of β coefficients inaccurate, which would result in misleading conclusions. When looking at the entire database containing 391 patients, 10 values of duration of hospital stay exceeded 100 days as illustrated by the distribution of hospital stay on Figure 2(c), which was right skewed. We conducted ln transformed linear regression with (Table III-7 (b) and without (Table-III-7 (a)) these values, which led to different results and affected the estimation of β coefficients. We anticipated that values of duration of hospital stay above 100 days would not be representative of the admission for the respiratory illness but probably more representative of some underlying conditions. We therefore deleted these 10 values exceeding 100 and more days and assumed that Table III-7(a) was a more accurate representation of the duration of hospital stay.

B. Clinical outcomes

Clinical outcomes of children presenting with a single viral acute respiratory tract infection as compared to HRV/ENT are presented in Table III-8. Two hundred and five patients were admitted to hospital: 85 (73.3% of all HRV/ENT patients) with HRV/ENT infections, 41 (39.8%) with RSV infections, 31 (37.8%) with FLU infections and the remaining 48 (60%) with any other single viral infection needed hospital admission. In univariable analyses, children with HRV/ENT had increased odds for admission in hospital compared to those with RSV (OR = 4.1; 95% CI: (2.3, 7.1); p<0.001) or FLU infections (OR = 4.5; 95% CI: (2.4, 8.3); p<0.001). Amongst children who were admitted to hospital, those with HRV/ENT infections had an increased length of stay compared to those with RSV (median of 8 days, IQR: (3,19.5) vs. 6 days, IQR: (3,9) ; p<0.001) or FLU infections (8 days, IQR : (3,20) vs. 4 days, IQR: (2,11) ; p=0.035). Children with HRV/ENT infections had significantly increased odds for admission to the intensive care unit (OR = 3.5; 95% CI: (1.4, 8.9); p=0.009) and oxygen requirements compared to those with FLU infections (OR = 3.3; 95% CI: (1.6, 7.1); p=0.001).

Mortality rates were the highest amongst children with HRV/ENT infections with four of the six fatalities in the study population (3.4%). Of the four fatalities with HRV/ENT infections, three presented with underlying immunosuppressive conditions and one with a cardiac co-morbidity. Two of the patients died from a sepsis-like picture with respiratory failure and possible underlying pneumonia with no other viral, bacterial, or

fungal pathogens identified, thus, suggesting that HRV/ENT may have potentially contributed to the mortality. The remaining two patients likely died of their underlying disease. Of the non-HRV/ENT related deaths, one presented with a progressive acute respiratory distress syndrome (ARDS) after ADV infection detected upon admission, and died four weeks after influenza A infection; and the remaining one presented with an HBoV infection.

C. Proportion of common respiratory illness

1. Upper respiratory tract infections

Comparison of the proportion of common respiratory illness (upper respiratory tract infections (URTI), pneumonia) between children presenting with HRV/ENT positive mid-turbinate swabs and the ones with RSV A/B, FLU A/B and other respiratory viruses positive mid-turbinate swabs detected by molecular assays are presented in TABLE III-9.

From the 381 children included in the study, 219 (57%) presented with URT symptoms of whom 30 (13.7%) also presented with pneumonia. A total of 65 (26.4%) children were diagnosed with pneumonia as defined in the method section. From the 219 children with URTI, 74 (63.8%) were detected positive for HRV/ENT, 66 (64.1%) for RSV, 42 (51.2%) for FLU and the remaining 37 (46.3%) with other respiratory infections.

In univariable analyses, children with HRV/ENT infection presented with significantly higher odds for URTI compared to those with FLU infections (OR = 2.0; 95% CI: (1.1, 3.2); p=0.025) or other single viral infection (OR = 2.1; 95% CI: (1.1, 4.0); p=0.017). Age (OR = 0.9 per year; 95% CI: (0.9, 1.0); p=0.012) was also a significant predictor for URTI. (Table III-9).

In multivariable analysis, adjusted for age, underlying immunosuppressive/metabolic co-morbidities and prematurity, HRV/ENT infected children were significantly more likely to present with URTI compared to those with FLU infections (OR = 1.7; 95% CI: (1.0, 3.1; p=0.035) or other infections (OR = 2.5; 95% CI: (1.3, 4.5); p=0.007). Age (OR = 0.9 per year; 95% CI: (0.9,1.0); p=0.028) also remained a significant predictor of URTI (Table III-9).

Assumption Testing and Model fit

Given the dichotomous dependent variable --the presence or absence of URTI used for this analysis, a logistic regression approach to modeling was chosen. From our analysis, our final model with all three predictors (viral status, age and prematurity) represented a good fit under the null hypothesis of good fit (HL χ^2 (df 8) of 7.1, p=0.528).

2. Pneumonia

From the 65 children with pneumonia, 28 (24.1%) were detected positive for HRV/ENT, 10 (9.7%) for RSV infections, 9 (11%) for FLU infections and 18 (22.5%) for any other single viral infection (Table III-10).

In univariable analyses, children with HRV/ENT infected presented with significantly higher odds for pneumonia compared to those with RSV infections (OR = 2.5; 95% CI: (1.1, 5.9); p=0.028). Age (OR = 1.1 per year; 95% CI: (1.0, 1.1); p<0.001) and underlying immunocompromised/metabolic conditions (OR = 3.0; 95% CI: (1.7, 5.5); p<0.001) were also significant predictors of pneumonia (Table III-10).

In multivariable analysis, adjusted for age and underlying co-morbidities, viral status did not remain a significant predictor of pneumonia. However, male gender, underlying prematurity and immunosuppressive/metabolic conditions remained significant predictors of pneumonia (Table III-10)

Assumption Testing and Model fit

Given the dichotomous dependent variable (presence or absence of pneumonia) used in this analysis, a logistic regression approach to modeling was chosen. From our analysis, our final model with all six predictors (viral status, age, male gender and all 3 underlying

conditions) represented a good fit under the null hypothesis of good fit (HL χ^2 (df 8) of 7.9, p=0.443).

Tables and Figures

Figure 1: Proportion of single viral infections

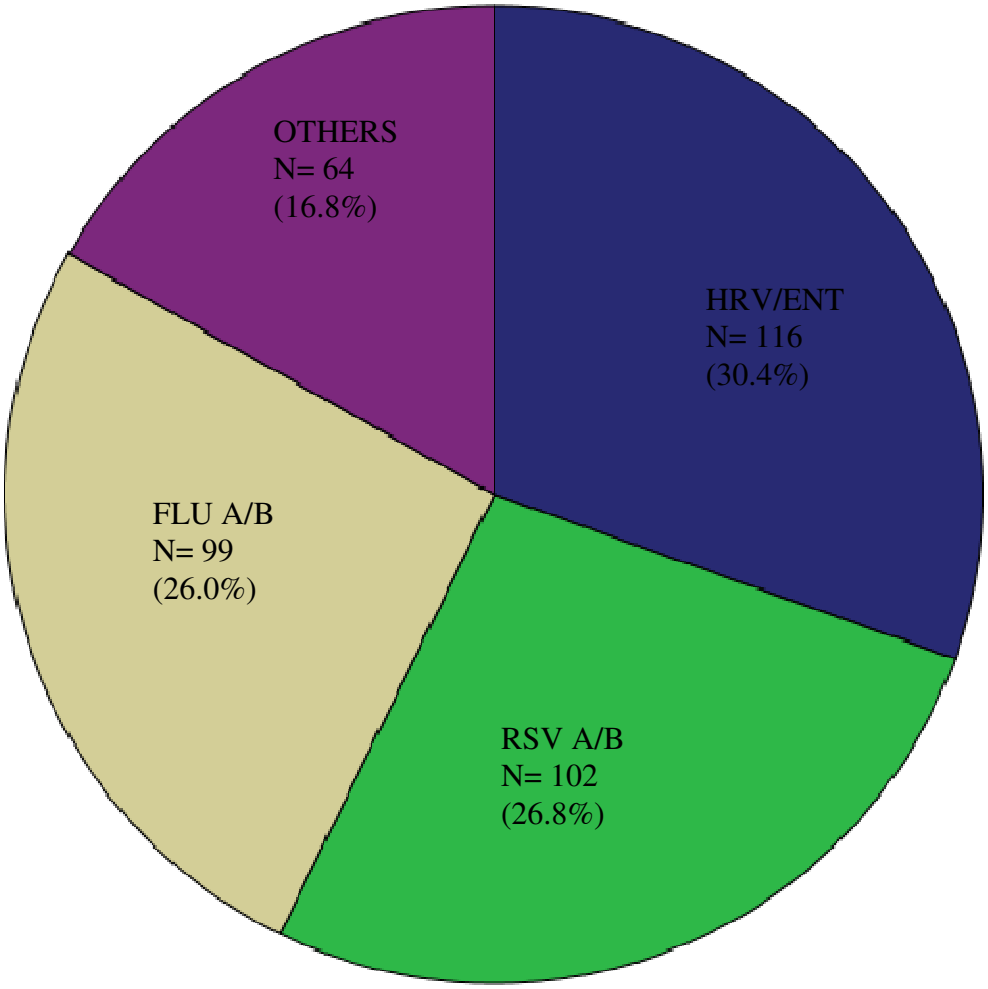

FIGURE 2. ASSUMPTION TESTING AND MODEL FIT

Figure 2 (a): Assumption of normality

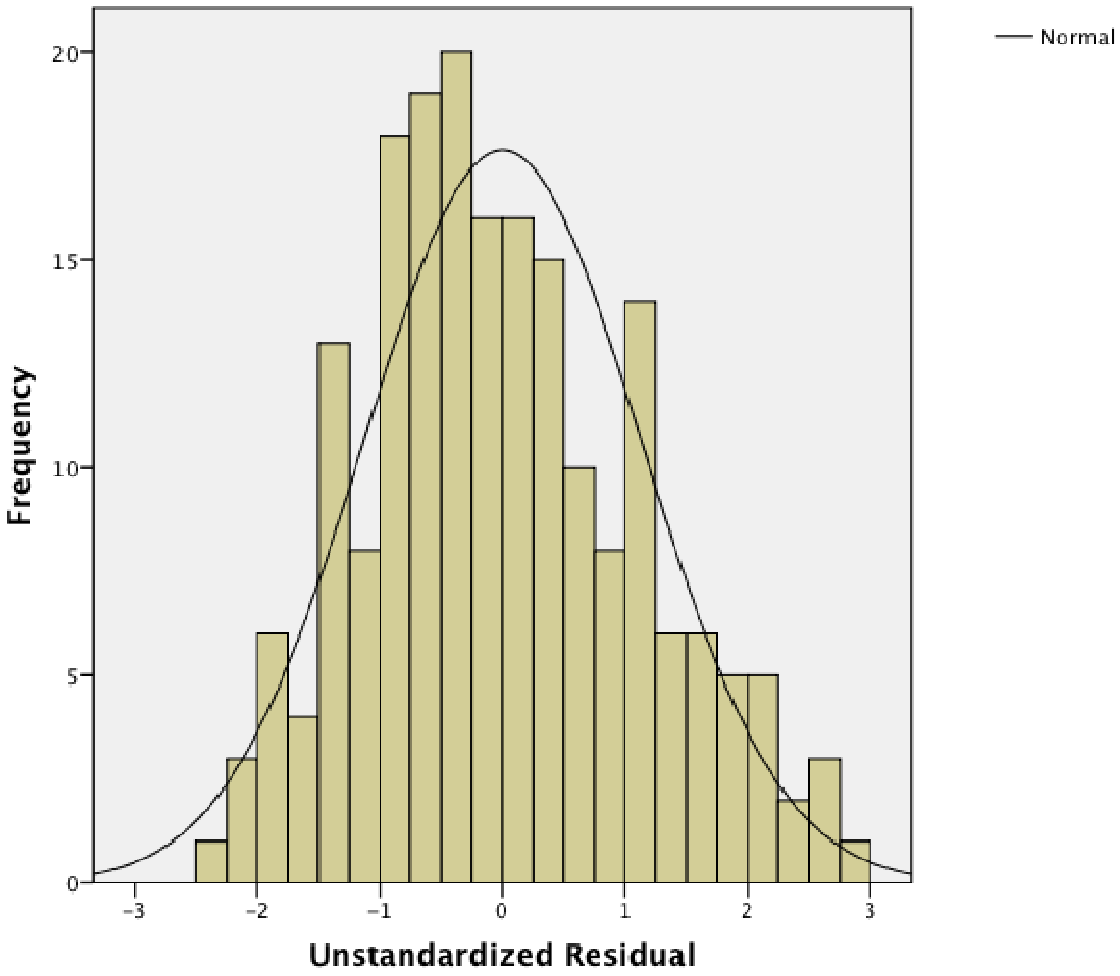

Figure 2(b): Assumption of homoscedasticity

Scatter plot of unstandardized Residuals against the predicted value of the ln transformed length of hospital stay

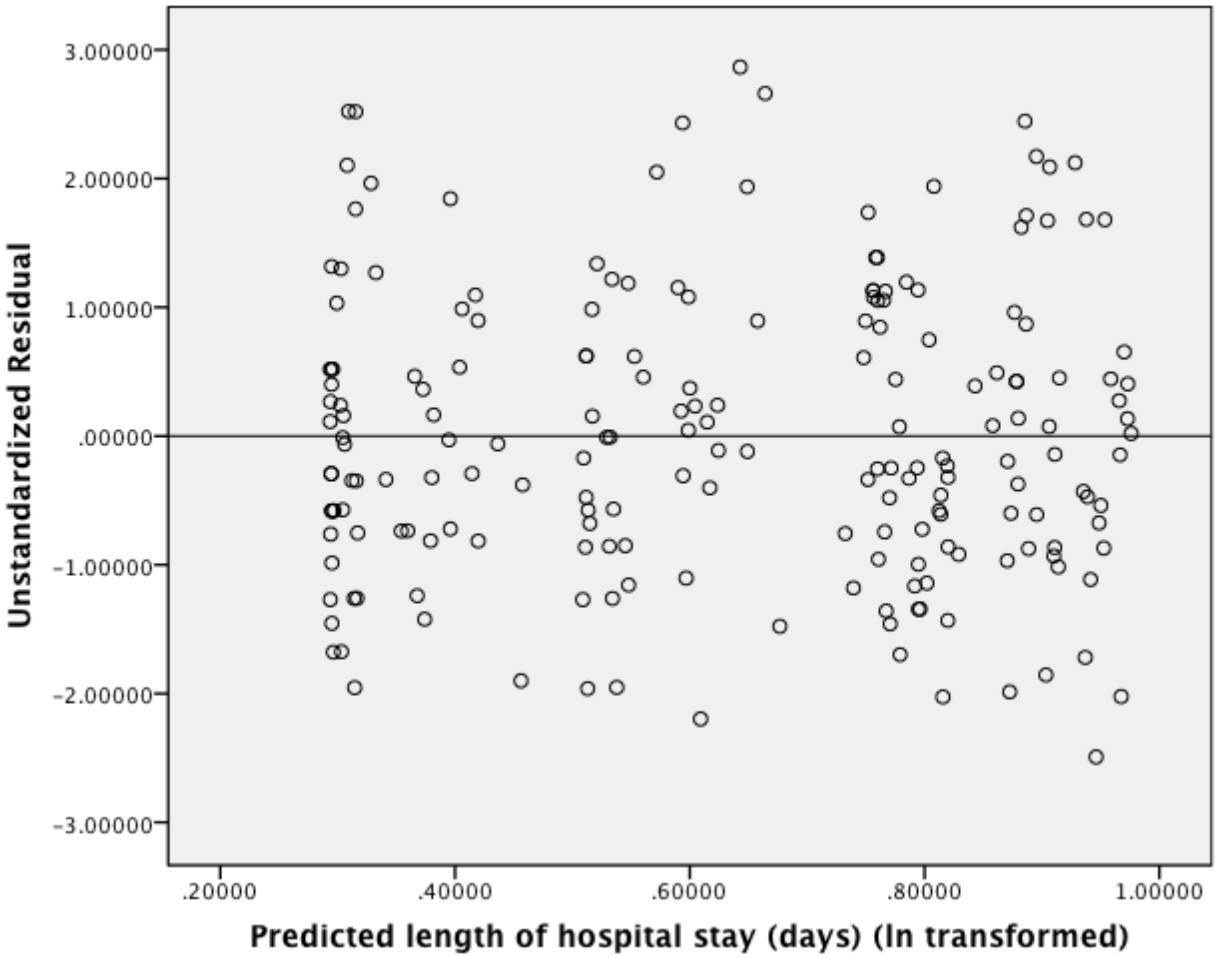

Figure 2(c): Distribution of hospital stay (number of days) of the 391 patients-including 10 leverage values

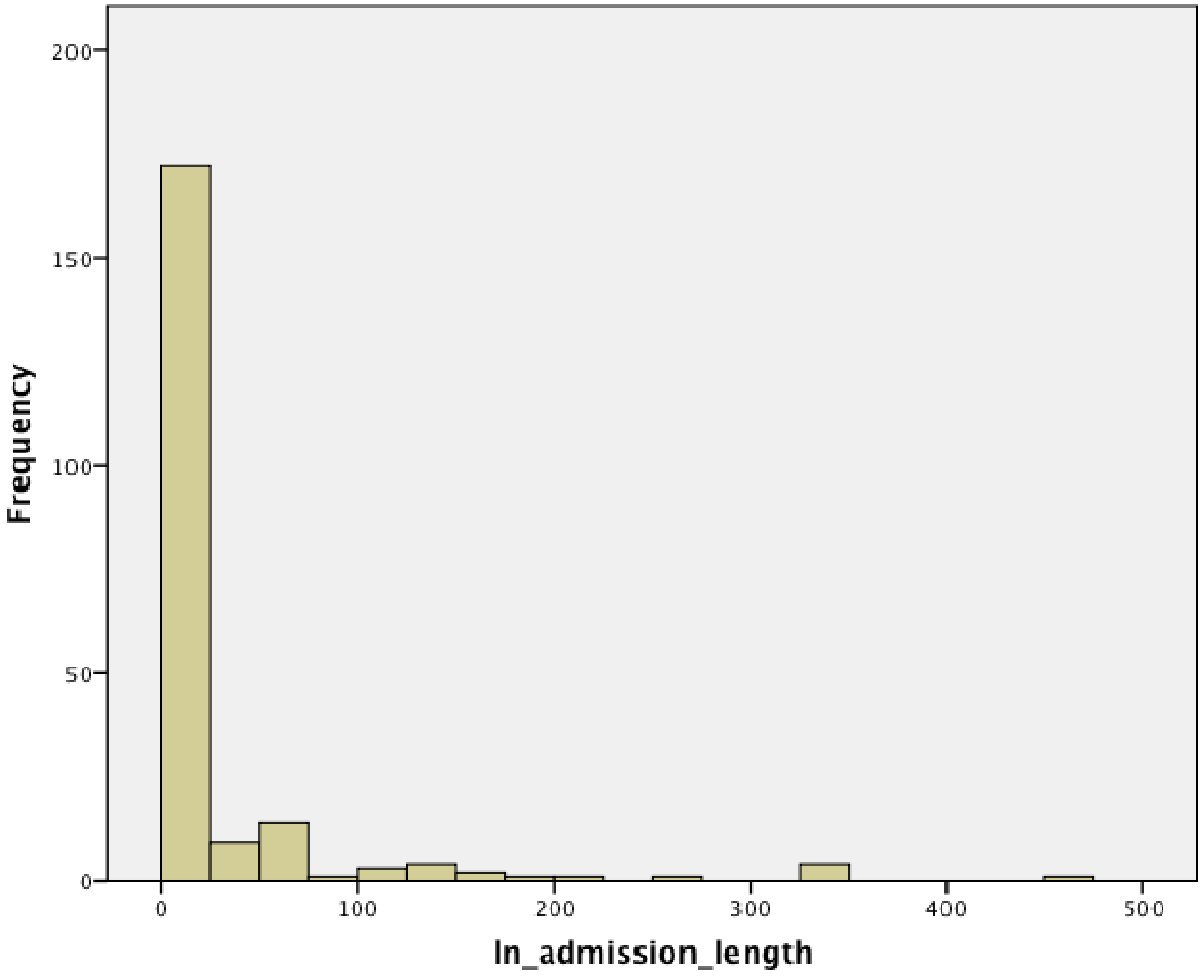

TABLE III-1: BASELINE CHARACTERISTICS OF CHILDREN PRESENTING WITH A SINGLE VIRAL ACUTE RESPIRATORY TRACT INFECTION AS COMPARED TO HRV/ENT

	HRV/ENT [a*] n=116	**RSV [b] n=102**	**FLU [c] n=99**	**Others [d] n=64**
Age in years, Median, IQR[£]	1.2 (0.4,5.8)	0.5 (0.1,1.8) **p<0.001**	3.7 (1.2,6.3) **p=0.001**	1.1 (0.5,3.5) p=0.663
Gender, Male N, %	64 (55.2)	63 (61.2) OR =1.3 95%CI: (0.8, 2.2) p=0.370	48 (58.5) OR = 1.1 95%CI: (0.6, 2.0) p=0.638	62 (96.9) OR 2.8 95%CI: (1.5, 5.3) **p=0.002**
Cardio-respiratory N, %	37 (31.9)	15 (14.6) OR =2.5 95% CI: (1.4, 5.0) **p=0.003**	8 (9.8) OR = 5.0 95%CI: (2.0, 10.0) **p=0.001**	10 (12.5) OR = 3.3 95%CI: (1.4, 7.1) **p=0.002**
Prematurity N, %	10 (8.6)	12 (11.7) OR = 0.7 95%CI: (0.3, 1.7) p=0.458	1 (1.2) OR = 10 95%CI: (1,50) p=0.055	6 (7.5) OR = 1.1 95%CI: (0.4, 3.3) p=0.778
Immunocompromised/ Metabolic N, %	44 (37.9)	14 (13.6) OR = 3.3 95%CI: (2.0,10.0) **p<0.001**	18 (22) OR = 2 95%CI: (1.1, 5.0) **p=0.018**	18 (22.5) OR = 2 95%CI; (1.1, 3.3) **p=0.024**

LEGEND
N= 462 patients

- HRV/ENT [a]: enterovirus/rhinovirus: risk estimates, 95% CI and P-values are indicated in each row for the comparison with HRV/ENT which was used; RSV[b]: respiratory syncytial virus; FLU[c]: Influenza; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV: adenovirus, coronaviruses, HBoV
- IQR[a] interquartile range

TABLE III-2: PREDICTORS OF HOSPITALIZATION

	Univariable analysis			**Multivariable analysis**		
	OR	**95 % CI**	**P -value**	**OR**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	4.3	2.4, 7.5	**<0.001**	2.6	1.4, 4.8	**0.003**
HRV/ENT vs. FLU [c]	3.2	1.8, 5.6	**<0.001**	3.0	1.6, 5.8	**0.001**
HRV/ENT vs. Others [d]	2.4	1.3, 4.6	**0.007**	1.7	0.8, 3.5	0.119
Age	1.1	1.1, 1.2	**<0.001**	1.1	1.0, 1.2	**0.009**
Cardio-respiratory	2.0	1.1, 3.4	**0.014**	1.5	0.8, 2.7	0.223
Prematurity	1.7	0.8, 3.8	0.192	-	-	**-**
Immunocompromised Metabolic	8.2	4.4, 15.4	**<0.001**	6.5	3.4, 12.5	**<0.001**

LEGEND
N= 205 inpatients

- HRV/ENT: [a] enterovirus/rhinovirus; RSV: [b] respiratory syncytial virus ; FLU: [c] Influenza ; Others :[d] PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV

TABLE III-3: PREDICTORS OF HOSPITALIZATION INCLUDING AN INTERACTION VARIABLE

(HRV/ENT STATUS AND UNDERLYING IMMUNOSUPPRESSION)

	Multivariable analysis		
	OR	**95 % CI**	**P-value**
HRV/ENT	2.6	1.5 4.6	**0.001**
Age	1.1	1.0, 1.2	**0.009**
Cardio-respiratory	1.5	0.8, 2.8	0.188
Immunocompromised Metabolic	7.0	3.2, 15.3	**<0.001**
Interaction HRV/ENT x Immunocompromised Metabolic	0.8	0.2, 3.1	0.706

Table III-4: ASSUMPTION TESTING AND MODEL FIT

	Multivariable analysis Model with 3 predictors			**Multivariable analysis Model with 4 predictors**		
	OR	**95 %CI**	**P -value**	**OR**	**95 % CI**	**P-value**
HRV/ENT [a] vs. RSV [b]	2.7	1.5, 5.0	**0.001**	2.6	1.4, 4.8	**0.003**
HRV/ENT vs. FLU [c]	3.3	1.8, 6.2	**<0.001**	3.0	1.6, 5.8	**0.001**
HRV/ENT vs. Others [d]	1.8	0.9, 3.6	0.085	1.7	0.8, 3.5	0.119
Age	1.1	1.1, 1.2	**<0.006**	1.1	1.0, 1.2	**0.009**
Cardio-respiratory	-	-	**-**	1.5	0.8, 2.7	0.223
Immunocompromised Metabolic	6.4	3.4, 12.3	**<0.001**	6.5	3.4, 12.5	**<0.001**
Deviance	441.4			439.9		
Hosmer-Lemeshow test	χ^2 (df 8) = 16.6; p=0.035			χ^2 (df 8) = 15.1; p=0.057		

	Multivariable analysis including an interaction variable between HRV/ENT and immunosuppression/metabolic underlying condition		
	OR	**95 %CI**	**P-value**
HRV/ENT	2.7	1.5 4.6	**0.001**
Age	1.1	1.0, 1.2	**0.009**
Cardio-respiratory	1.5	0.8, 2.8	0.188
Immunocompromised Metabolic	7.0	3.2, 15.3	**<0.001**
Interaction HRV/ENT x Immunocompromised Metabolic	0.8	0.2, 3.1	0.706
Deviance	442.4		
Hosmer-Lemeshow test	χ^2 (df 8) = 27.7; p=0.001		

LEGEND

- HRV/ENT [a]: enterovirus/rhinovirus; RSV [b]: respiratory syncytial virus ; FLU [c] Influenza ; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV

TABLE III-5 : ANALYSES OF PREDICTORS OF SEVERITY AS MEASURED BY A COMPOSITE ENDPOINT OF ADMISSION TO THE INTENSIVE CARE UNIT, HOSPITALIZATION > 5 DAYS, OXYGEN REQUIREMENTS OR DEATH

	Univariable analysis			**Multivariable analysis**		
	OR	**95 %CI**	**P -value**	**OR**	**95 % CI**	**P-value**
HRV/ENT [a] vs. RSV [b]	2.9	1.7, 5.1	**<0.001**	1.7	0.9, 3.2	0.082
HRV/ENT vs. FLU [c]	3.6	2.0, 6.4	**<0.001**	3.0	1.6, 5.6	**0.001**
HRV/ENT vs. Others [d]	2.6	1.4, 4.9	**0.003**	1.8	0.9, 3.6	0.094
Age	1.1	1.0, 1.1	**0.005**	1.0	1.0, 1.1	0.180
Cardio-respiratory	2.7	1.6, 4.6	**<0.001**	2.2	1.2, 4.0	**0.007**
Prematurity	1.2	0.6, 2.6	0.657	-	-	**-**
Immunocompromised Metabolic	5.3	3.2, 8.7	**<0.001**	4.7	2.8, 8.0	**<0.001**

LEGEND
N= 143 patients admitted in hospital with severe disease

- HRV/ENT [a]: enterovirus/rhinovirus; RSV [b]: respiratory syncytial virus ; FLU [c] Influenza ; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV

Table III-6: ASSUMPTION TESTING AND MODEL FIT

	Multivariable analysis Model with 3 predictors			**Multivariable analysis Model with 4 predictors**		
	OR	**95 % CI**	**P-value**	**OR**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	1.9	1.0, 3.5	**0.038**	2.6	1.4, 4.8	**0.003**
HRV/ENT vs. FLU [c]	2.8	0.8, 1.5	**0.001**	3.0	1.6, 5.8	**0.001**
HRV/ENT vs. Others [d]	1.9	0.9, 3.7	0.085	1.7	0.8, 3.5	0.119
Age	-	-	**-**	1.1	1.0, 1.2	**0.009**
Cardio-respiratory	2.3	1.3, 4.1	**0.005**	1.5	0.8, 2.7	0.223
Immunocompromised Metabolic	5	2.9, 8.3	**<0.001**	6.5	3.4, 12.5	**<0.001**
Deviance	434.4			432.6		
Hosmer-Lemeshow test	χ^2 (df 6) = 1.6 ; p=0.953			χ^2 (df 8) = 19.6 ; p=0.012		

LEGEND

- HRV/ENT [a]: enterovirus/rhinovirus; RSV [b]: respiratory syncytial virus ; FLU [c] Influenza ; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV
- 95% CI: confidence interval

TABLE III-7 (a): PREDICTORS OF ADMISSION LENGTH (Ln TRANSFORMATION)

	Univariable analysis			Multivariable analysis		
	β coefficients	**95 % CI**	**P -value**	**β coefficients**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	0.4	- 0.06, 0.9	0.085	0.3	-0.1, 0.7	0.189
HRV/ENT vs. FLU [c]	0.6	0.1, 1.0	**0.012**	0.6	0.1, 1.0	**0.009**
HRV/ENT vs. Others [d]	0.07	-0.4, 0.5	0.781	0.04	-0.4, 0.5	0.873
Age	0.008	-0.03, 0.05	0.687	-	-	-
Gender (Male)	0.06	-0.3, 0.4	0.740	-	-	-
Cardio-respiratory	0.3	-0.1, 0.7	0.160	-	-	-
Prematurity	0.6	-0.005, 1.1	0.052	0.5	-0.1, 1.0	0.113
Immunocompromised Metabolic	0.7	0.3, 1.0	**<0.001**	0.6	0.3, 0.9	**0.001**

LEGEND
N= 205 inpatients

- HRV/ENT [a]: enterovirus/rhinovirus; RSV [b]: respiratory syncytial virus ; FLU [c] Influenza ; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV
- 95% CI: confidence interval

TABLE III-7 (b): PREDICTORS OF ADMISSION LENGTH (Ln TRANSFORMATION) INCLUDING 10 LEVERAGE VALUES

	Univariable analysis			**Multivariable analysis**		
	β coefficients	**95 % CI**	**P- value**	**β coefficients**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	10.6	-13.5, 34.6	0.388	12.8	-10.6, 36.3	0.282
HRV/ENT vs. FLU [c]	5.1	-23.2, 28.2	0.846	5.1	-29.3, 19.0	0.675
HRV/ENT vs. Others [d]	19.1	-3.2, 41.4	0.093	16.6	-4.6, 37.8	0.125
Age	2.2	0.3, 4.1	**0.022**	2.8	0.9, 4.7	**0.003**
Gender	12.2	-30.3, 6	0.188	-	-	**-**
Cardio-respiratory	43.1	20.3, 65.9	<0.001	41	18.9, 63.1	**<0.001**
Prematurity	24.3	6.1, 54.7	0.116	-	-	**-**
Immunocompromised Metabolic	28.7	11.1, 46.3	**0.001**	28.8	11.6, 46.0	**0.001**

LEGEND
N= 214 inpatients

- HRV/ENT: [a]: enterovirus/rhinovirus; RSV: [b] respiratory syncytial virus; FLU: [c] Influenza ; Others: [d]:PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV

TABLE III-7(b): PREDICTORS OF ADMISSION LENGTH (Ln TRANSFORMATION) INCLUDING 10 LEVERAGE VALUES

	Univariable analysis			**Multivariable analysis**		
	β coefficients	**95 %CI**	**P- value**	**β coefficients**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	10.6	-13.5, 34.6	0.388	12.8	-10.6, 36.3	0.282
HRV/ENT vs. FLU [c]	5.1	-23.2, 28.2	0.846	5.1	-29.3, 19.0	0.675
HRV/ENT vs. Others [d]	19.1	-3.2, 41.4	0.093	16.6	-4.6, 37.8	0.125
Age	2.2	0.3, 4.1	**0.022**	2.8	0.9, 4.7	**0.003**
Gender	12.2	-30.3, 6	0.188	-	-	**-**
Cardio-respiratory	43.1	20.3, 65.9	<0.001	41	18.9, 63.1	**<0.001**
Prematurity	24.3	6.1, 54.7	0.116	-	-	**-**
Immunocompromised Metabolic	28.7	11.1, 46.3	**0.001**	28.8	11.6, 46.0	**0.001**

LEGEND

- HRV/ENT: [a]: enterovirus/rhinovirus; RSV: [b] respiratory syncytial virus; FLU: [c] Influenza ; Others: [d]:PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV

TABLE III-8: CLINICAL OUTCOMES OF CHILDREN PRESENTING WITH A SINGLE VIRAL ACUTE RESPIRATORY TRACT INFECTION AS COMPARED TO HRV/ENT

	HRV [a]; n=116	**RSV [b]; n=102**	**FLU [c]; n=99**	**Others [d]; n=64**
URTI **N, %**	74 (63.8)	66 (64.1) OR = 1.0 95% CI: (0.6, 1.7) p=0.965	42 (51.2) OR = 1.7 95%CI: (1.0, 2.9) p=0.078	37 (46.3)
Pneumonia **N, %**	28 (24.1)	10 (9.7) OR = 2.3 95%CI: (1.1, 5.3) **p=0.044**	9 (11) OR = 2.2 95%CI: (0.9, 5.0) p=0.073	18 (22.5) OR = 1.3 95%CI: (0.6, 2.6) p=0.515
Hospital admission N, %	85 (73.3)	41 (39.8) OR = 4.1 95%CI: (2.3, 7.1) **p<0.001**	31 (37.8) OR = 4.5 95%CI: (2.4, 8.3) **p< 0.001**	48 (60) OR = 1.8 95% CI: (1.0, 3.3) p=0.052
Length of hospital admission (days) Median, IQR [e]	8 (3-19.5)	6 (3,9) **p<0.001**	4 (2, 11) **p=0.035**	8 (3,19.5) p=0.858
Admission in the ICU **N, %**	25 (21.6)	14 (13.6) OR = 1.7 95%CI: (0.8, 3.6) p=0.127	6 (7.3) OR = 3.5 95%CI: (1.4, 8.9) **p=0.009**	15 (18.8) OR = 1.2 95%CI: (0.6, 2.4) p=0.633
Oxygen requirements **N, %**	40 (34.5)	28 (27.2) OR = 1.4 95%CI: (0.8, 2.5) p=0.245	11 (13.4) OR = 3.3 95%CI: (1.6, 7.1) **p=0.001**	23 (28.7) OR = 1.3 95%CI: (0.7, 2.4) p=0.399
Mortality** **N, %**	4 (3.4)	0	1 (1.2)	1 (1.6)

LEGEND
N=462 patients

- IQR [e] interquartile range
- HRV/ENT: [a]: enterovirus/rhinovirus; RSV: [b] respiratory syncytial virus; FLU: [c] Influenza ; Others: [d]:PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV:adenovirus, coronaviruses, HBoV
- HRV/ENT *: risk estimates, 95% CI and P-values are indicated in each row for the comparison with HRV/ENT for each categorical variable
- ** No risk estimates provided given the small number of events

TABLE III-9: PREDICTORS OF UPPER RESPIRATORY TRACT INFECTIONS

	Univariable analysis			**Multivariable analysis**		
	OR	**95 %CI**	**P -value**	**OR**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	1.0	0.6, 1.7	0.888	1.1	0.7, 2.0	0.618
HRV/ENT vs. FLU [c]	2.0	1.1, 3.3	**0.025**	1.7	1.0, 3.1	**0.043**
HRV/ENT vs. Others [d]	2.1	1.1, 4.0	**0.017**	2.5	1.3, 4.5	**0.007**
Age	0.9	0.9, 1.0	**0.012**	0.9	0.9, 1.0	**0.028**
Gender	0.9	0.6, 1.4	0.672	-	-	**-**
Cardio-respiratory	0.8	0.5, 1.3	0.302	-	-	-
Prematurity	2.1	0.9, 4.8	0.086	1.8	0.8, 4.4	0.167
Immunocompromised Metabolic	0.8	0.5, 1.2	0.277	-	-	**-**

LEGEND

- HRV/ENT [a]: enterovirus/rhinovirus: risk estimates, 95% CI and P-values are indicated in each row for the comparison with HRV/ENT which was used; RSV[b]: respiratory syncytial virus; FLU[c]: Influenza; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV: adenovirus, coronaviruses, HBoV
- 95% CI: confidence interval

TABLE III-10: PREDICTORS OF PNEUMONIA

	Univariable analysis			**Multivariable analysis**		
	OR	**95 %CI**	**P- value**	**OR**	**95 % CI**	**P -value**
HRV/ENT [a] vs. RSV [b]	2.5	1.1, 5.9	**0.028**	2.0	0.7, 5.0	0.187
HRV/ENT vs. FLU [c]	1.4	0.7, 3.3	0.257	1.3	0.6, 2.9	0.566
HRV/ENT vs. Others [d]	1.4	0.7, 3.3	0.310	1.4	0.6, 3.3	0.508
Age	1.1	1.0, 1.1	**<0.001**	1.1	1.0, 1.1	0.186
Gender	1.8	1.0, 3.4	**0.061**	2.0	1.0, 3.8	**0.049**
Cardio-respiratory	1.8	0.9, 3.4	0.075	1.4	0.7, 2.9	0.354
Prematurity	2.4	0.9, 6.4	0.079	3.2	1.1, 9.4	**0.035**
Immunocompromised Metabolic	3.0	1.7, 5.5	**<0.001**	3.0	1.6, 5.6	**0.001**

LEGEND

- HRV/ENT [a]: enterovirus/rhinovirus: risk estimates, 95% CI and P-values are indicated in each row for the comparison with HRV/ENT which was used; RSV[b]: respiratory syncytial virus; FLU[c]: Influenza; Others[d]: PIV: parainfluenza, hMPV: human meta-pneumovirus, ADV: adenovirus, coronaviruses, HBoV
- 95% CI: confidence interval

PART IV. Discussion

Summary of findings

We found that human rhinovirus/enterovirus (HRV/ENT) was the most frequent virus detected in single acute respiratory infections (ARIs) with 116 of the 382 patients (30.4%) testing positive for HRV/ENT. The majority of respiratory samples were collected between January-March, thus excluding the September-October and April periods, when HRV/ENT peaks are detected in the Northern Hemisphere. (Monto et al, 1994; Arruda et al, 1997). Therefore, it is likely that even more children may have been detected HRV/ENT positive if more samples were collected during fall or spring.

We also found that children with underlying cardio-respiratory, immunocompromised or metabolic conditions were more likely to present with HRV/ENT infections at the Hospital for Sick Children, Toronto, Ontario, Canada, thus suggesting that these patients may be at higher risk for HRV/ENT infections or for severe disease when infected with HRV/ENT. Conversely, patients with underlying conditions are known to be more fragile. A higher prevalence of HRV/ENT circulating in the community could also precipitate hospitalization of these more fragile patients, thus explaining a higher prevalence of HRV/ENT-related hospital admissions among this patient-population. In addition, we found that children with HRV/ENT infections had more severe outcomes compared to those with respiratory syncytial virus (RSV) or

influenza (FLU) infections, while controlling for underlying co-morbidities and age. These more severe outcomes included increased rates of hospital admission for out-patients and admission length > 5 days, intensive care unit (ICU) admission, any supplemental oxygen requirements or death in patients admitted to the hospital. Furthermore, we found significantly longer duration of hospitalization amongst children admitted with HRV/ENT infections compared to those admitted with FLU infections. We also found, higher rates of mortality among HRV/ENT positive children.

Finally, we found that HRV/ENT positive status was associated with increased odds of upper respiratory tract infections but not of pneumonia. In multivariable analyses, male gender, underlying prematurity or immunosuppression or metabolic conditions, were significant predictors of pneumonia.

Patient characteristics and viral detection

HRV/ENT was the leading virus identified among our patient-population presenting with ARIs with similar rates of detection compared to other studies conducted among children with viral ARIs detected by molecular assays (30.4% vs 27.2%)[13,14] despite the use of a more conservative definition (at least two or more positive molecular assays) for viral positivity. We also documented similar rates of RSV (26.8% vs 25%) and FLU (26% vs 22%) infections compared to recent published studies (Calvo et al,2007; 2010). Our study was conducted until March 2009, thus more than one third of

documented FLU infections belonged to the pandemic A(H1N1) 2009 (A(H1N1)pdm09) strains. We decided to rely on at least two or more positive molecular assays for viral detection to maximize the inclusion of recent infections and to balance the difference of sensitivities afforded by molecular assays. PCR increases the sensitivity of detection of respiratory viruses in children by 74.3% over DFA and viral isolation, while maintaining excellent specificity (Gharabaghi et al, 2011).

Mid-turbinate samples used in our study were collected to compare the performance of the four commercial multiplex assays (Resplex II Panel v2.0, Seeplex RV15, xTAG RVP and xTAG RVP Fast), direct fluorescent antibody (DFA) staining and viral isolation. Results were further published in a study by Gharabaghi et al, 2011. In each assay, the sensitivity and specificity for each target were determined against a composite reference standard. The same criteria for virus-positive samples were used in their study, which included for all targets except PIV4, BoV, coronaviruses, HRV/ENT, any positive viral culture or a positive result for a single target from any two of DFA and the four molecular assays was considered true positive. Because PIV4, BoV, coronaviruses, HRV/ENT could not be detected by DFA or viral isolation, a true positive for these agents was defined as a positive result by at least two of the three or four multiplex PCR assays. A single positive result in any assay, with the exception of viral culture, was considered false positive. Among all multiplex assays tested, Seeplex RV15 was the most sensitive for detecting all targets except for HRV/ENT. All four multiplex assays have good sensitivity for the detection of FLU A (93.7–98.4%) and FLU B (100%)

except for RVP Fast at 64.9%. The superior performance of Seeplex RV15 for RSV (100% sensitivity) was reflected by strong performance for both RSVA and RSVB, whereas the decreased sensitivity of other assays reflects a weaker performance for either target (i.e. Resplex II v2.0: RSVA 90.4%, RSV B 94.3%, and RVP: RSVA 85.5%, RSVB 98.3%). Similarly, variability in the sensitivity of individual targets of the four parainfluenza viruses resulted in variation in the overall sensitivity. Seeplex RV15 showed good sensitivity for all four types (85.7–100%), while Resplex II v2.0 had reduced sensitivity for PIV4 (60%), RVP had reduced sensitivity for PIV1 (71.4%) and 3 (71.4%), and RVP Fast had reduced sensitivity or PIV1 (46.7%), 2 (77.8%) and 3 (42.8%). Sensitivity for detecting hMPV was good for Seeplex RV15, RVP and RVP Fast (92.3–97.4%), and acceptable for Resplex II v2.0 (82%). However, performance for adenovirus, was very variable, ranging from 52.4% (RVP Fast) to 100% (Seeplex RV15), probably reflecting the variation in serotype coverage among the assays. Of the additional viral agents tested in the multiplex assays, coronaviruses were consistently detected across all assays except for CoV OC43 by RVP (53.8%) and CoV HKU1 by RVP Fast (16.7%). Seeplex RV15 and RVP Fast detected 100% of bocavirus infections, while the sensitivity of Resplex II v2.0 was only 75%. Detection of HRV/ENT was the most inconsistent as the highly conserved regions of the 5'NTR region of either HRV or ENT, also amplify members of the other genus. Thus, some assays, such as the RVP and RVP Fast assays, have combined the HRV and ENT targets, because developing specific targets for each genus outside of the 5'NTR region may compromise sensitivity of detection, especially of the HRV. As a result, RVP and RVP Fast may have resulted in increased sensitivity for

HRV/ENT detection but also in decreased specificity as a result of including previous infections or eventually asymptomatic infections. On the other hand, Resplex II v2.0 assay differentiates between HRV and ENT, however the occurrence of 38.4% of positive specimens testing positive for both targets, suggested that there may be cross-reactivity between them. Seeplex RV15 assay, separated HRV and ENT, which resulted in lower sensitivity compared to other assays (Gharabaghi et al, 2011).

Similar to others studies (Messacar et al, 2013; Papadopoulos et al, 2002; Calvo et al, 2013; Iwane et al, 2011), children infected by HRV/ENT alone were younger compared to those with FLU infections but older compared to those with RSV infections. Our patient-population included a significant number of children with underlying conditions: almost 1/3 presented with underlying immunosuppressive/metabolic conditions whereas 1/4 had underlying cardio-respiratory conditions, thus suggesting a more vulnerable population for severe respiratory infections. Children with HRV/ENT infections presented with higher rates of underlying cardio-respiratory and immunosuppressive conditions compared to those with any other single viral infection. Our findings are similar to those described in adults admitted with HRV/ENT respiratory infections, in whom high rates of underlying immunosuppressive conditions were identified, thus suggesting that these patients may be at higher risk for severe HRV/ENT infections, which may trigger hospital admission. However, the risk of getting infected with HRV/ENT remains similar to patients without any underlying conditions (Malcolm et al, 2001; Kaiser et al, 1999).

Clinical disease severity and outcomes

Despite the high proportion of underlying co-morbidities identified amongst our HRV/ENT population, HRV/ENT positive status was a significant independent predictor for hospital admission and resulted in an increased clinical disease severity amongst inpatients compared to either RSV or FLU, while controlling for underlying-comorbidities and age. Furthermore, we found significantly longer duration of hospitalization amongst children admitted with HRV/ENT infections compared to those admitted with FLU infections. Children with HRV/ENT infections had also significantly increased odds for admission in the intensive care unit and oxygen requirements compared to those with FLU infections. Treatment of influenza A (H1N1) pdm09 positive children with oseltamivir might be expected to improve the prognosis, however none of the children included in our study received oseltamivir, as specimens were collected before the implementation of guidelines advocating for oseltamivir treatment of high-risk children. This is important information as, had FLU-positive children been treated with oseltamivir, they would have been expected to present with less severe outcomes, which in turn would have biased our results towards an increased severity of HRV/ENT infected children. Finally, we documented that half of the deaths observed amongst the four HRV/ENT infected children with underlying conditions may have resulted from their HRV/ENT respiratory illnesses as no other viral, bacterial or fungal pathogens were documented. These findings contrast with recently published studies (Iwane et al, 2011; Messacar et al 2013; McCloskey et al, 2011), which reported equivalent disease severity between subjects with HRV/ENT

infections and those with other common viral infections. A recent study by Iwane et al. (2011) reported similar rates of intensive care unit (ICU) admission, mechanical ventilation use and supplemental oxygen requirements among children with HRV/ENT and any other single viral infection (Iwane et al, 2011). Another study by Messacar et al. (2013) conducted among hospitalized children with ARIs during the influenza A (H1N1) pdm09 reported that children hospitalized with HRV/ENT had significant shorter median length of stay, duration of fever and duration of hypoxemia than children with FLU virus. Also higher rates of mortality were reported among FLU infected children. However, they reported similar percentages of children with HRV/ENT and influenza virus admitted to the ICU with similar rates of mechanical ventilation thus suggesting equivalent disease severity between HRV/ENT and FLU infections. These discrepancies in severity may be explained by the inclusion of inpatients solely as they are expected to present with more severe disease thus suggesting a selection bias of the patient-population. Inpatients may present with more severe respiratory symptoms requiring admission or may eventually have underlying conditions, which could also favor their admission. Thus, the severity of their respiratory illness could either result from their more severe clinical presentation upon admission, from underlying conditions potentially affecting their outcomes or from the type of viral infection. Furthermore most of these studies (Iwane et al, 2011; McCloskey et al, 2011) compared HRV/ENT to all other viruses, which did not allow one-to-one comparison and did not adjust for underlying co-morbidities.

Several reports suggested that HRV-C causes more severe respiratory illness in adults and children as well as more asthma hospitalizations than HRV-A or HRV-B (Lau

et al, 2009; Miller et al, 2009; Piralla et al, 2009). In addition, other studies (Fuji et al, 2011; Iwane et al, 2011), which included measures of HRV/ENT viremia reported that among children with HRV-C infections, 31% were viremic, compared to 3% and 0% of children with HRV-A and HRV-B infections, respectively, thus indirectly suggesting a more severe disease among HRV-C infected children. Associations of HRV-C infections and severe respiratory illness may be the result of population differences, differences in methodology, or sample collection (Wark et al. 2005; Takeyama et al, 2012). More recent studies (Iwane et al, 2011; Xiang et al 2010) reported no differences in clinical manifestations and prevalence of lower respiratory tract infection among inpatients pediatric patients with HRV-A and HRV-C infections. Iwane et al, 2011 documented significantly higher rates of HRV-A and HRV-C when compared to asymptomatic control children, with no differences in clinical indicators of severity such as ICU admission between HRV/ENT species. Likewise studies by Calvo et al, 2010; Jacobs et al, 2013 conducted among children younger than 14 years of age with ARIs reported no difference in clinical severity between HRV-C and HRV-A ARIs.

The quantification of viral load may predict disease severity at higher levels (Jacobs et al, 2013; Piralla et al, 2012) reported that a viral load higher than 10^7 copies/ml in nasopharyngeal aspirate specimen was independently associated with lower respiratory tract infection among children and adults hospitalized for acute respiratory illness. Another study by Takeyama et al, 2012 also correlated viral load with illness severity scores for children above 11 months of age with ARIs. In contrast, a study by Costa et al,

2011 reported no correlation between viral load and symptomatic infections or more severe infections. Results from individual studies are difficult to interpret as a result of different real-time PCR methods and sampling techniques, which therefore may not be generalizable to other patient populations and clinical settings.

Based on these findings, neither HRV-C nor higher viral loads might be associated with more severe respiratory illness in hospitalized children. Future steps of our study will include genotyping of HRV/ENT species and measures of viral loads in order to determine the prevalence of HRV-A and HRV-C species among our HRV/ENT population and to assess if HRV-C or HRV-A and viral loads correlate with more severe disease.

In conclusion, HRV/ENT infected children presented with more severe clinical outcomes compared to RSV or FLU-infected children, even after taking their underlying conditions into account. Further investigations including HRV/ENT genotyping and measure of viral loads may provide more insights into our findings.

Proportion of common respiratory illness

HRV/ENT positive status was identified as a significant independent predictor for URTI compared to either FLU or other infections, while controlling for underlying-comorbidities and age. Our findings are consistent with published literature (Jacobs et al,

2013) stating that HRV/ENT is detected in more than one-half to two thirds of common colds.

We documented higher rates of pneumonia and hypoxemia amongst children with HRV/ENT infections compared to those with RSV infections. These rates were consistent with those reported from published studies (24.1% vs. 18 to 26%) (Jacobs et al, 2013). However, after adjusting for underlying comorbidities, age and gender, HRV/ENT positive status did not remain a significant predictor for pneumonia. No differences were found compared to FLU or other viral infections. These findings are controversial with our previous statement of more severe clinical outcomes reported from HRV/ENT infected children. We would have expected that the more severe clinical outcomes reported from HRV/ENT positive children would have resulted from progression of URT symptoms to CAP. This may suggest that other factors such as their increased underlying conditions or viral-bacterial co-infections may have still influenced the association between HRV/ENT positive patients and severe disease. Most studies reporting higher rates of HRV/ENT, CAP also reported high rates of bacterial co-infections (60%) (Xiang et al, 2010; Garcia-Garcia, 2012). Progression to HRV/ENT pneumonia has been mostly documented in adult lung transplant recipients (Kaiser et al, 1999) as these patients are more likely to undergo bronchoscopy and broncho-alveolar lavage (BAL) for graft rejection surveillance as well during episodes of respiratory illness and thus are more often investigated for respiratory viral illnesses. Hematopoietic stem cell transplant recipients (HSCT) also experience higher rates of progression to pneumonia with subsequent substantial morbidity and

mortality. However, in the majority of these reported cases of HRV/ENT in the lower airways among adult lung transplant or HSCT recipients, co-infection with one or more bacterial, viral, and/or fungal co-pathogens is present (Ison et al, 2003; Ghosh et al, 1999). In our, study HRV/ENT positive children presented high rates of underlying immunosuppressive co-morbidities but low rates of bacterial co-infections, and underlying immunosuppression was associated with pneumonia after adjusting for other important variables. Thus, it is possible that underlying co-morbidities and bacterial co-infections are more important predictors of progression to pneumonia than HRV/ENT positive status. Another hypothesis refers to our definition of CAP, which relied on infiltrates visualized on chest X-rays (CXR) upon. As there is usually a delay between clinical symptoms and the documentation of infiltrates on CXR, our estimation of CAP may have been underestimated which may have influenced our results. However, the discrepancy between increased clinical severity observed among HRV/ENT positive patients and the absence of association between HRV/ENT positive status and CAP remains unclear and should be stated as a limitation as discussed below.

Strengths and limitations

Important strengths of our study comprise the inclusion of outpatients, thus enabling the use of hospital admission as a proxy of clinical severity. Also, the adjustment for underlying co-morbidities and the inclusion of an interaction term between HRV/ENT positive status and underlying immunosuppressive/metabolic disease in multivariable

analysis reinforced the association between HRV/ENT and clinical outcomes. Finally our study enabled more extensive comparison groups (HRV/ENT vs. RSV, HRV/ENT vs. FLU and HRV/ENT vs. other common respiratory viruses) each with adequate sample sizes.

There are several limitations of this study. The first limitations relate to its retrospective design. However we believe that most of our patient-related important outcomes could be well assessed though chart review. Similarly, the observational nature of our study may have led to selection bias as not all consecutive patients were tested for respiratory viruses. However, the random selection of the respiratory samples tested by molecular assays reduced the risk of selection bias. Third, we assessed presence or absence of viruses, but did not measure their viral load; higher viral loads in acutely ill subjects compared to asymptomatic children might have further strengthened the association between HRV/ENT and severe disease as rhinoviruses are also commonly identified in community based-controls (Miller et al, 2009; Iwane et al, 2004). This is an important point as improved detection methods have increased the detection rates of asymptomatic HRV infections and raise questions regarding the clinical significance of positive test results (Jansen et al, 2011). The incidence of asymptomatic infections is estimated to be 20-30% in experimental challenge models (Brownlee & Turner, 2007) and varies from 5-40% in clinical studies as a result of different study populations, definitions of asymptomatic events, and detection methods (Peltola et al, 2008; Greenberg, 2011; Brownlee & Turner, 2007). A fourth limitation relates to the generalizability of the results. As the study was conducted in one tertiary-care center, the results may not be translatable

to other populations, especially to the community. Usually children with more severe symptoms or underlying conditions consult at tertiary care centers whereas those with less severe symptoms are seen in community settings. Thus, our population sample may have been biased in that sicker patients were overrepresented in our study. Furthermore, our conservative approach of only including virus-positive patients detected by two or more molecular assays may have selected patients with more severe symptoms and thus overrepresented sicker patients. An important information relates to the number of patients who were detected HRV/ENT positive by one molecular assay. Removal of an important number of HRV/ENT patients detected by only one molecular assay may have affected the correlation between HRV/ENT positive status and severe disease as these patients could have presented a milder disease. A more precise estimation of the correlation between HRV/ENT status and clinical severity would have been obtained by also including HRV/ENT positive patients detected by only one assay with subsequent sensitivity analyses being not affected by their removal. Finally, from the 4 molecular assays used, two did not distinguish HRV and ENT, which may have affected our findings as ENT is a common virus detected in children

PART V. CONCLUSIONS AND FUTURE STEPS

HRV/ENT infections were commonly detected in our patient-population and consisted of more than a third of viral infections. This reinforces the need for routine diagnosis in hospital and community settings as the use of molecular assays for viral detection is usually applied in tertiary care centers and often to patients with underlying co-morbidities. Furthermore, viral detection is often performed in children but not in adults in whom the spectrum of HRV/ENT illness is much less defined. Generalizing the use of molecular tests to community settings and various patient-population would enable a better understanding of the spectrum of illness. Furthermore routine screening for respiratory viruses may have an impact on infection control measures and thus on the prevention of viral spread in hospital settings and may rationalize antibiotic management. This may in turn have a substantial impact on the economic burden of viral infections in hospital settings and on the development of resistant bacterial organisms resulting from inappropriate antibiotic prescriptions.

We demonstrated that patients with HRV/ENT infections were more likely presenting with underlying cardio-respiratory and immunosuppressive and metabolic conditions. Despite a higher proportion of patients with underlying conditions, HRV/ENT positive status remained a significant predictor for clinical severity. Furthermore, children with HRV/ENT infections, presented with a longer duration of hospital stay, were more likely to be admitted to the intensive care unit (ICU) and presented with higher rates of

mortality.

Viral status was not an important predictor of pneumonia, whereas underlying immunosuppressive/metabolic conditions or prematurity were significant predictors of pneumonia.

Our findings provide new insight into the burden and severity of HRV/ENT infections as we demonstrated significant higher morbidities and mortality among HRV/ENT positive patients compared to RSV-positive and FLU-positive patients. On the other hand, host-related conditions rather than viral status were more important predictors of pneumonia. Our findings reinforce first the need for development and testing of specific antiviral drugs, as no anti-HRV drugs or prevention strategies are currently commercially available. Second, a better understanding of the pathogenesis of HRV/ENT-related infections should be performed as severity of illness may be correlated with the virulence of some strains, host related factors, or both.

As mentioned above, several antiviral molecules have been tested so far, although none have provided convincing results. Pleconaril, a capsid binding agent has been discontinued the Food and drug Administration (FDA) due to concerns about resistance and safety, including interactions with hormonal contraception and drugs used to treat HIV. Another capsid binding inhibitor, vapendavir is currently evaluated in a phase 2b treatment study involving asthmatic adults to assess the effect of vapendavir on upper respiratory tract infections (URTI) and asthma exacerbations, although results have not yet

been disclosed (Jacobs et al, 2013). Finally, pirodavir, an intranasal capsid-binding agent that reached phase 3 clinical trials for HRV/ENT prevention and treatment in the 1990', was discontinued as a result of no significant reduction in the duration or severity of symptoms (Jacobs et al, 2013). Rupintrivir, a proteolytic enzyme inhibitor, did not significantly affect viral loads and symptom severity and was therefore discontinued (Jacobs et al, 2013). Non-specific mucosal approaches such as interferons have antiviral, anti-proliferative and immunological effects that impact host cell susceptibility to infection. Other treatment options include *Echinacea* preparations, which demonstrated no conclusive evidence for benefit in treating or preventing the common cold in a Cochrane review (Linde et al, 2009) and Zinc for which benefits were limited to adults with no impact on symptom severity as assessed in a recent Cochrane Review (Singh et al, 2011). Despite these discouraging findings newer approaches have recently been suggested such as silencing RNA (siRNA) approaches. Silencing RNA are attractive targets for RNAi therapeutics for respiratory viruses, as they target other sequences than surface proteins which are most genetically variable and prone to viral resistance and are topically administered and thus delivered directly to the site of infection. Silencing RNA were designed to inhibit the replication of respiratory syncytial virus (RSV) by interrupting the synthesis of viral nucleocapsid protein (N-protein) (ALN-RSV01). A phase 2b study of inhaled ALN-RSV01 showed reduced incidence of progressive bronchiolitis obliterans syndrome (BOS) in RSV-infected lung transplant patients. Silencing RNA may be a valuable approach for treatment of HRV/ENT respiratory illnesses (DeVincenzo, 2008).

Efforts to develop prophylactic medications and vaccinations for HRV/ENT prevention have been unsuccessful so far. Alpha-2 and beta interferons use are limited by their local adverse reactions such as nasal irritation, mucosal friability and bleeding. Although intranasal alpha-2 interferon has shown some benefit for the prevention of HRV/ENT colds, studies focusing on treatment have yielded equivocal results (Jacobs et al, 2013). In a placebo-controlled trial including subjects with naturally occurring colds showed no benefit for symptom severity or duration and even showed that subjects receiving a higher dose of interferon nasal spray experienced a longer duration of symptoms and more severe sore throat and nasal congestion, likely as a result of a toxicity of the treatment. Given the limited clinical benefits and potential side effects, including nasal mucosal bleeding, intranasal interferon has not been adopted as a therapy for HRV/ENT infections. A Cochrane systematic review and meta-analysis of vitamin C for the prevention and treatment of the common cold (Hemilä et al, 2010) found no difference in the incidences of colds in subjects treated with vitamin C and those given placebo.

To date, there have been no HRV/ENT vaccines evaluated in clinical trials. Challenges to vaccine development include the presence of more than 100 different HRV/ENT serotypes, the lack of epidemiological data to identify the most commonly circulating HRV/ENT strains, the incomplete understanding of antigenic differences between the recently discovered HRV-C species and known serotypes, and limited animal models of HRV/ENT infection to understand viral pathogenesis (Rohde, 2011). More recently, in vitro studies have focused on deriving antigenic peptides from one of the viral

capsid proteins, VP1, which plays a central role in receptor binding and subsequent epithelial cell infection and is recognized by HRV/ENT-neutralizing antibodies (Edlmayr, 2011).

Given limited preventive and therapeutic strategies, prevention of person-to-person transmission through behavioral strategies is essential to reduce viral transmission. These include social distancing, the use of respiratory masks and hand hygiene.

The severity of HRV/ENT illnesses could result from the host immune response, from the virulence of some strains, or both. The recently published full-length genomic sequences of all known serotypes, including the group C viruses, suggested that HRV-A and HRV-C lead to more severe outcomes than HRV-B although these findings remain yet controversial (Kaiser, 1999; Ghosh et al, 1999). These assumptions will be tested further in our study with the inclusion of the measure of viral loads and the inclusion of genotyping analysis for HRV/ENT. The inclusion of outpatients and inpatients in our study will enable an eventual correlation between species, which could be more likely associated with admission and those circulating in the community. On another hand, the high prevalence of patients with underlying conditions identified among our HRV/ENT infected patients may suggest that host-related risk factors may also have an impact on the acquisition and severity of HRV/ENT infections. A better understanding of the mechanisms leading to manifestations of HRV/ENT infections and the role of the host immune response is needed to guide efforts at HRV/ENT prevention and treatment.

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