HUMAN RHINOVIRUS EPIDEMIOLOGY

ASPECTS OF HUMAN RHINOVIRUS INFECTION IN HOSPITALIZED AND NON-HOSPITALIZED INDIVIDUALS

By

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To my parents,

Marco and Elena

Abstract

Human rhinovirus (HRV) is a single-stranded RNA virus responsible for causing the common cold and exacerbating chronic respiratory diseases. HRV is the most common cause of acute respiratory illness. Unfortunately, difficult culturing conditions and perceived mild symptoms have limited our understanding of HRV. This thesis characterizes fundamental aspects of HRV such as viral load in different patient populations, prevalence and diversity of HRV, and severity and duration of infection.

Initially, we developed an HRV qPCR assay to quantitate HRV in clinical isolates. We used this assay to measure viral loads in hospitalized and community members. We found that HRV viral loads were similar regardless of age and need for hospitalization. Viral loads were significantly lower amongst individuals with asymptomatic HRV infection than symptomatic HRV infection. Next, we determined the prevalence and diversity of HRV in children and adults. We found that HRV is the most common respiratory pathogen in September-October in both children and adults. A broad range of HRV genotypes can be found circulating amongst children and adults; however HRV C is more prevalent in children. Furthermore, we investigated the association of HRV C duration and severity of illness. Among otherwise healthy individuals, HRV C did not persist longer than HRV A/B, nor was the viral load significantly different. In hospitalized children, HRV C was not more associated with an asthma or wheeze diagnosis. Overall, our data suggest that viral loads do not predict the severity of illness, HRV C commonly occurs in children, and behaves like other HRV species.

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List of Abbreviations and Terms

CAP	Community acquired pneumonia
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disorder
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
HEV	Human enterovirus
HRV	Human rhinovirus
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILI	Influenza-like illness
IRES	Internal ribosome entry site
LDL-R	Low-density lipoprotein-receptor
LFA-1	Lymphocyte function-associated antigen-1
LLOD	Lower limit of detection
LLOQ	Lower limit of quantitation
LR	Lu reverse primer
MDA-5	Melanoma differentiation associated gene-5
NO	Nitric Oxide
NPS	Nasopharyngeal
NS	Nasal swab
OR	Odds ratio
ORF	Open reading frame
PCR	Polymerase chain reaction
RAF	Rhinovirus A forward primer
RBF	Rhinovirus B forward primer
RIG-I	Retinoic acid inducible gene-I
RSV	Respiratory syncytial virus
RT-PCR	Reverse-transcriptase PCR
RVP	Respiratory viral panel
SD	Standard deviation
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
URTI	Upper respiratory tract infection
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VHS	Virus-Host shutoff

Chapter One

Introduction

1.1 Taxonomy

The *Picornaviridae* family consists of 12 genera and 28 species (Knowles et al, 2010). Human rhinoviruses (HRVs) belong to the Enterovirus genus which has 10 species (Table 1, adapted from Knowles et al, 2010). Until 2009, HRV was a genus, but was reclassified an Enterovirus to correct phylogenetic inconsistencies.

The picornaviruses are between 22-30 nm in diameter and contain no lipid envelope. The genome is a single-stranded RNA that is 7-9 kb in length. Piconaviruses have a limited host range and are transmitted horizontally by fecal-oral or respiratory routes. Enteroviruses infect humans and other primates, but certain species are known to infect other mammals such as cows, buffalo, sheep, and pigs (Knowles et al, 2010). Human rhinoviruses infect the respiratory tract of humans which results in local respiratory infections.

Table 1. Classification, natural host ranges and associated diseases of enteroviruses (Knowles et al, 2010)

Species	Genotypes	Natural host range	Associated disease(s)
Human enterovirus A	21	Humans, monkeys	Herpangina, paralytic disease, asceptic meningitis, encephalitis, ataxia, hand-foot-and-mouth disease
Human enterovirus B	59	Humans, monkeys, pigs	Epidemic pleurodynia, asceptic meningitis, paralytic disease, encephalitis, myocarditis, hand-foot-and-mouth disease, swine vesicular disease
Human enterovirus C	19	Humans	Acute anterior poliomyelitis, respiratory disease, asceptic meningitis, encephalitis, ataxia, acute hemorrhagic conjunctivitis
Human enterovirus D	3	Humans	Hand-foot-and-mouth disease, acute hemorrhagic conjunctivitis, paralytic disease
Simian enterovirus A	1	Monkeys	None known
Bovine enterovirus	2	Cattle, water buffalo, African buffalo, sheep, goats, deer, impala	Abortion, stillbirth, infertility, neonatal death, enteritis, respiratory disease
Porcine enterovirus B	2	Pigs	Skin lesions
Human rhinovirus A	74	Humans	Respiratory disease
Human rhinovirus B	25	Humans	Respiratory disease
Human rhinovirus C	60[a]	Humans	Respiratory disease

[a]Approximate number as of April 2012 (Bochkov & Gern, 2012)

1.2 Genetics

HRV have a single-stranded RNA genome that is translated into a single polyprotein, and is cleaved to form P1 (containing structural proteins VP1-4), P2 (nonstructural proteins 2A-C), and P3 (nonstructural proteins 3A-C) (Hershenson, 2010). In addition, HRV contain a 5' untranslated region (5'UTR) for virus replication, a type I internal ribosomal entry site (IRES) that binds to the 40S ribosomal subunits in order to find the initiating AUG codon, and a 3'UTR to terminate translation of the open reading frame (ORF) (Palmenburg et al, 2010).

The most complex region of the HRV genome is the 5'UTR because of its many secondary and tertiary motifs (Palmenburg et al, 2010). At the 3' end of this region is the VpG which serves as a primer for genome replication (Palmenburg et al, 2010).

Previously, HRV diversity was not thought to arise from recombination events. Palmenburg et al (2009) identified 23 recombination events in the 5' UTR and protein capsid regions among 99 reference strains. When analyzing field isolates, recombination events were more frequent and were thought to be the result of co-infections within the host (Palmenburg et al, 2009). HRV C, while recently discovered, has most likely been circulating undetected for a long time. There are more rates of recombination events in the HRV C genome compared to HRV A or B (Bochkov & Gern, 2012).

There are 60 copies of each of the four structural proteins that form 12 pentamers around the virion (Palmenburg et al, 2010). Within VP1 is a hydrophobic 20 Å canyon which is believed to be the location of receptor attachment and is isolated from immune pressures (Olson et al, 1993). The VP4 region is located internally and is responsible for the assembly of the virus and infection of new cells (Bella & Rossman, 2000; Fuchs & Blaase, 2010).

The 2A protease cleaves the P1 and P2/P3 portion of the polyprotein, inactivates the *elF-4F* complex of the host cell in order to inhibit cellular translation of mRNA, and initiates virus host shutoff (VHS) to translate viral proteins with host machinery by stopping the normal cap-dependent mode of host translation (Dreschers et al, 2007). The

3C and 3D proteases are responsible for post-translational maturation of the VpG and RNA polymerase (Dreschers et al, 2007).

1.3 Pathogenesis

HRV is transmitted to humans horizontally by coughs, sneezes, respiratory secretions, and fomites (e.g. door knobs) (Bella & Rossman, 2000; Gwaltney, 2002). The majority (90%) of HRV A and B genotypes utilize intracellular adhesion molecule-1 (ICAM-1) as their cell receptor (Bella & Rossman, 2000; Bella & Rossman, 1999). Ten HRV A genotypes utilize low-density lipoprotein receptor (LDL-R) (Kennedy et al, 2012). Structural modeling studies indicate that HRV Cs use a distinct receptor to gain entry into the cell, although it remains unidentified (Bochkov et al, 2011). HRV binds to ICAM-1 near the site of lymphocyte function-associated antigen-1 (LFA-1) attachment on ciliated epithelial cells in the upper respiratory tract and as a result the capsid uncoats allowing the viral RNA to endocytose (Kennedy et al, 2012).

HRV does not cause cytopathology like other respiratory viruses (i.e. influenza, respiratory syncytial virus). However, it does disrupt tight junctions in the epithelial barrier causing increased vascular leakage and mucus secretions resulting in rhinorrhea (Kennedy et al, 2012). Coughing occurs in 30% of episodes and is possibly the result of direct infection of the large airways (Kennedy et al, 2012). Increased vascular permeability can lead to secondary bacterial infections in the sinus openings and Eustachian tube resulting in acute, secondary bacterial sinusitis and otitis media respectively (Kennedy et al, 2012).

HRV predominantly replicates at 33˚C, and thus was thought to be restricted to the upper respiratory tract. It is now known that HRV can infect the lower respiratory tract and has been cultured from secretions in the lower respiratory tract (Halperin et al, 1983). HRV RNA has been identified by *in situ* hybridization 4 days after an experimental infection in lower airway epithelium (Papadopoulos et al, 2000).

1.4 Immune Response

Once HRV infects the epithelium, the innate immune system is induced. Ngamtruakulpanit et al (2003) found that type I interferon production and a decrease in airway pH occurs within 24 hours of experimental exposure to HRV. 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 results in release of type I and type III interferons to inhibit virus replication. Cytokines like interleukin (IL)-12 and IL-15 recruit natural killer cells. Chemokines secreted (e.g. CXCL8 and CXCL10) early in the infection recruit neutrophils (Kennedy et al, 2012). Neutrophils in nasal secretions are one of the characteristic features of a symptomatic cold and also induce the production of bradykinin and kallidin and increase vasodilation (Kennedy et al, 2012).

The humoral immune response to HRV infection is incompletely understood. Patients with humoral immune failures experience severe episodes of HRV (Kainulainen et al, 2010). In experimental HRV infections of individuals with previous exposure,

serotype-specific IgA is detected by day 3 and IgG at day 7-8 (Message & Johnston, 2001). Antibodies contribute to viral clearance by preventing the virus from entering the host cell, opsonizing the virus, and by initiating natural killer cell-mediated antibody-dependent cellular cytotoxicity (Kennedy et al, 2012).

Viral titres begin to decline approximately 72 hours post-infection and cell-mediated immune responses reflect the activation of pre-existing memory T cells. Memory T cells respond to shared epitopes on the capsid of the infecting HRV (Kennedy et al, 2012; Edlmayr et al, 2011). The role of $CD8^+$ T cells in HRV infections is not well documented. However, they are thought to drive the eradication of the virus by the production of IFN-γ (Kennedy et al, 2012; Sun et al, 2011).

1.5 Clinical Presentation and Complications

The prototypic illness associated with HRV is the common cold. The incubation period of a typical infection is 8-12 hours, and the initial symptom is a sore throat commencing 10-16 hours post infection (Gwaltney, 2002). Symptoms include: rhinitis, rhinorrhea, sneezing, sore throat, cough, and fever. The mean duration of symptoms is 7.5 days (Gwaltney, 2002).

Otitis media complications from HRV infections are caused by mucociliary damage, blocked middle ear ventilation, increased mucus in the Eustachian tube and secondary bacterial infections (Greenberg, 2011). In a prospective study of 121 children prone to otitis media, HRV was identified in 30% of specimens, and was co-infected with

Moraxella cattarhalis and/or *Streptococcus pneumoniae* (Alper et al, 2009). In addition, abnormalities in the maxillary and ethmoid sinuses can result in rhinosinusitis (Greenberg, 2011). Less than 20% of infections have complications from a secondary bacterial infection (Greenberg, 2011; Gwaltney et al, 2000).

HRV infections are associated with exacerbations of chronic respiratory diseases. HRV infections are common in individuals with underlying asthma or chronic lung conditions like chronic obstructive pulmonary disorder (COPD) (Kennedy et al, 2012). In asthma, innate immune responses such as decreases in IFN-α, IL-12 and IFN-γ are thought to be defective in bronchial epithelial cells (Greenberg, 2011). HRV infections also induce vascular endothelial growth factor (VEGF), nitric oxide (NO), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), and neutropine to induce airway remodeling (Greenberg, 2011). For cases of COPD, the mechanism behind HRV induced COPD exacerbations is unknown. It is possible that it is the result of ICAM-1 up-regulation in the bronchial mucosa or the adherence of bacteria to HRV-infected epithelial cells causing ICAM-1 and TLR-3 up-regulation leading to increased HRV binding and an increased HRV-induced cytokine response (Sajjan et al, 2006).

1.6 Epidemiology

HRV infections are the most common cause of respiratory illness in children and adults. They can occur all year long but are most common in spring and autumn (Winther et al, 2006). Within the first two years of life incidence of HRV is reported to be 0.7-0.8 infections per person per year (Blomqvist et al, 2002; van Benten et al, 2003).

In children 3 months-15 years, Winther et al (2006) identified 6 picornavirus infections per person per year. Adults have been documented to have 2-3 infections per year (Madigan et al, 2003). The home is the most common location for transmission of virus, as school-age children frequently introduce the infection (Hendley & Gwaltney, 1988). Secondary infection rates range from 25-70% (Greenberg, 2011). Transmission of virus can occur by close contact, autoinoculation, fomites, and aerosols (Greenberg, 2011).

The incidence of asymptomatic infections is estimated to be 20-30% in experimental challenge models (Brownlee & Turner, 2007). The prevalence of asymptomatic HRV infections varies from 5-40% because of different study populations, definitions of asymptomatic events, and detection methods (Peltola et al, 2008; Greenberg, 2011; Brownlee & Turner, 2007). 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).

HRV has been detected in blood and nasal secretions. Case reports are increasingly identifying HRV in pericardial fluid, stool, plasma, and urine in neonates (Tapparel et al, 2009a; Broberg et al, 2011). Viremia in HRV is rare, although common in human enteroviruses (HEV) (Tapparel et al, 2009a). Unlike viremia, HRV is commonly shed through nasal secretions and can spread to uninfected individuals. A review of current literature indicates that HRV can be shed in nasal secretions for 11-days post-infection (Cate et al, 1964; van Elden et al, 2008; Hendley & Gwaltney, 2004).

Initial reports of HRV C suggested that it resulted in more severe lower respiratory tract infections in young children compared to HRV A or B (Bochkov & Gern, 2011; Lau et al, 2007; Tapparel et al, 2009a; Khetsuriani et al, 2008; Arden & Mackay, 2010). The role of HRV C in severe disease presentation is unclear and its predominance in severe clinical presentations has conflicting evidence. Among a cohort of 528 children with community acquired pneumonia (CAP), HRV was detected in 17.9% of children (Xiang et al, 2010). Among these patients, 51.5% were positive for HRV A, 10.1% for HRV B, and 38.4% for HRV C (Xiang et al, 2010). HRV C strains did not circulate at a higher prevalence and severity of illness was comparable to HRV A (Xiang et al, 2010). In addition, 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).

HRV is also an important factor in the development of recurrent wheeze and asthma and short-term mortality in children >12 months; although its role is not completely understood (Kieninger & Regamey, 2012). The seasonality of asthma exacerbations is associated with the seasonality of HRV infections. Johnston et al (1996) compared seasonal patterns of respiratory infections and hospital admissions for asthma in children and adults and identified a strong correlation between the two ($r=0.72$, $p<0.0001$). Hospital admissions for asthma and respiratory infections were more common in the fall and spring when children return to school from vacations (Johnston,

1995). Johnston et al (1995) reported that 80-85% of asthma exacerbations were associated with viral infections in children 9-11 years with a history of asthma. Of the viruses detected, rhinoviruses accounted for 66% (147/226) of detected virus (Johnston et al, 1995). In a cohort of 259 infants followed from birth to 6 years, HRV-associated wheezing at birth to age 3 was a more likely predictor of asthma at age 6 (OR 9.8) than RSV (OR 2.6) (Jackson et al, 2008). At age 3, previous HRV infections were associated with an increased risk of asthma (OR 31.7) compared to RSV (OR 13.6) (Jackson et al, 2008). In adults with asthma, virus was identified in 57% of subjects with colds and asthma exacerbations (Nicholson et al, 1993). HRV was the most frequently identified pathogen isolated in 33% (76/229) of nasal swabs (Nicholson et al, 1993).

The role HRV plays in the development of asthma is unknown. Only some infants with HRV-associated bronchiolitis develop wheezing or asthma later in life, suggesting other factors are involved (Kieninger & Regamey, 2012). Midulla et al (2012) suggests the highest risk factors for asthma development include recurrent viral infections, atopic history (early allergen sensitization or maternal history of atopy), and pulmonary remodeling, which may all interact to increase the risk of asthma development.

1.7 Diagnosis of HRV Infection

Our increased knowledge about the biology and clinical effects of HRV are due in large part to improved detection methods. Traditional diagnostic methods include culture in primary pulmonary fibroblast cells and acid lability testing (inactivation of virus at low pH) (Santti et al, 1999). These methods are largely insensitive due to many strains and

require separate equipment (incubator set at 33˚C) (Lu et al, 2008). The diversity of HRV makes serology unfeasible; as a result molecular testing such as reverse transcription-PCR (RT-PCR) is the preferred method for HRV detection (Santti et al, 1999).

Numerous molecular assays have been described for HRV since 1988, primarily targeting the 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). The region contains many conserved motifs required for translation and replication, and there are also conserved regions in both entero- and rhinoviruses (Santti et al, 1999). However, they do not distinguish between HEV and HRV without sequencing the amplicon, hybridization with an internal HRV-specific probes, or use of nested PCR (Mahony, 2008; Savolainen et al, 2003). In addition, no currently published HRV assay is able to detect all know HRV genotypes. Faux et al (2011) compared the relative sensitivity of 10 5'UTR primer pairs and determined that none of the primers could detect all 99 HRV A/B genotypes and HRV C clinical isolates tested. The primer pairs did not exhibit any species bias by preferentially amplifying one species over another (Faux et al, 2011). However, specimens with a lower RNA concentration were less likely to be detected than those with higher concentrations (Faux et al, 2011). This illustrates that multiple primer pairs would be required not to miss any divergent variants (Faux et al, 2011; Bochkov & Gern, 2012). There are currently no published assays that utilize multiple primer pairs in the 5'UTR in order to detect all HRV genotypes.

The capsid proteins VP2/4 and VP1 have been utilized for explicit species classification; however, they are more variable than the 5'UTR hindering the development of universal primer pairs (Bochkov & Gern, 2012). Commercial diagnostic assays like 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) (EraGen Bisosciences Inc., Madison, WI) have included both 5'UTR and capsid gene primers in their platforms 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 sensitivity of PCR tests complicates the interpretation of clinical results as the presence of HRV detection may not correlate with hospitalization (Jansen et al, 2011). The use of PCR has increased HRV by 85% in recent years (van Der Zalm et al, 2009). The amount of genome copies or viral load in a clinical sample measured by qPCR could be utilized to determine if the presence of HRV in a clinical specimen is related to the hospitalization and is causing complications. qPCR assays are available for other viruses such as HIV and RSV, and the amount of virus in these examples do correlate with disease severity (Falsey, Criddle, & Walsh, 2006). Commercial assays for HRV are not able to quantitate viral load and there are limited published assays for HRV quantitation.

The few studies that have investigated HRV viral loads and have only looked at hospitalized children (Utokaparch et al, 2011; Franz et al, 2010). There are no

publications concerning HRV viral load in older populations, viral load over the course of a typical infection, or asymptomatic HRV viral load in adults. Comparisons of in viral typical HRV infections, asymptomatic infections, and HRV infections requiring hospitalization should be conducted to determine if there are significant differences in viral load between the three groups. Jansen et al (2011) conducted a prospective case-control study in asymptomatic and symptomatic children and found significant differences in viral load in symptomatic children compared to the controls ($p<0.01$). This indicates that it is possible to define a minimum amount of HRV that contributes to symptomatic illness and hospitalizations in children. However, different factors will need to be assessed first for each patient population such as timing of sampling, co-morbidities, sample quality, and standardization of sampling (Jansen et al, 2011).

As a final note, the development of a quantitative PCR assay for HRV could aid in the future development of HRV anti-virals by measuring drug efficacy over the course of an infection.

1.8 Treatment

There are no specific treatments for HRV currently available. The vast number of serotypes makes a vaccine against HRV unrealistic. The search for HRV inhibitors began in the late 1980s when the crystal structure of HRV 14 was first identified (Rossmann et al, 1985). The target of choice was the protein capsid (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 put into clinical trials in 1996 but was rejected by the FDA (Food and Drugs Administration) in 2002 due to safety concerns (Hershenson, 2010). Capsid binding agents like pleconaril readily select for drug resistance because the HRV structural proteins are less conserved than non-structural proteins (Thibaut et al, 2012).

Alternatives to the capsid binding agents are proteolytic enzyme targets. The proteases 2A, 3C, and 3CD are essential for viral replication and share little sequence similarity with cellular proteases (Thibaut et al, 2012). Enzymes can be inhibited by irreversibly trapping the enzyme or competing with the substrate by reversibly binding on the enzyme's active site (Rawlings et al, 2004; Fear et al, 2007). The most potent inhibitor developed was rupintivir, but it did not decrease the clinical severity or viral load in natural infections (Thibaut et al, 2012). Another protease inhibitor is LVLQTM, targeting the 2A protease (Falah et al, 2012). It has been shown to inhibit HRV 2 and 14 replication in A549 cells 500- and 150-fold respectively, although further evaluation is required (Falah et al, 2012).

Recently, the capsid structure of HRV C was modeled and was identified to have a shorter VP1 protein and a more restricted hydrophobic pocket than either HRV A or B (Basta et al, 2012; Sgro et al, 2012). Novel capsid-binding agents will need to be developed for HRV C genotypes.

Hypothesis

HRV is the prime cause of the common cold and as a result they were neglected by the medical community for 40 years. There are now many unanswered questions regarding HRV epidemiology. HRV molecular assays require continual improvements in order to detect newly discovered genotypes. Increased detection of HRV also raises questions about the importance of HRV to clinicians and its role in disease severity. The development of qPCR assays and investigations of viral load in symptomatic infections and asymptomatic infections will determine its need to be included in routine diagnostic testing. HRV C was identified in 2006 and there is still much that is not understood such as the prevalence in individuals of different ages or its role in the development of chronic respiratory disease.

This thesis will answer the following hypotheses: 1) viral loads are lower in the community versus hospitalized patients, 2) HRV C is more common in children than adults, and 3) HRV C does not result in more severe or prolonged infections compared to HRV A/B.

Foreward

HRV is a common viral pathogen that infects every individual and whose clinical spectrum of disease has recently expanded to include exacerbations of chronic respiratory diseases and lower respiratory tract infections. While the specific research objectives are defined in each of the research chapters 2 through 4, the comprehensive objectives of this

research project were to investigate HRV viral loads amongst different patient populations, prevalence of HRV C in children and adults, and the severity and duration of HRV C infections compared to HRV A/B.

This thesis is organized into three major research chapters (chapters 2 through 4) comprising three manuscripts, together with introductory and concluding chapters. I was the principal author of all of these papers and conducted the experiments conducted herein unless otherwise stated. Chapter two describes the development of an HRV qPCR assay and presents data on the quantitation of clinical samples from three distinct patient populations. This work has been submitted to Diagnostic Microbiology and Infectious Disease and is presented in manuscript format as outlined by the Journal. To understand how common HRV is in a student population and to compare the viral loads of a symptomatic population, we conducted a 10% random sampling of asymptomatic university students stratified by week. These data are presented in Chapter three in manuscript format. Chapter four investigates the genetic diversity of HRV in children and adults and the association of HRV and asthma development in children. Drs. Nancy Nashid and Jeffrey Pernica, at McMaster University, Department of Pediatrics performed the medical chart reviews used for this study. These data have are presented in manuscript format. Dr. Mahony guided my experiments throughout the entire duration of my studies and provided invaluable comments on all manuscript drafts, and in doing so, helped me improve the final versions presented here.

Chapter Two

Author's Preface to Chapter Two

In this chapter, I describe a novel qPCR assay used to quantitate HRV in different patient populations. This work identifies the first use of HRV qPCR assays to report viral loads in adult populations and to measure HRV viral loads over seven days in healthy adults. The overall implications of these findings are that (i) HRV viral loads do not differ between the community and hospitalized patients and (ii) viral loads significantly decrease following symptom onset in healthy individuals.

The material in Chapter two has been submitted for publication in the peer-reviewed journal Diagnostic Microbiology and Infectious Disease. The experiments described in this chapter are my original contributions. Dr. Samira Mubareka at Sunnybrook Health Sciences Centre provided clinical samples from an HRV outbreak at that institution. Emma Goodall and Lisa Banh provided clinical samples from university students. Sylvia Chong and Kathy Luinstra conducted xTAG^{TM} RVP (Luminex, Toronto, ON) testing on pediatric and university samples respectively. Drs. James Mahony and Marek Smieja provided comments and helped with the experimental design.

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Use of an improved qPCR assay to determine differences in human rhinovirus viral loads in different populations

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Running Title: Rhinovirus viral loads in three populations

Abstract

Human rhinoviruses (HRV) frequently cause acute respiratory infections and chronic respiratory disease exacerbations. However, testing is not generally offered. We developed a modified HRV quantitative PCR (qPCR) assay to assess viral loads in the community and hospital patients. The assay had a lower limit of detection (LLOD) of 2 $\log_{10}$ viral copies/ml, displayed linearity over 5 $\log_{10}$ viral copies, with a lower limit of quantitation (LLOQ) of 4 $\log_{10}$ viral copies/ml. Mean viral loads (95% CI) for hospitalized children, university students, and institutionalized elderly respectively, were 7.08 $\log_{10}$ viral copies/ml (6.7, 7.5), 6.87 $\log_{10}$ viral copies/ml (6.5, 7.2) and 7.09 $\log_{10}$ viral copies/ml (6.9, 7.3), ($p=0.67$). Serial specimens of 14 university students showed a decrease of mean viral loads from 6.36 $\log_{10}$ viral copies/ml on day 1 to 2.32 $\log_{10}$ viral copies/ml 7 days past symptom onset ($p<0.001$). Using an HRV qPCR we showed that viral loads did not differ between the community and hospitalized populations and significantly decreased following symptoms onset in healthy individuals.

Keywords: Human rhinovirus, quantitative, PCR, viral load

1. Introduction

HRVs cause two to three self-limited episodes of the common cold per person each year (Hershenson, 2010). It is a leading cause of respiratory virus infections causing hospitalizations in children: some 25-50% of children with respiratory symptoms test positive for HRV (Van der Zalm et al, 2011; Legg et al, 2005). HRV exacerbate chronic respiratory diseases and increase morbidity rates in the elderly (Gerna et al, 2009; Jartti and Korppi, 2011; Longtin et al, 2010a). Viral infections cause 85% of asthma exacerbations in children, and HRV infections are responsible for 50-80% of these exacerbations (Kelly & Busse, 2008; Wark et al, 2005). Surveillance of long-term care facilities in Ontario from July 1-December 31, 2009 found that of 297 respiratory outbreaks, HRV was identified in 59% (Longtin et al, 2010b). Despite its increasing clinical importance in chronic respiratory disease, HRVs are not usually diagnosed by clinical laboratories. Traditional detection methods (i.e. antigen detection) are not feasible due to the large number of serotypes. Recently, molecular assays targeting the 5'UTR and commercial multiplex assays detecting human enterovirus (HEV) and HRV have been described (Gambarino et al, 2009; Jokela et al, 2005; Lu et al, 2008). Molecular tests targeting the conserved 5'UTR have improved sensitivity and increase the coverage of picornaviruses; however, they do not distinguish between HEV and HRV (Mahony, 2008). Not all resources, such as locked nucleotide analog primers which stabilize melting temperatures and allow for shorter primer sequences are available to everyone as there are limited manufacturers limiting the possibilities of developing HRV

specific assays (Lu et al, 2008). qPCR assays have recently been developed for some respiratory viruses (Gianella et al, 2011; Hohaus et al, 2011; Ward et al, 2004); including HRV (Franz et al, 2010; Utokaparch et al, 2011) and are used to assess responses to antiviral agents and predict patient outcomes. However, there are no commercial qPCR assays currently available.

The objectives of this study were to develop a modified qPCR assay for HRV from Lu et al (2008), evaluate viral load in three symptomatic patient populations, and monitor viral loads in a student population for seven days following symptom onset.

2. Methods

2.1 Clinical Specimens

Nasopharyngeal swab (NP) and mid-turbinate nasal flocked swabs (NS) were collected by experienced healthcare professionals or self-collected from three patient populations including hospitalized children, university students, and institutionalized elderly. This study was approved by the McMaster University Research Ethics Board (Hamilton, ON).

One hundred NP specimens were submitted in universal transport medium (UTM) (Copan Italia, Brescia, Italy) to the Regional Virology Laboratory; Hamilton, ON between September-December 2009 from children (0-16 years) represented the first population. Symptoms were recorded upon admission. The NP specimens were tested by xTAG^{TM} RVP v.1 (Luminex, Toronto, ON).

McMaster University undergraduate students (n=422) participating in a cohort study between September-October 2009 and September-October 2010 represented the second population. Mid-turbinate flocked nasal swabs (NS) in CyMol^{TM} transport medium (Luinstra et al, 2011; Smieja et al, 2010) (Copan Italia, Brescia, Italy) were self-collected at symptom onset and for up to 7 days. Students submitted electronic questionnaires of upper respiratory tract infection (URTI) symptoms weekly. NS were tested with xTAG^{TM} RVP v.1 (Luminex, Toronto, ON).

The third group included 59 residents in a long-term care facility at Sunnybrook Health Sciences, Toronto, ON. NP specimens were collected in UTM (Copan Italia, Brescia, Italy) from August 27-October 13, 2010. Symptoms were recorded upon hospital admission. Samples were positive for HRV using the Resplex II v.2 assay (Qiagen, Mississauga, ON).

2.2 Total nucleic acid extraction

Total nucleic acid was extracted from the NP and/or NS of all three populations (200 µl) using the easyMAG^{TM} automated extractor according to the manufacturer's instruction (bioMeriéux, Montreal, QC). Twenty microliters of MS2 bacteriophage (Luminex, Toronto, ON) were added to each sample.

2.3 Quantitative PCR

A 210 bp region of the 5' UTR of HRV was selected for amplification. Two modified forward primers (RAF and RBF), a reverse primer (LR) and a dual-labeled

probe (Rhino Probe) were used in the qPCR (Table 1). Primers were synthesized at Mobix (Hamilton, ON) and the probe labeled with 6-carboxyfluorescein (5') and Black Hole Quencher-1 (3') was synthesized at Biosearch Technologies Inc (Novato, CA) (Table 1).

qPCR was performed using the QuantiTectProbe RT-PCR kit from Qiagen (Mississauga, ON). The 20 µl reaction volume contained 1.5 µM each of forward and reverse primers, 0.2 µM of probe, 5 µl of nucleic acid and amplification were performed using a LightCycler 2.0 (Roche, Laval, QC) as follows: reverse transcription, 30 min at 50˚C, polymerase activation, 15 min at 95˚C, and 45 cycles of 30 s at 95˚C and 60 s at 60˚C.

RNA transcripts were prepared with a representative HRV strain for analytical sensitivity and specificity studies. PCR amplicons were prepared from HRV 60 (American Type Culture Collection (ATCC), Manassas, VA) using primers that bracketed the qPCR region (Kiang et al, 2008). The transcripts were synthesized with the MEGAscript T7 *in* vitro Transcription kit (Applied Biosystems, Carlsbad, CA) and purified with PureLink PCR purification kit (Invitrogen, Burlington, ON). Transcripts were quantified using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Positive-sense transcripts were 400 bp in length with yields of $1.50x10^{13}$ viral copies/µl. A standard curve was generated with replicate serial 10-fold dilutions in easyMAG Extraction Buffer 3 (bioMérieux, Montreal, QC) and stored at -70˚C.

The specificity of the HRV qPCR assay was assessed using RSV, PIV 1-3, adenovirus, human metapneumovirus, human coronavivrus 229E and OC43, influenza A H1N1 and H3N2, influenza B, herpes simplex virus 1, cytomegalovirus AD169, *Chlamydophila pneumoniae, Legionella pneumophila*, *Streptococcus pneumoniae,* and *Mycoplasma pneumonia* from clinical isolates. To assess the ability of the qPCR to detect a range of picornaviruses, 22 HEV from clinical specimens were tested.

2.4 Sequencing

Sanger sequencing was used to distinguish between HEV and HRV targeting 400 bp of the 5'UTR of the HEV/HRV genome (Kiang et al, 2008). To identify species, a partial sequencing of the VP1 gene was amplified (Nix et al, 2006). Amplicons were purified using PureLink PCR purification, (Invitrogen, Burlington, ON) and 5 µl were sequenced on the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) (Mobix, Hamilton, ON).

2.5 Statistical Analysis

ANOVA was performed using GraphPad InStat 3 (GraphPad Software, La Jolla, CA). A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Development of qPCR assay for HRV

3.1.1 Sensitivity and Specificity of qPCR

A qPCR assay amplifying 210 bp of the 5'UTR was developed to assess HRV viral loads. A panel of 18 different respiratory viruses and bacteria were tested to assess the specificity of the qPCR. All specimens tested negative by qPCR.

A panel of 22 recent HEV isolates was tested to assess cross-reactivity with HEV. Only 3/22 HEV (13.6%) (mean threshold cycle (C_T) 32.9; 95% CI, 31.7-34.0) were detected by the HRV qPCR assay.

3.1.2 Lower Limit of Detection and Quantitation

HRV 60 RNA transcripts were used to assess amplification efficiency. Amplification was linear over a 5-log dynamic range (Figure 1), an R^2 value of 0.996 and efficiency (Bustin et al, 2009) of 104.5%. The LLOD was determined by testing 15 replicates of HRV 60 RNA transcripts (1.9 $\log_{10}$ to 3 $\log_{10}$ viral copies/ml each). Between 2 $\log_{10}$ to 3 $\log_{10}$ viral copies/ml, 15/15 replicates were positive, while, at 1.9 $\log_{10}$ viral copies/ml, only 3/15 replicates were positive. The LLOD was 2 $\log_{10}$ viral copies/ml. The LLOQ was determined by plotting mean C_T for dilutions 1.9 $\log_{10}$ to 3 $\log_{10}$ viral copies/ml as shown in Figure 1. Linearity was lost below 4 $\log_{10}$ viral copies/ml indicating an LLOQ of 10^4 copies of HRV RNA.

3.1.3 Reproducibility

Next we assessed inter- and intra-assay reproducibility using serial dilutions of RNA transcripts ranging from 2 $\log_{10}$ to 8 $\log_{10}$ viral copies/ml by testing on 5 consecutive days. Mean C_T (SD) from 2 $\log_{10}$ to 8 $\log_{10}$ ranged from 33.0 (1.1) to 17.5 (0.03) within runs, and 32.6 (0.7) to 16.5 (0.4) between runs, respectively. The assay was reproducible between 10^3 and 10^8 copies of RNA.

3.1.4 HRV viral loads of clinical specimens

A sampling of 187 HRV clinical isolates were tested using the HRV qPCR assay, comprising 81 HRV A, 54 HRV B, and 52 HRV C confirmed by Sanger sequencing. All were detected and quantified by the assay.

3.2 Comparison of HRV viral loads in different populations

To determine HRV viral loads we tested HRV positive samples from 18 hospital admitted children, 19 university students and 18 institutionalized elderly on day 1 of illness. Mean viral loads from children, students, and elderly were 7.1 $\log_{10}$ viral copies/ml (95% CI 6.7, 7.5), 6.9 $\log_{10}$ viral copies/ml (95% CI 6.5, 7.2) and 6.9 $\log_{10}$ viral copies/ml (95% CI 6.5, 7.3), respectively (p=0.67). Twelve children reported symptoms of asthma exacerbations (33.3%), wheezing (8.3%), fever (50.0%), rhinitis (8.3%), and cough (41.7%). Eighteen students reported symptoms of rhinitis (77.8%), fever (22.2%), cough (66.7%), sore throat (38.9%), and wheezing (16.7%). Eighteen institutionalized elderly residents reported fever (25.0%), rhinitis (15.0%), and cough (90.0%). No

individual in the three populations was co-infected with another respiratory virus as determined by xTAG^{TM} RVP v. 1 (Luminex, Toronto, ON).

3.3 Serial daily sampling of HRV viral loads in university students

To determine the duration of HRV shedding, 14 undergraduate students with symptoms of URTI self-collected NS for a period of 7 days from the onset of illness, and then once weekly. Virus was detected on days 1-7, but none had detectable virus on day 14. Mean viral load decreased from 6.4 $\log_{10}$ viral copies/ml on day 1 to 2.3 $\log_{10}$ viral copies/ml on day 7 ($p<0.001$) (Figure 2). Sanger sequencing of HRV positives identified 5 HRV A, 5 HRV B, and 4 HRV C species. There was no significant difference in viral load between species ($p=0.81$). In addition, no significant difference in viral load decline was detected between species ($p=0.86$).

4. Discussion

In this study, we used an improved qPCR assay to evaluate viral loads in three patient populations and investigate HRV shedding in students. This assay detects a wider range of HRV genotypes due to the addition of a second forward primer. We found no significant difference in viral load between the three study populations ($p=0.67$). Serial observations of students for seven days post onset of symptoms demonstrated a significant decrease in viral load ($p<0.001$).

The HRV qPCR assay targeted a 210 bp region in the 5' UTR. This is a highly conserved region with lower rates of recombination compared to HEV (Lu et al, 2008).

The modified primers adapted from Lu et al (2008) are effective at detecting all three HRV species, although not all genotypes were tested and future modifications to the primer and probe design may be necessary as novel genotypes emerge (Faux et al, 2011). While the assay does not detect other respiratory pathogens, it detected some HEV (3/22 positives) at a low copy number (mean C_T, 32.86). The relevance of low viral loads of HEV in clinical isolates is not known and will require further investigation. In addition, it is not known whether HRV viral loads below our LLOQ are clinically relevant and result in clinical presentations. We are currently investigating HRV viral loads in asymptomatic individuals.

We used the qPCR to test different symptomatic patient populations and expected to see different quantities between children, university students, and the elderly to correlate viral load with severity of clinical presentation. To our surprise the mean titres of HRV were the same for these three populations. Sampling with NS and NPS is comparable and cannot account for differences in viral titres (Lambert et al, 2008; Larios et al, 2011). Timing may have affected our results as adult specimens were collected on day one following symptom onset, whereas the time of collection for children and symptom onset was not available. This is the first report, to our knowledge, of HRV viral loads in adult populations. Two previous reports measured HRV viral loads in hospitalized children (Franz et al, 2010; Utokaparch et al, 2011). Utokaparch et al (2011) tested archived samples and reported lower viral loads (mean viral load, 1.07 $\log_{10}$ viral copies/ml) for children than we detected (Utokaparch et al, 2011). Lower viral loads in their study may

have been due to prolonged specimen storage with potential RNA degradation, different assay methodology, or collection method. Future studies will be required to determine the range of viral loads in children as well as how viral load changes over time.

We evaluated the duration of infections in the community by testing otherwise healthy university students. There was no difference in the rate of decline for viral load of HRV A, B, or C ($p=0.86$), although our sample size was small. Students were not infected with a distinct HRV genotype during the symptomatic episode nor did they experience a second episode. Additional studies with larger numbers of healthy individuals will be required to determine if there are differences in viral loads across difference HRV species.

The qPCR assay described here conducts rapid, sensitive, and precise quantitation of HRV. To date, there are no data on viral loads at various times following infection of hospitalized patients. Observations of hospitalized patients allowing comparisons to community infections may be warranted. qPCR tests provide accurate tools for investigation of HRV viral loads and could be used to measure the efficacy of novel antivirals currently under development.

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Figure Legends

Figure 1. Dynamic range of the HRV qPCR assay and lower limit of quantitation. Mean threshold cycle (C_T) values were plotted against the copy number. The lower limit of quantitation is 4 $\log_{10}$ viral copies/ml and is indicated by an arrow.

Figure 2. Longitudinal surveillance of HRV viral loads in university students. Self-collected mid-turbinate nasal swabs (n=98) were serially submitted over a seven-day period by undergraduate students (n=14) following the onset of upper respiratory tract infection. Fourteen nasal swabs were tested per time point. Data is plotted as mean viral loads ($\log_{10}$ viral copies/ml) and SEM for various days following onset of symptoms ($p<0.001$).

Table 1. **Primers and probe for HRV qPCR assay and detection assay**

Name	Sequence (5' → 3')[a]	Position[b]
RAF, forward[c]	GACAGTGTTCYAGCCTGCG	342-360
RBF, forward[c]	RACHGTGTCYYAGCCTGCG	342-360
LR, reverse[d]	GAAACACGGACACCCAAAGTA	556-536
Rhino Probe[d,e]	TCCTCCGGCCCCTGAATGYGGC	437-458
DK001[f]	CAAGCACTTCTGTTTCCC	151-168
DK004[f]	CACGGACACCCAAAGTAGT	539-521

[a]R= dA or dG, H= dA, dC, or dT, Y= dC or dT

[b]The nucleotide numbering is based on that of the HRV 60 (1473) sequence (Accession no. FJ445143.1)

[c]Primers for HRV qPCR modified from Lu et al (2008)

[d]Adapted from Lu et al (2008)

[e]The probe was 5'-end labeled with 6-carboxyfluorescein and 3'-end labeled with Black Hole Quencher-1 (Biosearch Technologies Inc, Novato, CA)

[f]HRV/HEV detection primers adapted from Kiang et al (2008)

Figure 1

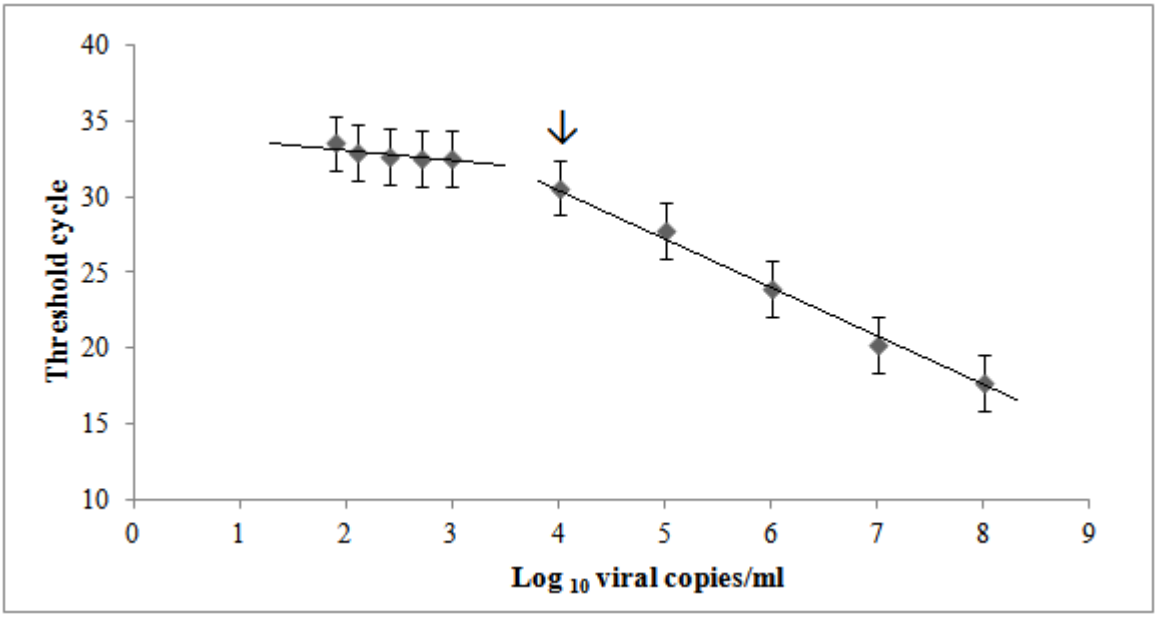
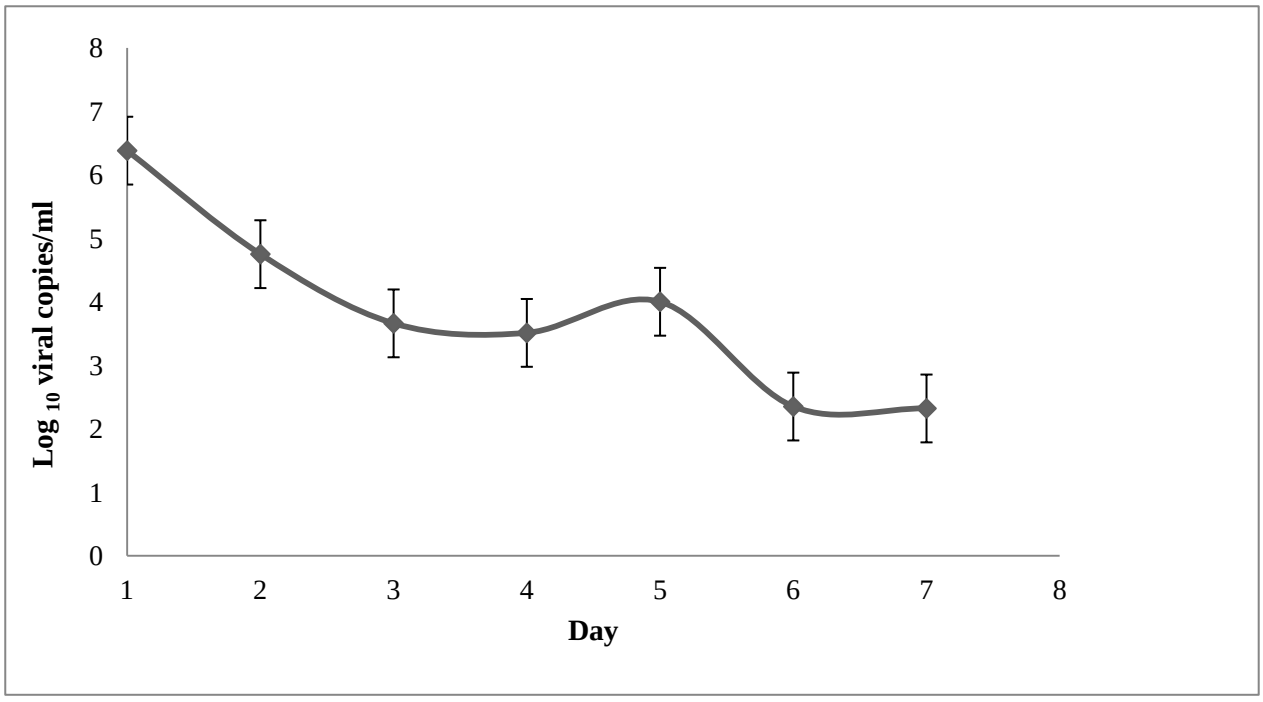

40
35
30
25
20
15
10
Threshold cycle
0
1
2
3
4
5
6
7
8
9
Log 10 viral copies/ml

Figure 2



Chapter Three

Author's Preface to Chapter Three

In this chapter, I describe a cohort study of university students to determine the prevalence of asymptomatic HRV infections, to compare viral loads of symptomatic and asymptomatic individuals, and to determine the persistence of viral shedding in asymptomatic individuals. Few studies have investigated the prevalence and viral load of asymptomatic HRV infections in adults. The overall implications of these findings are that (i) asymptomatic HRV infections occur frequently in university students, (ii) viral loads in asymptomatic HRV infections are significantly lower than in symptomatic HRV infections, and (iii) asymptomatic HRV infections can persist for 8-10 days.

The experiments described in this chapter are my original contributions. Emma Goodall provided clinical samples from university students as well as symptom diaries. Kathy Luinstra conducted xTAG^{TM} RVP testing (Luminex, Toronto, ON) on all samples. Drs. James Mahony and Marek Smieja provided comments and helped with the experimental design.

Asymptomatic human rhinovirus infections in university students

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Abstract

Objective: To determine the prevalence, viral load and duration of shedding of asymptomatic human rhinovirus (HRV) infections in two cohorts of undergraduate students. **Methods:** Students (n=545) enrolled for the McFlu2 COLD3 clinical trial in September-October 2010 (n=271) and 2011 (n=274) and self-collected mid-turbinate nasal flocked swabs weekly in CyMol^{TM} transport medium (Copan) regardless of symptoms. Weekly electronic questionnaires assessed upper respiratory tract infection (URTI) symptoms. To identify asymptomatic HRV, a 10% random sampling of swabs from students who did not report URTI in the 8 study weeks, stratified by week was conducted. Nucleic acid was extracted by easyMag^{TM} (bioMeriéux) and enterovirus/rhinovirus RNA was detected by RT-PCR using the One Step RT-PCR kit (Qiagen), sequencing confirmed HRV. Virus quantitation was performed by qPCR using QuantiTect Probe RT-PCR kit (Qiagen). **Results:** Thirty-three HRV infections (n=400) (8.25%) were found from swabs sampled. Mean viral load for asymptomatic and symptomatic infections were 5.27 $\log_{10}$ copies/ml, and 6.43 $\log_{10}$ copies/ml respectively (mean difference= 1.15, 95% CI: 0.59, 1.72, $p<0.001$). There was no relationship observed between viral load and HRV species ($p=0.22$), however, viral load did correlate with symptom presence ($p=0.004$). Longitudinal follow-up of asymptomatic individuals identified virus shedding for 8 days in 10/33 (30.3%) participants. **Conclusion:** Our cohort studies demonstrate that asymptomatic HRV infection occurs frequently, and is associated with lower viral loads than symptomatic infections ($p<0.001$), and HRV may

be persistently shed for 1-2 weeks. HRV viral loads may be useful for monitoring responses to treatment and for evaluating novel antivirals.

Introduction

Human rhinoviruses (HRV) are responsible for the common cold and the majority of viral respiratory illnesses in the spring and fall. More recently, HRV has also been associated with exacerbations of chronic respiratory disease[1-3]. This expansion of the HRV clinical spectrum has largely been the result of improved detection. The use of PCR assays has increased the rate of HRV detection by 85% compared with traditional methods such as culture[4]. Increased sensitivity raises questions about the significance of positive PCR test results. PCR cannot distinguish between viable and non-viable virus, but it can be used to detect HRV shedding in symptomatic or asymptomatic individuals.

Few studies have investigated the prevalence of asymptomatic HRV infections[4-11]. In experimental HRV challenge models, 20-30% of volunteers are asymptomatically infected[12]. In previous natural history studies of HRV infections prevalence varies widely from 5-40%[10,12-13]. The variance results from different study populations, definitions of asymptomatic infections, and viral detection methods implemented in each study [13]. We investigated two cohorts of university students to determine the prevalence of asymptomatic HRV infections, to compare viral loads in symptomatic and asymptomatic individuals and to determine persistence of viral shedding in asymptomatic individuals.

Methods

Clinical specimens

Mid-turbinate flocked nasal swabs (NS) (Copan Italia, Brescia, Italy) were self-collected from McMaster University undergraduate students and used to determine asymptomatic and symptomatic HRV infections. NS were extracted (200 µl) using the easyMAG™ automated extractor according to the manufacturer's instruction (bioMeriéux, Montreal, QC). Twenty microliters of MS2 bacteriophage were added to each sample as an internal extraction control. This study was approved by the McMaster University Research Ethics Board (Hamilton, ON).

We enrolled 545 McMaster University undergraduate students from September to October 2010 (n=271) and September to October 2011 (n=274). Study participants self-collected NS in CyMol™ transport medium (Copan Italia, Brescia, Italy) and submitted electronic questionnaires assessing symptoms of upper respiratory tract infections (URTI) weekly[14-15]. A symptomatic event was determined if a student reported at least two symptoms, one of which must be stuffy nose, sneezing, cough, sore throat, or wheezing. To identify asymptomatic individuals, a 10% random sampling of NS specimens from students who did not report URTI during the 8 week study period, stratified by week was conducted each year (n=200 in 2010; n=200 in 2011).

Detection of entero-rhinovirus infections

Clinical specimens were amplified using the One-Step RT-PCR kit from Qiagen (Mississauga, ON) targeting a 400 bp region of the human enterovirus (HEV) and HRV 5'untranslated region (5'UTR) (see Chapter 1, pg 34)[24]. Positives were confirmed using the xTAG™ RVP (Luminex Molecular Diagnostics, Toronto, ON).

Human rhinovirus qPCR assay

To determine the viral load of symptomatic and asymptomatic HRV infections, we quantitated HRV in positive specimens using a HRV qPCR assay targeting a conserved 210 bp region of the 5'UTR using the QuantiTectProbe RT-PCR kit from Qiagen (Mississauga, ON). The final 20 µl reaction volume contained 1.5 µM each of forward and reverse primers, 0.2 µM of the probe, and 5 µl of nucleic acid (see Chapter 1, pg. 34). Amplification was performed using a LightCycler 2.0 (Roche, Laval, QC) and the following steps: reverse transcription, 30 min at 50˚C, polymerase activation, 15 min at 95˚C, and 45 cycles of 30 s at 95˚C and 60 s at 60˚C. Each run included RNA transcript standards (3 $\log_{10}$ to 8 $\log_{10}$ viral copies/ml).

Sequencing HRV

Sequencing was used to determine the species of positives (ie. HEV or HRV A, B, C). For sequencing, 400 bp of the 5'UTR of the HEV/HRV genome was amplified[24] (see Chapter 1, pg. 34) using the One-Step RT-PCR kit (Qiagen, Mississauga, ON) targeting 400 bp of the 5'UTR of the HEV/HRV genome[24]. Amplicons were purified

using PureLink PCR purification, (Invitrogen, Burlington, ON) and 5 µl were sequenced on the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) (Mobix, Hamilton, ON). To confirm HRV species (A, B, C), 400 bp of the VP1 gene was sequenced using HotStarTaq (Qiagen, Mississauga, ON)[25]. Amplicons were purified using PureLink PCR purification, (Invitrogen, Burlington, ON) and 5 µl were sequenced on the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) (Mobix, Hamilton, ON).

Statistical analysis

ANOVA and linear regression analyses were performed using SPSS v.20 (SPSS Inc, Chicago IL), $p<0.05$ was considered to be statistically significant.

Results

Comparison of asymptomatic and symptomatic HRV infections

We investigated the prevalence of asymptomatic and symptomatic HRV infections in university students. Through our 10% weekly random sampling, 33/400 (8.25%) asymptomatic specimens were identified as positive for HRV. One hundred and fifty-seven symptomatic episodes of URTI were reported, and 76 (13.9%) were identified as HRVs. We compared the weekly prevalence of positive symptomatic (n=250/week) and asymptomatic (n=25/week) HRV infections for weeks 36-43 in 2010 and 2011(Figure 1). Incidence rates of asymptomatic and symptomatic infections were 8.25%/week and 1.83%/week, respectively.

Comparison of viral loads in asymptomatic and symptomatic individuals

We compared the difference in viral load of asymptomatic and symptomatic HRV infections. Mean viral load (SD) in asymptomatic students was 5.3 (± 1.5) $\log_{10}$ viral copies/ml and 6.4 (±1.3) $\log_{10}$ viral copies/ml in symptomatic students. The mean difference was 1.15 $\log_{10}$ viral copies/ml (95% CI 0.59, 1.72) (Figure 2).

To determine if a correlation exists between HRV species and symptom presence we used VP1 sequencing. Sequencing identified 50 HRV A, 45 HRV B, and 8 HRV C. A linear regression of species (HRV C versus HRV A/B) adjusting for symptom presence was conducted. There was no relationship between viral load and HRV species (beta= -0.39, p=0.37). However, viral load did increase with symptom presence (beta=0.92, p=0.004).

Persistence of HRV in ten asymptomatic individuals

We investigated the duration of HRV shedding amongst asymptomatic individuals. Of the 33 individuals identified, 10 (30.3%) were positive for a period of at least 8 days (Table 1). Mean viral load on day 1 was 6.53 $\log_{10}$ viral copies/ml (±1.74) and 2.56 $\log_{10}$ viral copies/ml on day 8 (±1.23) (Mean difference= 4.0 $\log_{10}$ viral copies/ml, 95% CI 1.15, 3.98, paired t-test p= 0.001). Based on sequencing of the VP1 gene, 4/10 (40.0%) individuals were infected with the same genotype for at least 8 days, 4/10 (40.0%) individuals were infected with a different genotype by day 8, and 2/10 (20.0%) had an unresolved genotype.

Discussion

In this study, we identified a high incidence of asymptomatic HRV infections in university students. The rate of novel HRV infections was 8.20% per week; from there, we can infer that 60.5% of the cohort was infected during an eight week period. Comparisons of asymptomatic and symptomatic infections indicated that asymptomatic individuals had lower viral loads. Sequencing identified that 40.0% of asymptomatic individuals were infected with a distinct genotype 8 days later.

HRV is often found in students, and in one study of 165 students followed prospectively over nine months, the number of symptomatic episodes was 1.6 colds per student[16]. For rates of asymptomatic infections, HRV challenge models in university students estimate a prevalence rate of 20-30%[12]. This compares to 12%-68% for natural infections, however, most populations investigated were children[6-11,17]. To our knowledge, our study is the first to study asymptomatic HRV rates in an adult population.

Few studies have investigated viral loads in asymptomatic individuals. Peltola et al (2008) did not find a statistical difference between asymptomatic and symptomatic HRV viral loads[10]. However, their methodology differed as both adults and children were analyzed together[10]. Jansen et al (2011) found a significant difference between asymptomatic and symptomatic HRV viral loads in children 0-6 years[18]. We hypothesized that the viral loads of asymptomatic students should be lower compared to symptomatic students and indeed found that there was a significant difference ($p<0.001$) with lower viral loads in asymptomatic students. However, the confidence intervals of

each population overlapped. A clear cut-off value for a symptomatic infection based on viral load could not be defined in this population. Jansen et al (2011) and Gerna et al (2009) found that levels greater than or equal to 4.5-5 $\log_{10}$ viral copies/ml were indicative of a symptomatic infection in children 1-6 years of age. This would indicate that viral load is not the only factor involved in the development of symptomatic infections. They are likely protected by acquired immunity against many HRV genotypes[10]. Further studies should be conducted to determine the significance of low level HRV detection in PCR assays.

Previous studies only collected asymptomatic specimens at one time point[8,10]. Our study design allowed us to investigate the duration of virus shedding however we could not accurately identify the start of an infection. Persistent HRV infection was found for at least 8 days in 30.3% of asymptomatic individuals with viral loads that significantly decreased by day 8 ($p=0.001$). This is consistent with other reports that state asymptomatic HRV was not detected beyond day 11 [13,17]. This also agrees with our previous findings that symptomatic HRV persists for at least 7 days in university students but not longer than 14 days[23]. Genotyping of asymptomatic HRV on day 8 indicated 40.0% were a different genotype, no student in our symptomatic category were identified to have a distinct genotype during their URTI.

Few studies have investigated the length of HRV shedding in asymptomatic infections. Reports of children infected with HRV RNA demonstrated detection of HRV RNA 5-6 weeks and infections with multiple genotypes[11,13]. In our study, there was no

relationship between HRV species and viral load (p=0.37). Previous reports indicated that HRV C can cause severe illness in certain populations[19-21]. Only 8 HRV C infections (6 symptomatic, 2 asymptomatic) were identified in our student cohorts. The low numbers in our student population could signify that HRV C is more prevalent in children than in healthy adults as suggested by Bochkov and Gern (2012) [22]. HRV B was frequently identified in our asymptomatic student population. Reports have stated that HRV A and C are associated with acute respiratory disease in hospitalized adults[22]. It is possible that HRV B is associated with more mild or asymptomatic infections.

In summary, this study demonstrates the high extent of asymptomatic HRV in healthy adults. HRV viral loads could be used to measure novel antivirals. This will prevent asymptomatic transmission of HRV infections in the community and hospitals.

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Figure Legends

Figure 1. Distribution of asymptomatic and symptomatic HRV infections by week from weeks 36-43. Specimens from asymptomatic (n=25/week) and symptomatic (n=250/week) patients were tested weekly in 2010 (a) and 2011 (b). Vertical bars represent standard error.

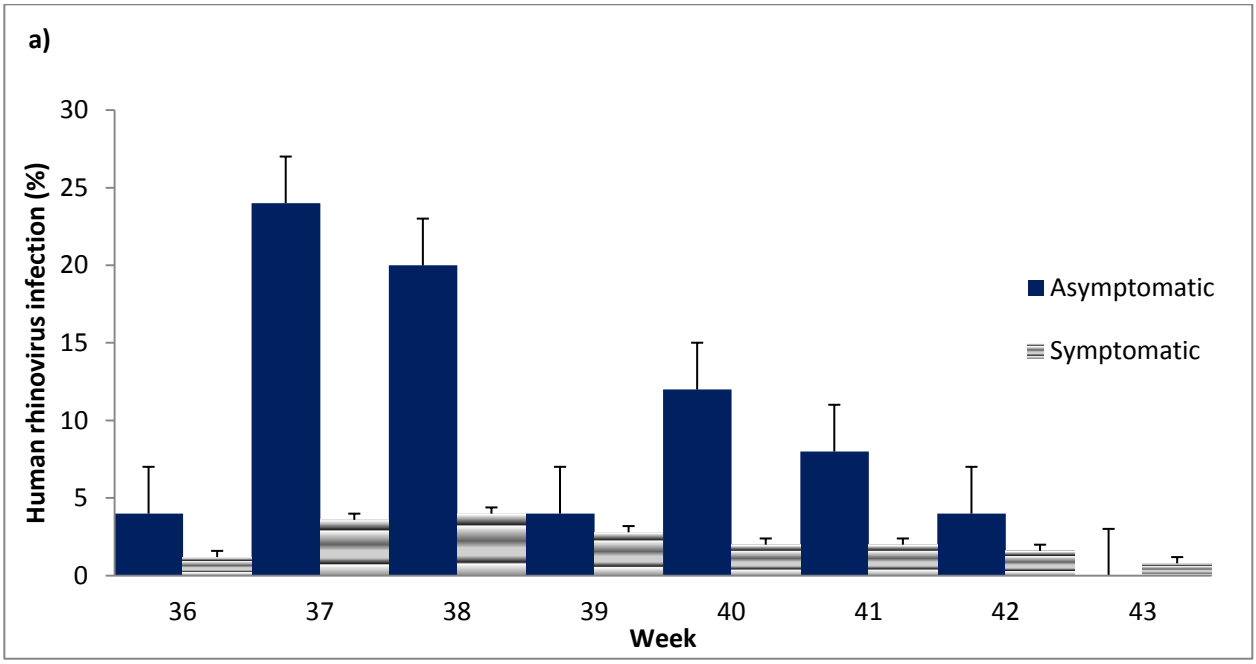
Figure 2. Box and whiskers plot of human rhinovirus (HRV) viral loads (log_{10} viral copies/ml) in NS specimens from symptomatic and asymptomatic university students. Symptomatic students were self-reporting upper respiratory tract infections (Symptomatic, n=85), and asymptomatic students were identified from a 10% random sampling (Asymptomatic, n=33) and (t-test, $p<0.001$). The solid line represents the median; the bottom and top of the box represent 25^{th} and 75^{th} percentiles, and the whiskers represent the minimum and maximum data points, respectively.

Table 1. **Human rhinovirus viral loads on day 1 and 8 in ten asymptomatic individuals**

Individual	Day 1[a]	Day 8[a]	Change in viral load[a]
1	5.88	3.32	-2.56
2	7.14	3.85	-3.29
3	4.38	3.14	-1.24
4	7.51	4.07	-3.44
5	8.04	6.56	-1.48
6	8.81	4.60	-4.21
7	7.09	3.44	-3.65
8	3.82	5.22	1.40
9	8.00	3.26	-4.74
10	4.66	2.24	-2.42

[a]Viral load, $\log_{10}$ viral copies/ml

Figure 1


a)
Human rhinovirus infection (%)
30
25
20
15
10
5
0
36
37
38
39
40
41
42
43
Week
Asymptomatic
Symptomatic

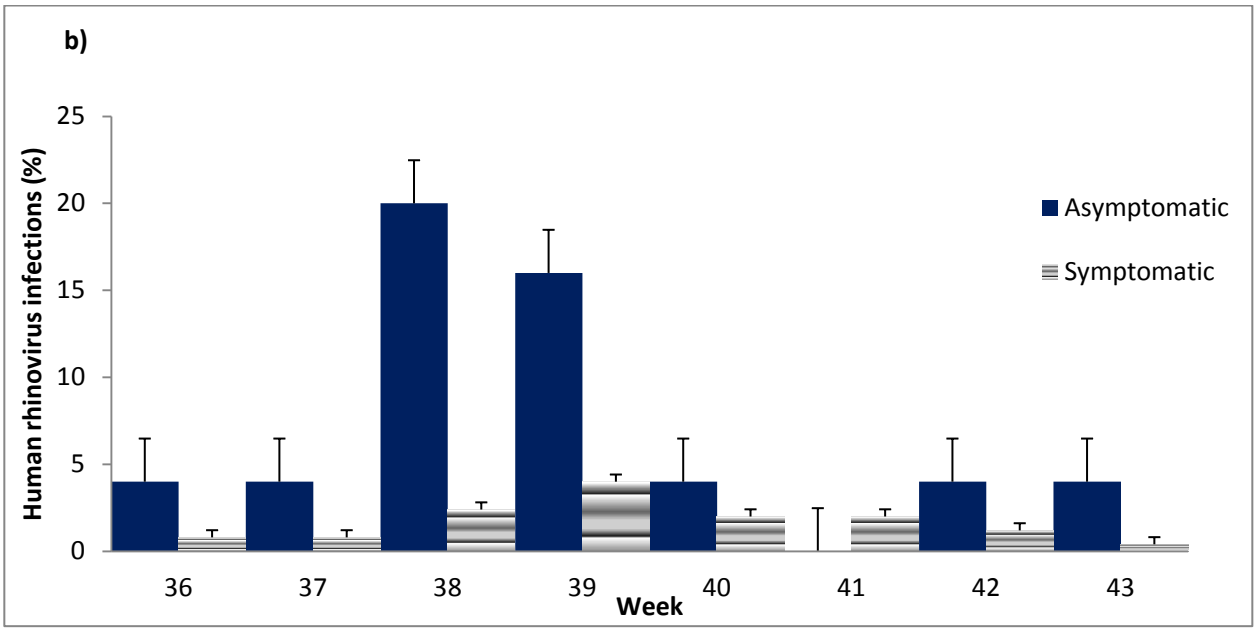
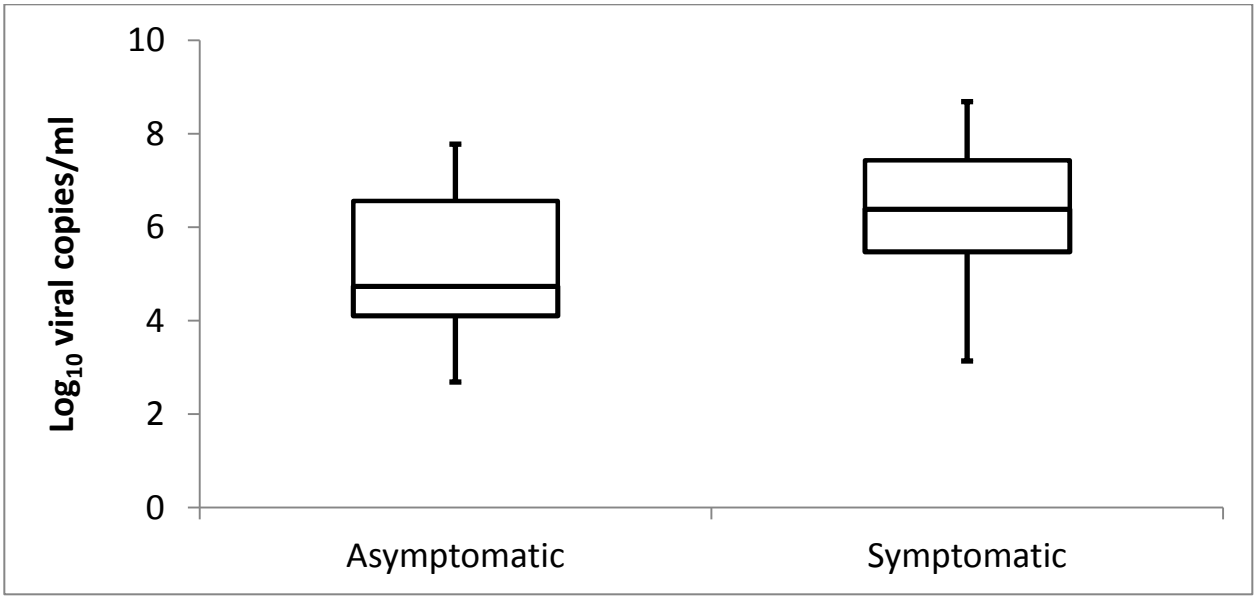

b)
Human rhinovirus infections (%)
25
20
15
10
5
0
36
37
38
39
40
41
42
43
Week
Asymptomatic
Symptomatic

Figure 2



Chapter Four

Author's Preface to Chapter Four

In this chapter, I describe a retrospective study comparing the genetic diversity of HRV in hospitalized children and adults in the community. This work identifies the first investigation of HRV diversity in children and adults in Canada. It also determines an association between HRV and asthma. The overall implications of these findings are that (i) HRV occurs frequently in September and October in children and adults (ii) HRV C is 14.5 times more likely to occur in children than adults, and (iii) HRV is associated with a diagnosis or history of asthma or wheezing in children.

The experiments described in this chapter are my original contributions. Emma Goodall recruited university students in 2010 and 2011. Drs. Nancy Nashid and Jeffrey Pernica performed the medical chart reviews used for this study as well as assisted in the experimental design. Drs. James Mahony and Marek Smieja provided comments and helped with the experimental design.

Analysis of human rhinovirus species (A, B, C) in children presenting to an emergency department and in symptomatic university students in the community

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Abstract

Objective: To determine the frequency and diversity of HRV in hospitalized children and university students over two 8-week periods. **Methods**: Nasopharyngeal swab samples that were negative for conventional respiratory viruses (n=116) were collected from children (3-59 months) admitted to McMaster Children's Hospital, Hamilton, ON from September-October 2010 and September-October 2011. University students were enrolled in the McFlu2 COLD3 clinical trial, a randomized trial of vitamin D or gargling for prevention of respiratory infection, in September-October 2010 (n=271), and 2011 (n=274) and self-collected mid-turbinate nasal flocked swabs weekly in CyMol^{TM} transport medium (Copan, Brescia, Italy). HRV was identified and quantitated by an in-house HRV-specific RT-qPCR targeting 210 bp of the 5'UTR using the QuantiTect Probe RT-PCR kit (Qiagen, Mississauga, ON) on a LightCycler 2.0 (Roche, Laval, QC). Partial sequencing of the VP1 gene was used to confirm HRV infection. Retrospective blinded chart reviews were conducted on clinical specimens from the children. **Results**: HRV was identified in 65.5% in hospitalized children and 50.9% of university students. The children had 25 HRV A, 9 HRV B, and 41 HRV C. University students had 37 HRV A, 31 HRV B, and 6 HRV C. HRV C was 14.5 times more likely to occur in children than in adults. Among children, HRV C did not occur more frequently than HRV A or B (p=0.18). In children, HRV was associated with a diagnosis or history of asthma or wheezing (p=0.025) (OR 4.3, 95% CI 1.3, 14.3). **Conclusion**: Our results have identified that HRV C infects children more often than adults compared with other HRV

species, HRV C is not associated with severe illness, and all HRV species are associated with a diagnosis of asthma in children.

Introduction

HRVs are the primary causes of the common cold in individuals of all ages. HRV has also been isolated in the lower respiratory tract and is the leading cause of respiratory virus illness causing hospitalizations in children under 5 years[1]. HRVs are responsible for 50-80% of viral-induced asthma exacerbations, and HRV in three-year old children was a stronger predictor of asthma by age six than was RSV (HRV OR 9.8 versus RSV OR 2.6)[2-7]. Studies have found inconsistent difference in illness severity among HRV species A, B, and C[8-9]. In a cohort of 128 children with a moderate to severe episode of asthma, 87.5% were infected with HRV[10]. HRV C was present in 59.4% of episodes and was associated with more severe presentations[10]. Other reports have found no difference in illness severity amongst the HRV species[11-12]. Similarly, other investigators found that HRV A and C are responsible for more episodes of acute respiratory illness in hospitalized adults and children than HRV B[16].

Our increased understanding of HRV has improved during recent years due to the advent of molecular testing. Molecular tests enable the detection of all HRV species, including HRV C, which cannot be grown in cell culture. While HRV is associated with hospitalizations, its role in severe illness presentation is not completely understood[14]. The objectives of the current study are to delineate the association of HRV A, B and C with severe illness in hospitalized children and to compare the frequency and genotypic diversity of HRV in hospitalized children versus adults in the community.

Methods

Clinical specimens

Nasopharyngeal swabs (NPS) and mid-turbinate flocked nasal swabs (NS) were collected from hospitalized children and university students respectively (Flocked™ swabs, Copan Italia, Brescia, Italy). This study was approved by the McMaster University Research Ethics Board.

We conducted a retrospective screen of 116 NP (nasopharyngeal) specimens for HRV infections in children 3 to 59 months admitted to the emergency room of McMaster University Children's Hospital from September and October 2010 and in September and October 2011. They were tested in the Regional Virology Laboratory at St. Joseph's Healthcare (Hamilton, ON) and determined to be negative by direct fluorescence antibody for influenza A and B (before Nov 1, 2010) and PCR (after Nov 1, 2010) for parainfluenza 1-3, adenovirus, respiratory syncytial virus, and metapneumovirus.

We enrolled 545 McMaster University undergraduate students from September to October 2010 (n=271) and September to October 2011 (n=274). Participants self-collected NS in CyMol™ transport medium (Copan Italia, Brescia) and submitted electronic questionnaires assessing symptoms upper respiratory tract infection (URTI) weekly[14-15]. We identified 74 entero-rhinovirus infections (43 in 2010, 31 in 2011).

Detection of enterovirus/rhinovirus by RT-PCR

Total nucleic acid was extracted from clinical specimens (200 µl) using the easyMAG™ automated extractor. Twenty microliters of the MS2 bacteriophage were added into each sample. HRV was detected using an in-house RT-PCR targeting 400 bp of the 5'UTRof the human enterovirus (HEV) and HRV genome [18] (see Chapter 1, pg. 34). Clinical specimens were amplified using One-Step RT-PCR kit from Qiagen (Mississauga, ON). The 25 µl reaction contained 1x buffer, 0.4 mM of dNTP, 0.6 µM of primers, and 2 µl of enzyme mix. Amplification conditions including reverse transcription, 50˚C for 30 min, amplification, 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 30 s for 40 cycles, and extension for 72˚C for 1 min were utilized on a PTC 200 Thermocycler (MJ Research, Minneapolis, MN). Products were visualized on a 2% agarose gel with ethidium bromide staining. HRV positives were confirmed by the xTAG™ RVP (Luminex, Toronto, ON).

Sequencing

To distinguish HEV from HRV, Sanger sequencing was conducted on the specimens previously described. Primers targeted 400 bp of the HEV and HRV 5'UTR (See Chapter 1, pg. 34)[18]. To identify species, a partial VP1 gene was sequenced[17]. Amplicons were purified using the PureLink PCR Purification kit from Invitrogen (Burlington, ON), and 5 µl was sequenced on the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) (Mobix, Hamilton, ON). Phylogenetic trees of the VP1 gene were constructed by the Neighbour-Joining Method and 1000 bootstrap replicates.

Sequencing analysis was analyzed by Mega v.5 (Centre for Evolutionary Medicine and Informatics, The Biodesign Institute, Tempe, AZ).

HRV qPCR

To determine HRV viral loads, HRV was quantitated by using an HRV RT-qPCR targeting 210 bp of the HRV 5'UTR (see Chapter 1, pg 34). The assay used the QuantiTect Probe RT-PCR kit (Qiagen, Mississauga, ON). The 20 µl mixture contained 1x buffer, 1.5 µM each of forward and reverse primers, 0.2 µM of the probe, and 5 µl of nucleic acids. Amplification was performed using a LightCycler 2.0 (Roche, Laval, QC) under the following conditions: reverse transcription 50˚C for 30 min, and amplification of 94˚C for 30 s, and 60˚C for 60 s for 45 cycles. Each run included RNA transcript standards (3 $\log_{10}$ to 8 $\log_{10}$ viral copies/5 µl).

Statistical analysis

Chi-square tests were performed using Minitab v. 16 (Minitab Inc., State College, PA), and $p<0.05$ was considered to be statistically significant.

Results

HRV in children and students

For hospitalized children, 116 nasopharyngeal swabs met our criteria of a patient ages 3-59 months and negative for conventional respiratory viruses, of which 99 were positive for a respiratory pathogen. HRV was identified in 76 episodes (65.5%). Mean

viral load (SD) for children (n=76) was 6.46 (±1.5) $\log_{10}$ viral copies/ml. For the university student population, there were 167 episodes of URTI reported and HRV was identified in 85 (50.9%) cases. The mean HRV viral load (SD) for university students (n=85) was 6.37 (±1.3) $\log_{10}$ viral copies/ml. No significant difference in viral load was seen between the children and adults (t-test, p=0.86).

No university student was re-infected with HRV during the 8-week study period, however, 5 children were tested more than once and 3 children were re-infected during the study period with a distinct genotype.

Distribution and diversity of HRV species between hospitalized children and university students

Sequencing of the VP1 gene was used to determine HRV species. Table 1 shows the distribution of species for children and students. HRV A and C were the most common in hospitalized children making up 90.4% of the total infections, whereas HRV A and B comprised the majority of HRV species (91.9%) identified amongst the students. We compared the incidence of HRV C to HRV A or B and identified a significant increase in the frequency of HRV C in children compared to students ($p<0.001$, OR 14.5, 95% CI 5.2, 42.7).

To evaluate relatedness of HRVs in each population, phylogenetic trees (Figure 1) were constructed. Isolates with >75% nucleotide identity were considered to represent strains of the same serotype[17]. There were a total of 33 genotypes identified amongst the

76 children specimens and 32 genotypes amongst the 85 students. Furthermore, there were 16 HRV C genotypes in the children and only two in the students ($p<0.0001$, OR 14.1, 95% CI 2.6, 101.5).

Clinical history and association between HRV and asthma history in children

Age and clinical history of the hospitalized children are shown in Table 2. For the 116 NPS evaluated, 109 patient histories were available. Between HRV positive and HRV negative children, there was no significant difference in the presence of a co-morbidity ($p=0.84$) (Table 2). In addition, there was no difference in the occurrence of HRV C compared to HRV A and B in children ($p=0.18$) (Table 2).

Clinical characteristics were compared between children with HRV and children without HRV (Table 3). Length of stay and diagnosis of pneumonia/bronchiolitis did not differ in children regardless of HRV presence ($p=0.19$ and $p=0.35$ respectively), however HRV was more common in children with a history or diagnosis of asthma, reactive airway disease or wheezing ($p=0.025$; OR 4.3; 95% CI 1.3, 14.3). Presence of HRV C was not associated with asthma, reactive airway disease or wheezing compared to HRV A/B ($p=0.37$).

Discussion

Our study investigated the association of HRV with severe illness in hospitalized children and compared the frequency and genotypic diversity of HRV in hospitalized children and university students. We identified that HRV is associated with a

history/diagnosis of asthma (OR 4.3), HRV C occurs more frequently in children than adults (OR 14.5), and HRV C is not more associated with a history/diagnosis of asthma than HRV A/B (p=0.18).

To our knowledge, this is the first study to investigate the genetic diversity of HRV in hospitalized children and university students. During the study period, HRV was the principal pathogen circulating among children and students. Phylogenetic analysis demonstrated that all three species circulated in both populations. However, children were more likely to be infected with HRV C than were students (OR 14.5) and had significantly more HRV C genotypes (OR 14.5) than students. Cross-sectional studies have found that HRV A and C are the most common respiratory virus pathogens in children with acute respiratory illness[16]. However, in our study, HRV A and B were more frequently detected than HRV C in university students in our study. This is consistent with other studies[16]. It is possible that in childhood HRV C infections elicit a stronger immune response than HRV A or B reducing the likelihood for subsequent HRV C infections later in life.

In children <5 years of age admitted to the hospital, there were similar rates in hospital stay, co-morbidity, and diagnosis of pneumonia/bronchiolitis across all three species. Furthermore, we found that rates of HRV C infection in asthma were similar HRV A/B infections in children which is consistent with other studies[15,19].

Our data supports an association between HRV and asthma/wheezing (OR 4.3). While the association is consistent with previous findings[3,20], we cannot compare these

predictors directly as we only investigated the correlation in the months of September and October.

Our study has some limitations. First, HRV infections may not reflect the cause of hospitalization. Second, as a retrospective study, we did not follow up on individuals after their visit to the hospital to track asthma onset. Finally, asthma is difficult to diagnose in young children because pulmonary function tests are not recommended in children <5 years of age; to account for this we used definitions described by the Asthma Predictive Index to determine the likelihood of asthma development and the modified Pediatric Respiratory Assessment Measure to assess severity of illness[21]. A strength of our study was the use of qPCR, which may be useful in future studies attempting to correlate HRV viral load with disease severity.

In summary, our study provides evidence that HRV C is more likely to occur in children than adults ($p<0.001$), HRV infection is associated with a history or diagnosis of asthma in the months of September and October ($p=0.025$) and HRV C is associated with similar rates of illness severity in children as HRV A/B ($p>0.05$). Longitudinal studies will be required to understand the role HRV infection in children with severe respiratory illnesses and to determine specific whether certain species or genotypes are responsible for severe respiratory disease.

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Figure Legend

Figure 1. Phylogenetic trees of partial human rhinovirus isolates in hospitalized children (a) and university students (b). The phylogenetic trees were constructed by the Neighbour-Joining method and 1000 bootstrap replicates using partial VP1 gene sequence. Reference strains of each species were obtained from GenBank. Analysis was conducted by Mega v.5 (Centre for Evolutionary Medicine and Informatics, The Biodesign Institute, Tempe, Az).

Table 1. **Human rhinovirus distribution by sequence in children and students**

Human Rhinovirus Species	Children (n=73)[a]	Students (n=74)[b]
A	25 (34.2%)	37 (50.0%)
B	9 (11.0%)	31 (41.9%)
C[c]	41 (56.1%)	6 (8.1%)

[a]Children (3-59 months) admitted to McMaster Children's Hospital Emergency Room in Sept-Oct 2010 and Sept-Oct 2011

[b]Student (18-24 years) recruited in Sept-Oct 2010 and Sept-Oct 2011

[c] (chi square test, $p<0.001$), OR 14.5 (95% CI 5.2, 42.7)

Table 2. **Age and medical history of children**[a]

Characteristic	HRV A (n=19) (%)	HRV B (n=5) (%)	HRV C (n=37) (%)[b]	HRV Negative (n=37) (%)
Age, mo				
0-5 (n=19)	7 (36.8)	0 (0.0)	5 (13.5)	7 (18.9)
6-23 (n=43)	6 (31.5)	2 (40.0)	15 (40.5)	20 (54.1)
24-59 (n=36)[c]	6 (31.5)	3 (60.0)	17 (45.9)	10 (27.0)
Male (n) (%)	14 (73.7)	3 (60.0)	22 (55.9)	17 (45.9)
Co-morbidity[d]	5 (26.3)	2 (40.0)	10 (27.0)[e]	11 (29.7)[f]
Premature birth (>1 mo early)	2 (10.5)	1 (20.0)	2 (5.4)	3 (8.1)

[a]Data are for September-October 2010 and September-October 2011

[b] (chi-square, p=0.18) for comparison between HRV C and HRV A/B

[c] (chi-square, p=0.39) for comparison of HRV C and HRV A/B in children 24-59 months and children 0-23 months

[d]Includes history of cancer; diabetes mellitus; sickle cell disease; immunodeficiency; disease of the heart, kidney, or lung (including asthma); and neuromuscular conditions, such as seizures, cerebral palsy, or muscular dystrophy

[e] (chi-square, p=0.86) for comparison of HRV C and HRV A/B in children presenting with a co-morbidity

[f] (chi-square, p=0.84) for comparison of HRV positive and HRV negative children presenting with a co-morbidity

Table 3. **Clinical course of hospitalized children**[a]

Characteristic	HRV A (n=19) (%)	HRV B (n=5) (%)	HRV C (n=37) (%)[g]	Total HRV[b] (n=61) (%)	HRV Negative (n=37) (%)
Length of hospital stay, median, IQR	1 (2)	0 (1)	1 (2)	1 (2)[d]	1 (3.25)
Stay >3 days	5 (26.3)	1 (20.0)	13 (35.1)	19 (31.1)[d]	11 (29.7)
Discharge diagnosis					
Pneumonia/bronchiolitis	6 (31.5)	1 (20.0)	7 (18.9)[e]	14 (23.0)[d]	5 (13.5)
Asthma, by age[c,f]					
0-11 mo	2 (10.5)	0 (0.0)	3 (8.1)	5 (8.2)	0 (0.0)
12-23 mo	1 (5.3)	1 (20.0)	3 (8.1)	5 (8.2)	2 (5.4)
24-59 mo	3 (15.8)	0 (0.0)	9 (24.3)	12 (19.7)	2 (5.4)
Croup	1 (5.3)	0 (0.0)	0 (0.0)	1 (1.6)	3 (8.1)
Otitis media	0 (0.0)	0 (0.0)	1 (2.7)	1 (1.6)	2 (5.4)

[a]Data are for September-October 2010 and September-October 2011

[b]Data are for children in whom only HRV was detected

[c]Asthma is defined as a history or diagnosis of asthma or wheezing, which includes asthma, reactive airway disease, and chronic or recurrent wheezing

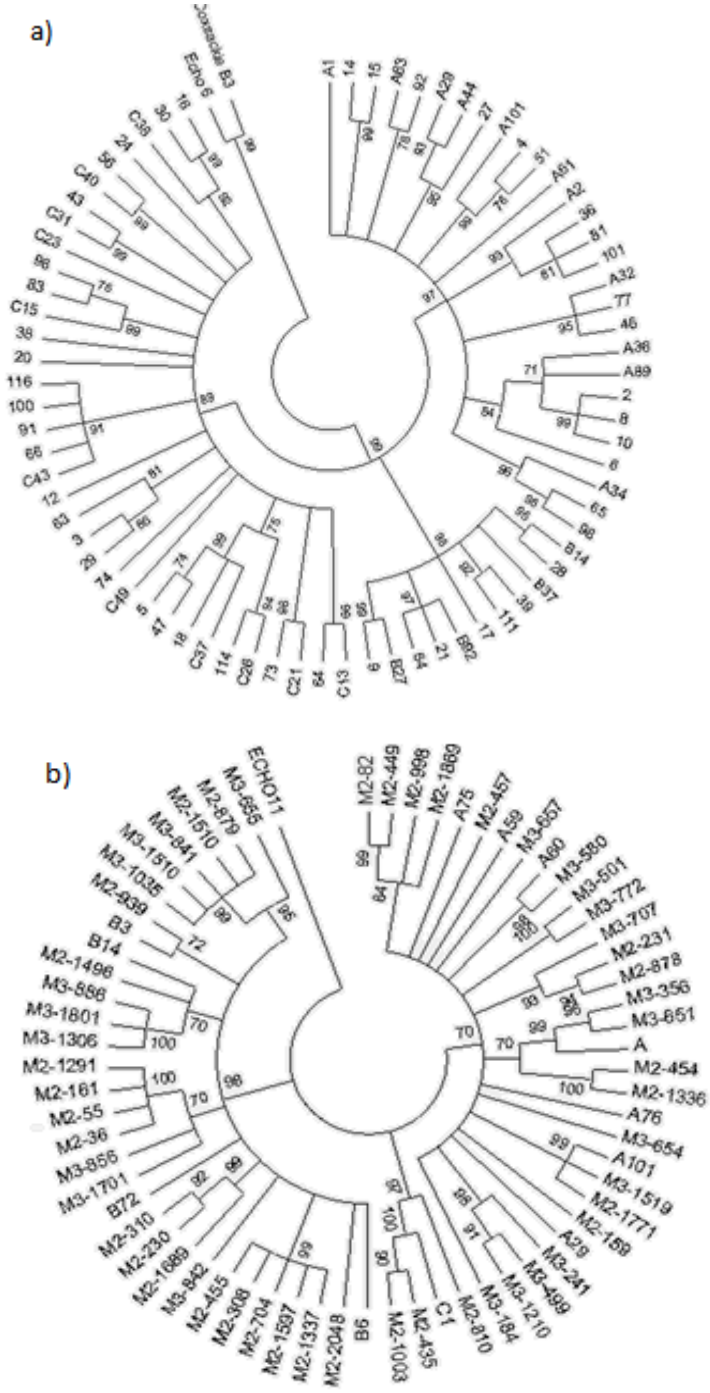
[d]$p>0.05$ for comparison HRV positive and HRV negative

[e]$p>0.05$ for comparison between HRV C and HRV A/B

[f](Mansel-Haensel, $p=0.025$) (OR 4.3, 95% CI 1.3,14.3) for comparison of HRV positive and HRV negative patients with asthma

[g](Chi-square, $p=0.37$) for comparison of HRV C and HRV A/B in children with asthma

Figure 1


a)
Coxsackie B3
Echo 6
C39
30
16
24
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C40
A3
C31
C23
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20
116
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C37
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C26
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C21
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B14
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A34
6
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8
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A89
A36
46
77
A32
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81
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A2
36
A51
4
A101
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A44
A29
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92
15
14
A1
b)
ECHO11
M3-885
M2-879
M2-1510
M3-841
M3-1510
M3-1035
M2-939
B3
B14
M2-1496
M3-888
M3-1801
M3-1306
M2-1291
M2-161
M2-55
M2-36
M3-856
M3-1701
B72
M2-310
M2-230
M2-1689
M3-842
M2-455
M2-908
M2-704
M2-1537
M2-1337
M2-2048
B6
M2-1003
M2-435
C1
M2-810
M3-184
M3-1210
M3-499
M3-241
A29
M2-159
M2-1771
M3-1519
A101
M3-654
A76
M2-1336
M2-454
A
M3-651
M3-356
M2-878
M2-231
M3-707
M3-772
M3-501
M3-580
A60
M3-657
A59
M2-457
A75
M2-1869
M2-998
M2-449
M2-82

Chapter Five

Discussion

The human rhinovirus was thought to cause a disease with a simple solution, but in reality, rhinovirus complex and there is no easy answer for its treatment. The impracticality of HRV detection between the 1960s through the 1990s led to the misconception that HRV played a minor role in serious respiratory illness (Arden & Mackay, 2009). Unlike other viruses, there are many questions left unanswered about HRV epidemiology, immunobiology, strain characterization, and clinical impact (Mackay, 2008). The last decade has seen a significant increase in our understanding of the epidemiology of HRV, mainly due to the development of molecular tests.

My initial goal was to develop a qPCR for HRV to detect and quantitate the virus in clinical specimens. I further expanded my work to answer the following hypotheses: 1) viral loads are lower in community versus hospitalized patients, 2) HRV C is more common in children than adults, and 3) HRV C does not result in more severe or prolonged infections compared to HRV A/B. We determined that HRV viral loads in the community did not differ from hospitalized patients, HRV C occurs more frequently in children than adults ($p=0.025$, OR 14.3), and HRV C is not more frequently associated with severe or prolonged infections compared HRV A/B.

In this chapter, I discuss potential inter-relationships between the data described in chapters 2 through 4. I have drawn on recent advancements to identify the implications of my work.

5.1 HRV qPCR assay design

Molecular diagnostic tools like RT-PCR are the method of choice for detecting HRV infections. In order to detect and quantify HRV in our experiments we designed a quantitative PCR assay for HRV. In this section I discuss the validation of our assay and its use in viral load quantification in clinical specimens.

We modified an existing real-time assay from Lu et al (2008) into a quantitative PCR assay. An HRV qPCR is necessary as there are no commercial HRV quantitative assays available. This assay was chosen because it was able to detect all 99 prototype HRV genotypes and is utilized in various studies (Do et al, 2010; DeByle et al, 2012; Faux et al, 2011; Pierce et al, 2012; Miller et al, 2012). A one-step RT-PCR approach was chosen for HRV detection because it saves time and results in a lower risk of technical errors (Schibler et al, 2012).

Lu et al (2008) (Centres for Disease Control, Atlanta, GA) utilized a locked nucleotide analog in their forward primer design to increase the specificity of HRV detection. These analogs increase thermodynamic stability and balance melting temperatures between forward and reverse primers and allow for a shorter sequence (Lu et al, 2008). However, they are not readily available everywhere. Therefore we re-designed the forward primer converting it to two primers with degenerate positions in order to detect all prototype HRV genotypes and HRV C. The addition of multiple primers increases the possibility of HRV genotype detection (Faux et al, 2011). The primer set did not miss any HRV infections detected by the xTAG^{TM} RVP assay (an FDA

approved commercial assay that detects 8 respiratory viruses and subtypes) (Luminex, Toronto, ON).

Serial dilutions of HRV 60 RNA transcripts demonstrated that the assay was linear over 5 Log_{10} viral copies and had a LLOD of 2 Log_{10} viral copies/ml. This is comparable to other HRV assays described in other studies (Lu et al, 2008; Schibler et al, 2012). The assay had a LLOQ of 4 Log_{10} viral copies/ml. The coefficient of variation of RNA transcripts at values below 4 Log_{10} viral copies/ml were less than 15.0% (data not shown). This is within the acceptable range of variation described by Bustin et al (2009). It is currently unknown if viral loads below the LLOQ are clinically relevant. The assay did detect lower levels of HEV therefore sequencing is necessary to confirm results.

There are factors that could lead to inaccurate measurements in clinical specimens. First, there is no international reference for HRV standard to establish standard curves (Schibler et al, 2012). However, reproducible and quantitative RNA transcripts such as those described in Chapter 2 allow for precise quantitation. PCR efficiency may vary between experiments therefore, it is important to include standard RNA transcripts in each experiment.

Sampling techniques for respiratory virus detection can differ between individuals depending on where in the nasal cavity the sample is collected and how much force is applied, thus the amount of viral RNA collected can vary. There is no correlation between HRV RNA and the amount of human cells tested (Schibler et al, 2012). However, few cells present in the specimen may indicate poor sampling and the viral load

may be underestimated. An internal cellular control will assess specimen quality and reproducibility. We did not include an internal cellular control in the assay; however we did include an extraction control to ensure there was no RNA inhibitors in the specimen.

The HRV qPCR assay that was developed improved the original Lu et al (2008) assay because of the addition of another forward primer increasing the range of HRV genotype detection, and synthesis of primers using reagents that are widely available to all researchers. However, there are some limitations to the assay. The LLOQ was 4 Log_{10} viral copies/ml and clinical samples with quantities below would not be accurately measured. The assay did not have an internal cellular control to monitor for quality of specimen collection. In the future, adjustments to the primer design can be conducted to improve detection as novel HRV genotypes arise, and the inclusion of an internal cellular control.

In summary, the HRV qPCR assay previously described (Chapter 2) is recommended for diagnostic use and RNA quantification in clinical specimens.

5.2 Similar viral loads in different patient populations

The clinical importance of rhinovirus infection has been questioned for some time. In this section, I discuss the potential role of viral load in the clinical diagnosis of HRV and possible factors responsible for severe clinical presentations.

To use viral load for the detection of HRV in clinical isolates a greater understanding of its asymptomatic prevalence, duration of infection and evaluation of

assay designs need to be determined. Molecular tests such as PCR significantly increase detection of viruses that are difficult to culture (Mahony, 2008; Jansen et al, 2011). As such, clinicians must question the relevance of a positive result for viruses such as HRV, human bocavirus, and human metapneumovirus. Low amounts of detected virus could signify asymptomatic colonization or shedding post-infection. We identified that among university students, asymptomatic HRV was more prevalent than symptomatic HRV infections (Chapter 3). By contrast, viruses such as RSV are rarely identified in asymptomatic individuals; it should be considered the causative pathogen when it is detected by PCR (Jansen et al, 2011, Falsey, Criddle, & Walsh, 2006; Franz et al, 2010). Since asymptomatic HRV is commonly found in adults as well as children, (14% in children <1-6 years) a positive result will not necessarily indicate that it is the causative pathogen (Jansen et al, 2011). We compared viral loads in asymptomatic and symptomatic university students and identified a significant decrease in viral load amongst the asymptomatic individuals (Chapter 3). Viral loads among the two groups demonstrated some overlap and cut off values of clinical significance cannot be determined from this data set alone. In children under 6 years, Jansen et al, 2011 has been able to determine that values above 4.5 $\log_{10}$ copies/ml contribute to illness presentation. Rates of asymptomatic infection increase with age. Subclinical infections could be the result of an adaptive immune response to past exposures of that particular genotype or a related genotype.

Several reports have identified persistent HRV infections among hospitalized children 5-6 weeks post-infection, but not in adults (Jartti et al, 2004; Greenberg, 2011). We investigated the persistence of HRV infections and identified that both asymptomatic and symptomatic infections persist for up to 7 days, but not longer than 14 days (Chapter 2-3). Re-infections with distinct HRV genotypes were very common among the asymptomatically infected students, but only lasted for 7-14 days. We looked at the duration of a natural infection among 14 infected individuals (Chapter 2). Viral loads did significantly decrease by day 7; however, the largest decrease in viral load occurred after day 3 (Chapter 2). Further studies will be required to evaluate the clinical utility of HRV viral load tests in hospitalized patients.

Viral load is used as a measure of disease severity in HIV patients and more recently, higher viral loads have been correlated to disease severity in infants presenting with RSV (Mutasa et al, 2012; Scagnolari et al, 2012). We have identified that across three age groups HRV viral loads do not differ significantly (Chapter 2). We repeated this with larger study populations of students and children and agreed with our previous findings (Chapter 4). While these were the first studies conducted on adult community and hospitalized populations, there have been investigations conducted in children (Utokaparch et al, 2011; Franz et al, 2010). With no obvious difference in viral load to account for hospitalizations in children and the elderly, these results imply that other factors such as immune responses are involved. This is in agreement with the finding that HRV is less cytopathogenic than other respiratory viruses (Kennedy et al, 2012). In

children, immature immune responses and the small diameter of the airways contribute to the need for hospitalizations (Takeyama et al, 2012). By comparison, in adults, the adaptive immune response is stronger in women <50 years old than in men or the elderly (Carroll et al, 2010). Kraft et al (2012) compared admission rates of pH1N1 and HRV in adults between September 9 and October 9, 2009 and found that 40% of adults with HRV were admitted, however, 71% presented with a co-morbidity compared to 62% for pH1N1. Adults presenting to hospital with HRV are likely to be immunocompromised or with a co-morbidity already.

5.3 HRV C in children and adults

HRV prevalence has not been investigated in Canada. In this section, I discuss HRV species occurrence in the hospital and community. HRV circulates all year round, but peak activity occurs in early spring and fall, coinciding with the start of school (Winther, 2011). Children commonly spread HRV to family members (Winther, 2011). Consistent with the literature, we found that HRV was the most frequently detected pathogen in September-October (Chapter 4). Children have about six HRV infections per year, decreasing to about 2-3 when they are adults (Winther, 2011). The frequency of HRV detection among our university student population occurs because the students are largely crowded indoors similarly to school-aged children.

Phylogenetic analysis of HRV circulating among children and university students over two 8 week periods found that over 30 genotypes were circulating in each population. HRV A was most commonly found in both populations; however children

were 14.5 times more likely to be infected with HRV C than university students (Chapter 4). Within the population of children, HRV C infections occurred more frequently (56.1%), however the frequency was not significantly different compared to HRV A/B ($p=0.18$). This finding is not unusual as Watanabe et al (2010) found that children 5 years and under were commonly infected with HRV A (72.8%) and C (27.2%). Although, they found that in adults with HRV (n=154, 23.9%), median age of 30 years, HRV C (22.1%) occurred at intermediate frequencies compared to HRV A (60.7%) and B (17.2%) (Watanabe et al, 2010).

Looking closer at the diversity of HRV genotypes, there were 16 different HRV C genotypes circulating among the hospitalized children and only two among the students ($p<0.001$) (Chapter 4). Iwane et al (2011) has also found a high diversity of HRV C in children, comparable to that of HRV A. In the elderly, Watanabe et al (2011) found two HRV C and 6 HRV A genotypes, from among 14 HRV-positive individuals; although they were only able to successfully sequence 9 isolates. Based on these findings we can conclude that HRV C is more diverse and likely to occur in children than adults. The reason behind this is currently unclear and is possibly the result of immune selective pressures and recombination events creating new HRV C variants.

As a final note, we were able to show that HRV B occurred frequently in both symptomatic and asymptomatic university students (Chapter 3 and 4). HRV B is proposed to cause mild infections because it is rarely identified in hospitalized patients (Bochkov & Gern, 2012). Further studies investigating asymptomatic HRV infections in

children and the elderly are warranted to determine if asymptomatic HRV B commonly occurs in these populations, and to determine if HRV B is responsible for mild or sub-clinical infections.

5.4 HRV C and severe respiratory disease

HRV C is the most recently identified species and its role in disease presentation have raised questions, but its role in the community, has not been previously investigated. In this section, I discuss the role of HRV C in clinical presentations in symptomatic and asymptomatic adults and in hospitalized children.

Very little is known about HRV C in the community; we investigated its role in clinical presentation in a university student population with URTI. Looking at the duration of a typical HRV infection, HRV C does not have a significantly higher viral load, nor does it have a longer rate of decline than HRV A/B (Chapter 2). A typical HRV infection has been identified to last from 7-14 days (Greenberg, 2011; Johnston et al, 1993). HRV C did not have a significantly higher viral load than HRV A/B in asymptomatic individuals (Chapter 3). Based on these results, we determine that HRV C does not contribute to prolonged infections and behaves no differently than HRV A or B in healthy adults. Watanabe et al (2010) found that HRV A and B were more likely to cause an influenza-like illness (ILI) in adults in the general community and health care workers. HRV C (68.5%) was more likely to cause a non-ILI than HRV A or B (Watanabe et al, 2010).

HRV is strongly associated with the development of asthma in children (Jackson et al, 2008). We conducted a retrospective study of children ages 3-59 months to determine the association of HRV and HRV species and asthma.

We determined that HRV is associated with a diagnosis or history of asthma/wheezing in children 3-59 months during September-October (OR 4.3). This ratio is consistent with previous studies (Iwane et al, 2011; Kotaniemi-Syrjanen et al, 2003; Jackson et al, 2008). However, we cannot compare this predictor directly since previous investigations have followed HRV infections over at least 12 consecutive months.

There was no association between HRV C infections and a diagnosis of asthma/wheezing. In addition, indicators of illness severity (i.e. length of stay, discharge diagnosis, and co-morbidities) did not differ between species. MacKay et al (2012) also found that HRV produced similar clinical presentations regardless of species in 138 pre-school children in the community observed over 12 months. Previous 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).

In summary, HRV C is not associated with more severe respiratory illness in hospitalized children nor does it contribute to more prolonged infections in healthy adults compared to HRV A/B.

Summary

The field of human rhinovirus has expanded in the last decade. Advancements in technology have allowed us to discover new species and a novel appreciation for the virus. Arguably, we have learned more about HRV infections over the past decade than in the four preceding decades. My study has demonstrated that viral loads are similar regardless of the need for hospitalization, HRV C occurs frequently in children, and that HRV C is not associated with severe illness. Although these findings are significant, there are many other HRV epidemiological questions that are yet to be answered.

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