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Rhinovirus-associated wheezing and asthma in young children

Zofia Piotrowska

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Zofia Piotrowska

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RHINOVIRUS-ASSOCIATED WHEEZING AND ASTHMA IN YOUNG CHILDREN

Zofia Piotrowska (Supported by Jeffrey S. Kahn)
Section of Pediatric Infectious Diseases
Yale University School of Medicine, New Haven, CT

Human rhinoviruses (HRV) are a common cause of the common cold, and are thought to be associated with asthma exacerbations in both children and adults. Recently, HRV have been identified as a major cause of hospitalization in children < 5 years old. The purpose of this study was too determine whether HRV are a cause of either wheezing and/or hospitalization in children < 2 years old.

We used a PCR assay to screen for HRV infection in children < 2 years old: 1) with symptoms of upper or lower respiratory tract disease without wheezing; 2) with wheezing; 3) who were asymptomatic. A group of children who had a respiratory specimen submitted to a diagnostic laboratory for whatever reason and who tested negative for four common viruses in the clinical lab were also screened. All specimens were collected between January 1 and December 31, 2004. Phylogenetic analyses were performed on a majority of HRV isolates.

Overall, 28 (17%) of 165 children with symptoms of respiratory traction infection without wheezing; 21 (26.3%) of 80 children with symptoms of respiratory tract infection and wheezing; 3 (3%) of 93 asymptomatic children and 47 (23.3%) of 202 children with specimens submitted to the diagnostic laboratory tested positive for HRV. The difference between the rate of infection in the asymptomatic group and each of the three other groups was statistically significant ($p \leq 0.01$). Among children with samples submitted to the diagnostic laboratory in whom HRV was the only identified pathogen, 55% were hospitalized. This rate was similar to that observed for respiratory syncytial virus (52.7%) among children of a similar age group and time period (P=0.85). Diverse groups of HRV were circulating during the one-year study period.

We conclude that HRV are important pathogens among young children < 2 years old and are responsible for a significant proportion of wheezing this age group. Among a group of children with a respiratory specimen submitted to the diagnostic laboratory in whom a rhinovirus was the only identified pathogen, a majority were hospitalized.

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I. BACKGROUND

Clinical significance of respiratory viruses

Viruses are an important cause of human disease worldwide. In particular, viral respiratory tract infections are a major cause of illness in both the adult and pediatric populations. Respiratory infections of viral etiology are particularly prevalent among children and are a major cause of emergency room visits, hospitalizations and significant medical costs in this population. In 1991, one study estimated that parainfluenza virus infections in children lead to 250,000 emergency room visits, 70,000 hospitalizations and $190 million in medical costs annually[1]. Likewise, approximately 100,000 children are hospitalized annually due to respiratory syncytial virus (RSV) infection, resulting in an annual cost of $300 million[2, 3].

Although they are common among all age groups, viral respiratory tract infections are particularly prevalent among young children. In a report from the New Vaccine Surveillance Network, the rate of hospital admissions for acute respiratory illness in children less than 5 years old was reported to be 180 per 10,000 children, and a respiratory virus was identified in 61% of these hospitalizations (approximately 110 per 10,000 children)[4]. To compare the rates of viral infection among children and adults, Hammond et al looked at the percentage of lower respiratory tract disease which could be attributed to a viral pathogen among patients of different ages[5]. In infants, viruses are responsible

for up to 90% of cases of pneumonia. In children age 2 to 5 years 58% of lower respiratory tract infections are viral, whereas in children older than 5 years viral pathogens account for 37% of lower respiratory tract disease. In the adult population, this percentage is much lower-- only 12% of lower respiratory tract infections are associated with a viral etiology[5]. Although adults frequently suffer from mild viral infections of the upper respiratory tract, virus-associated pneumonias are relatively uncommon in this population. It is postulated that the relative immaturity of the infant immune system and a lack of adaptive immunity may lead to increased susceptibility to viral infections among our youngest patients[5].

Although the majority of viral respiratory tract infections cause disease of mild to moderate severity, viruses can also be a significant cause of morbidity, and even mortality. Among children, one fifth of the five million deaths due to respiratory tract disease reported annually are associated with a viral infection[6]. The mortality of viral illness is even higher among children in the developing world, where it is estimated that 4-5 million children die of acute respiratory infections each year, with viruses accounting for over half of these deaths[7, 8].

A wide variety of viruses infect the human respiratory tract

There are over 200 viruses known to infect the respiratory tract, representing a variety of viral families. Many of these pathogens were discovered during a boom in the study of virology that occurred during the 1950s and 1960s.

Prior to that time, the influenza viruses were the only known, filterable respiratory viruses. The first influenza A virus was discovered in swine in 1931, and the first human isolates of influenza A were identified two years later.[9] The B and C strains of influenza were discovered in 1940 and 1947, respectively. In 1953 Rowe, Huebner and Hilleman identified the first adenovirus and established its significance in acute febrile respiratory tract disease[10, 11]. These discoveries paved the way for the identification of other important respiratory viruses during this decade, including the parainfluenza viruses, which were first isolated from children with croup in 1954 and 1958[12]. In 1954, Morris et al identified a chimpanzee coryza agent, which was subsequently linked to isolates identified in infants with bronchopneumonia and laryngotracheobronchitis, leading to the identification of RSV in 1957[13, 14]. In the same year, the first human rhinovirus was isolated from the nasal washings of naval recruits with mild cold-like symptoms[15].

Many of the viruses discovered during this time are now known to represent the most common respiratory pathogens. For example, influenza virus is the most commonly-isolated viral respiratory pathogen among adults. Among children, RSV causes the majority of clinically-significant viral infections of the respiratory tract. In particular, RSV represents 24-36% of community-acquired pneumonia in children, which can also be associated with parainfluenza virus 1, 2 and 3, human metapneumovirus (hMPV), influenza virus and adenovirus[5]. The human rhinoviruses are the most common agents associated with the common cold, and are known to cause up to 80% of cases of mild upper respiratory tract

infections. The HRV are also associated with otitis media, sinusitis and, in particular, with asthma exacerbations in both children and adults.

Epidemiology of respiratory viruses

The respiratory tract is a major portal of entry for human pathogens, making viral respiratory tract infections extremely common around the world. By definition, respiratory viruses are transmitted by airborne droplets. An infected human or animal host can shed viral particles by any mechanism which spreads respiratory secretions, including coughing, sneezing, or talking. Sneezing, in particular, disperses up to a million tiny aerosolized virus-filled droplets less than 10µm in diameter[6]. Due to their small size, these droplets can remain suspended in the air for minutes and be transmitted by inhalation to others in the vicinity. Coughing, spitting and speaking, on the other hand, produce larger droplets (>100 µm) which fall to the ground more rapidly, affecting only those in close proximity immediately after they are released. Viral particles can also be transmitted directly by passing oral and nasal secretions via hands or other direct contact to another person's nose or mouth.

While viral respiratory tract infections are common throughout the year, a seasonal distribution of the "common cold" has been described. In particular, rhinovirus-associated infections have been noted to peak in spring and fall months[16]. Interestingly, Johnston et al noted an increase in HRV infection during the academic school year, with decreased rates of infection during times of

school rescess[17]. The association between viral infections and school attendance suggests that such small, enclosed environments facilitate the transmission of respiratory viruses through aerosolized particles or hand-mouth contact. This is also supported by outbreaks of respiratory tract infections which are so commonly seen in other closed communities such as long-term care facilities[18].

Respiratory viruses, in particular, are likely to be spread by close contact because large numbers of viral particles, 10^3-10^9 particles per ml of respiratory secretions, are shed during the peak of infection[6]. In general, this peak infectivity coincides with the period during which the patient is most likely to be sneezing and coughing frequently, readily spreading contaminated respiratory secretions. Furthermore, respiratory viruses are highly infectious; even a single viral particle may be enough to initiate a respiratory tract infection. In this way, respiratory viruses can spread easily and rapidly across populations, leading to frequent outbreaks of respiratory tract infections.

Mechanisms of viral pathogenesis in the respiratory tract

Once inhaled, viral particles settle at different locations within the respiratory tract depending on particle size. Large droplets (those >10μm in diameter) may be deposited in the nasal turbinates while smaller particles may travel to the lower respiratory tract and be trapped in the trachea or bronchioles. Infection is initiated when a virion attaches to a susceptible epithelial cell via appropriate ligand-receptor interactions. The virus then enters the cell, replicates,

and, several hours later, may be released from the apical surface of the cell into the lumen of the respiratory tract. Human defense mechanisms against viral infection include alveolar macrophages, which phagocytose and inactivate the viral particles before infection occurs. A thick layer of mucus lining the respiratory tract traps viral particles leading them to be swallowed or expelled by coughing. The mucus layer also contains virus-inactivating substances such as glycoprotein inhibitors which neutralize influenza, while lung surfactant contains mannose-binding lectins which also have anti-viral activity. Finally, virus-infected epithelial cells may produce and secrete interferon, which binds to interferon receptors on neighboring cells, thereby protecting them from viral infection[6].

In cases of re-infection, when pre-formed virus-specific antibodies are already present in the respiratory tract, these antibodies bind to viral antigens, blocking the pathogen's ability to bind to and infect epithelial cells. In cases of primary infection, viral infection may continue, relatively unhindered, for several days before a humoral response is mounted. During this lag time, non-specific immune mechanisms such as natural killer (NK) cells are activated by interferon and act to lyse cells infected with virus. Shortly thereafter, T-lymphocytes are recruited and trigger lymphokines to stimulate further immune response, eventually leading to the production of virus-specific antibodies by B-lymphocytes.

Methods of Viral Detection:

Most common respiratory pathogens identified during the 1950s and 1960s were first characterized and studied by propagating the viral particles in live cell lines. Such traditional cell culture methods remain an important tool used by virology researchers, but they are now complemented by a variety of molecular and antibody-based methods used to identify, diagnose and study viral pathogens. Each of these methods has significant advantages and disadvantages, outlined below. Together, these techniques provide scientists with a variety of tools with which to study the epidemiology and molecular characteristics of previously-known and newly-discovered viruses.

Cell culture

Traditionally, propagation of viral particles in cell culture served as the main tool of researchers studying respiratory viruses. The main advantage of traditional cell culture methods is the ability to identify a wide variety of viruses and detect multiple viruses within a single sample. Furthermore, cell culture requires no *a priori* knowledge of the virus which is being propagated, making it a useful diagnostic tool in the clinical setting. By using cell lines of different origins and different growth conditions, researchers can propagate a multitude of viruses. Viruses which have been shown to propagate in cell culture are numerous and include common pathogens such as adenovirus, cytomegalovirus (CMV), polioviruses, coxsackieviruses, echoviruses, herpes simplex virus (HSV),

influenza A and B viruses, measles virus, mumps virus, parainfluenza virus types 1 to 4, RSV, rhinoviruses, and varicella-zoster virus (VZV), as well as Ebola virus, severe acute respiratory syndrome coronavirus (SARS-CoV) and hMPV[19].

The proliferation of viruses is detected by identifying cytopathic effects (CPE) of the viral particles on cells in the culture. CPE can include a variety of morphologic changes, including swelling, shrinking, rounding or clustering of cells, syncytium formation or even destruction of the monolayer. The degree of CPE varies with the viral species being studied; some viruses, such as HSV, produce easily-visible CPE within 24 hours of culture inoculation[19]. Most viruses, however, take days to weeks to develop CPE, and the effects they produce can be subtle. Thus, the detection of viral propagation in cell culture by identification of CPE can be a slow, time-consuming and operator-dependent process. Moreover, cell culture can have poor sensitivity in detecting viruses present in low titers.

While these techniques have lead to many important discoveries in the field of virology and cell culture remains an important scientific tool, the introduction of antibody-based and molecular probe-based methods of viral detection are important new tools which have revolutionized the study of viral pathogens.

Viral antigen detection and serologic assays

In techniques such as immunofluorescence (IF), fluorescence-labeled antibodies specific to particular viral antigens are used to detect even small

amounts of viral particles in cell culture and directly within tissues. Fluorescence-labeled antibodies were first used as a diagnostic tool in clinical virology in 1956, when Liu used these antibodies to detect influenza virus antigen in nasal smears[20]. In the 1970s, the introduction of monoclonal antibodies allowed scientists to create antibodies specific to individual viruses. This development revolutionized our ability to detect and study pathogens which had been difficult to detect through traditional cell culture methods. In the 1980s, monoclonal antibodies were introduced as a diagnostic method and improved our ability to detect a variety of viral antigens in the clinical setting.

Clinically, serologic assays to detect human antibodies to a desired virus are an alternative to the direct detection of viral antigens. While serologic assays are an important tool in the clinical laboratory, these methods are not without their own limitations. First, not all clinically significant viral infections will generate a detectable serologic response in the human host. For example, as many as 30% of culture-positive cases of influenza infection are serologically negative[1]. Moreover, many antibodies may cross-react with viral antigens from multiple related viruses; for example, antibodies to the parainfluenza viruses display significant cross-reactivity between the different types of parainfluenza, making it difficult to distinguish between them[21]. Finally, serologic assays may not be useful in the acute setting, as it may take up to 2-4 weeks for increased serologic titers to a particular virus to be detectable.

Molecular Methods

Although cell culture techniques are still considered, by many, to be the "gold standard" of viral diagnostics, the introduction of polymerase chain reaction (PCR) in 1985, and the subsequent development of other molecular probe-based methods of viral detection, have revolutionized the study of viruses. In particular, a variety of techniques commonly known as nucleic acid amplification tests (NAATs) allow researchers to rapidly and efficiently detect viral particles with a high degree of sensitivity. These methods also avoid the need for highly-experienced observers to detect subtle effects of CPE. Perhaps most importantly, unlike cell culture, the detection of virus by NAAT does not require viable viral particles. Finally, NAAT techniques can detect viral particles even before viral antigens are present in sufficient quantities to be detected by immunofluorescence techniques. A variety of techniques can be classified in the NAAT category, including polymerase chain reaction (PCR), reverse-transcriptase polymerase chain reaction (RT-PCR,) the nucleic acid sequence-based amplification (NASBA), real-time PCR techniques where amplification and detection occur simultaneously, and many others.

Most authors agree that molecular diagnostic assays are rapidly becoming the new gold standard in virus detection. Their sensitivity is unparalleled, reported to be 12-30% higher than cell culture techniques when appropriate controls are included to ensure the validity of the assay[22]. PCR-based techniques utilize short oligonucleotide primers that specifically target known segments of DNA within a desired viral genome. These sequence serve as the basis for

amplification of the desired fragment. PCR-based assays are extremely sensitive, capable of detecting even 1 to 10 copies of target DNA[23]. Moreover, PCR amplification is rapid, easy and relatively inexpensive, making it an ideal target for the development of new diagnostic tests. By including a reverse transcriptase-mediated amplification step, PCR can also be used to detect RNA viruses via RT-PCR.

Multiplex PCR includes primers targeting nucleic acid sequences of multiple viruses and allows for the detection of several viruses within a single PCR reaction. Recently, researchers have been working to develop commercially-available multiplex PCR kits for use in the clinical virology laboratory. For example, the Hexaplex PCR kit is capable of detecting seven of the most common respiratory viruses—RSV A and B, influenza A and B, hPIV1, 2 and 3—with 100% sensitivity and 98% specificity.[24]

Finally, PCR and RT-PCR-based assays allow researchers to determine the nucleic acid sequence of amplified nucleic acid fragments. This sequence data can then be used to perform phylogenetic analyses of viral isolates and to determine their molecular epidemiology. In this study, RT-PCR screening for the human rhinoviruses and sequencing of amplified fragments will be used to determine the molecular epidemiology of the HRV circulating in New Haven, Connecticut during 2004.

The Human Rhinoviruses

As previously mentioned, the human rhinoviruses have long been known to be the primary pathogen responsible for mild upper respiratory tract infections known as the “common cold.” Recent studies suggest that rhinoviruses are responsible for up to 80% of cold cases [25]. The majority of common cold cases are brief and mild in nature; the median duration of illness is seven days, with symptoms usually peaking 1-3 days after onset of infection, persisting for 2-3 days and then gradually improving[26]. Symptoms generally associated with HRV infection are mild and may include sneezing, nasal obstruction and/or discharge, sore throat, headache, cough and malaise. The physical exam is usually normal, with the exception of nasal congestion. Laboratory studies may show mild neutrophilia and leukopenia on the first day of illness[27], but these values typically normalize within 1-2 days and thus are unlikely to be detected during routine medical visits. In addition to the common cold, HRV are also associated with otitis media and sinusitis in children, as well as with more serious infections of the upper and lower respiratory tract [28, 29].

The first HRV was isolated in 1957 by exposing rhesus monkey kidney tissue cultures to nasal washings from persons with colds[15]. The genus name was suggested in 1960, and was based upon the location of primary infection in the upper respiratory tract. A variety of culture conditions and cell lines quickly permitted researchers to appreciate the vast variety of rhinovirus serotypes in circulation and within seven years of the initial characterization of rhinoviruses

over 30 serotypes had been identified[30]. To date, over 100 genotypically and serotypically diverse HRV are known to be circulating throughout the world. Moreover, new HRV continue to be discovered [31], suggesting that this genus may be even more diverse than previously thought.

Despite the large diversity of known HRV serotypes, recent work has demonstrated that many of these viruses share common means of entry into the host cell. As a result, some researchers divide the rhinoviruses into major and minor groups based on cellular receptor usage[32]. The major group, comprising about 90% of known HRV serotypes, is known to enter cells via the intercellular adhesion molecule-1 (ICAM-1) receptor[33]. However, a small minority, about 10% of HRV serotypes, use the low-density lipoprotein (LDL) receptor to enter cells and deposit genomic material[34].

The HRV are members of the *Picornaviridae* virus family. As the name suggests, members of this family are non-enveloped viruses containing a small (pico = small) RNA genome. The single-stranded, positive-sense genome is surrounded by a protein shell to create a simple, spherical virion with a diameter of about 30nm. In addition to the HRV, this large family contains multiple other important human pathogens including hepatitis A virus, poliovirus, as well as many human cosxackieviruses, echoviruses and enteroviruses. Among these, rhinoviruses are related most closely to the enteroviruses. However, HRV can be easily distinguished from other related viruses by their instability in acidic conditions (pH values of less than 6.0.) This is in contrast to the enteroviruses, hepatoviruses and cardioviruses, whose members are acid stable and retain

infectivity at pH values of 3.0 and below[35]. This characteristic influences the sites of replication of these pathogens. For example, the acid-stable enteroviruses are able to pass through the acidic environment of the stomach and retain their infectious nature in the intestinal tract. The rhinoviruses, however, cannot replicate in the alimentary tract and their primary site of infection is the respiratory tract. Moreover, in contrast to enteroviruses, many serotypes of rhinovirus are thermostable at temperatures up to 50°C, and can survive for days exposed to the environment at ambient temperatures of 24 to 36°C[26].

The single-stranded, positive-sense RNA genome of the *Picornaviridae* is translated immediately upon entry into the cell to produce all the proteins required for viral replication. The size of the genome varies in length among the *Picornaviridae,* ranging from 7,209 to 8,450 bases. The length of the HRV genome is among the shortest of the family, about 7,200 nucleotides. Like other members of the family, the HRV genome contains a single open reading frame (ORF) that is translated into one long polyprotein and subsequently processed into mature proteins by virus specific proteases. The HRV genome (see figure 1A) contains four structural proteins, VP1-4. The structure of the human rhinovirus is very simple, with an icosahedral capsid composed of 60 identical subunits arranged into 12 pentamers (see figure 1C). The four structural proteins VP1-4, make up each subunit (see figure 1D), with the VP1, 2 and 3 proteins on the capsid surface and the VP4 protein closely associated with the viral RNA genome on the inner surface of the capsid. Accordingly, VP1-3 are antigenic and are known to be the part of the genome with the highest variability[36].

In addition, all *picornaviridae* contain a 5'-untranslated region (5'-UTR) which contains sequences that control genome replication and translation, including a complex structure known as the internal ribosome entry site (IRES), which can bind ribosomal subunits directly. The 5'-UTR of the HRV is about 620 basepairs in length and is highly conserved among the different HRV serotypes, with about 90% sequence homology between any two HRVs[37]. Regions of the 5'-UTR have been used to design molecular probes capable of detecting multiple rhinovirus serotypes within a single assay. Oligonucleotide PCR primers targeting the 5'UTR of HRV were used in this study.

The role of rhinoviruses in asthma exacerbation

In addition to their role as a major cause of the common cold, HRV have also been implicated in exacerbations of asthma among both adults [38] and children [39]. In their study of children seen in the emergency department with wheezing, Duff et al demonstrated that in young children (those younger than 2 years) the major risk factors for acute wheezing episodes were RSV infection and cigarette smoke exposure[40]. However, for children older than two years, viral infections, and in particular HRV infection, were the greatest risk factors for wheezing. A history of respiratory allergies was also identified as a risk factor for wheezing in older children, suggesting that respiratory allergies and HRV infection may have a synergistic effect in increasing the likelihood of wheezing episodes[40, 41]. Furthermore, Johnston et al demonstrated that 80-85% of school-

aged children with a history of asthma were infected with a virus during wheezing episodes, and that the most commonly-isolated virus in this population was HRV[39]. Finally, in adults, about one half of asthma exacerbations can be attributed to rhinovirus infection[42]. However, given the fact that HRV are among the most common respiratory viruses, the association between HRV and asthma exacerbation merits further investigation. Does the presence of HRV necessarily cause disease? While these data suggest that viral infections, particularly HRV infections, are associated with the development of asthma exacerbation or wheezing in children and adults with pre-existing asthma, few studies have compared the rates of infection among wheezing patients to those of asymptomatic controls.

The molecular mechanisms underlying rhinovirus-induced asthma exacerbations remain an area of active research. Most researchers agree that the pathologic effects of HRV infection occur mainly at the level of the bronchial epithelium. Patients with asthma, in particular, are thought to have an especially fragile bronchial epithelium which is susceptible to the cytotoxic effects of HRV infection. Some studies suggest that the major HRV cell surface receptor molecule, ICAM-1, may also play a role in allergic inflammation of the airways[43], and that HRV may, in fact, act to upregulate ICAM-1 receptors via release of the chemokine NF-kappa B[44]. NF-kappa B also up-regulates other proinflammatory cytokines and chemokines. Specifically, HRV infection of airway epithelial cells is known to increase levels of interleukins involved in B- and T-cell maturation and recruitment of neutrophils. Moreover, HRV infection increases levels of IL-16, an

interleukin known to be involved in chemotaxis of eosinophils and monocytes.[45] Finally, airway epithelial cells infected by an HRV are also known to product the chemokines eotaxin and RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), which are both known to be involved in the chemotaxis of eosinophils leading to airway inflammation[45].

In addition to inducing a chemokine profile favoring an allergic, proinflammatory response, infection with an HRV is also believed to promote a type 2 inflammatory response, characterized by high IL-4 levels. Researchers have noted that asthmatic individuals may have a deficient type 1 inflammatory response, with low levels of characteristic cytokines such as interferon-gamma and low interferon-gamma:IL-4 ratios, which may lead to low antiviral activity and a predisposition to HRV infection in these patients[46]. Therefore, asthmatic patients may clear HRV infections more slowly, allowing for the development of airway inflammation and, potentially, asthma exacerbation.

The current study

Recently, Miller et al reported an association between HRV infection and hospitalization in children < 5 years old [47]. While these studies suggest that HRV infections are common and are likely the cause of a substantial proportion of respiratory tract infections, the prevalence of this pathogen among asymptomatic children, for the most part, has not been studied suggesting that the presence of HRV does not necessarily cause disease. Therefore the role of these viruses in

respiratory tract disease remains poorly defined.

In order to further investigate the association between HRV and lower respiratory tract disease, including wheezing, among young children, we screened specimens obtained from children < 2 years old between January and December 2004 for HRV and compared these results to results from a group of asymptomatic controls whose specimens were obtained during the same year. We also screened specimens submitted to a clinical diagnostic laboratory for HRV. Lastly, we defined the molecular epidemiology of HRV during a 1 year period in New Haven, Connecticut.

II. STATEMENT OF PURPOSE

Hypothesis:

Rhinovirus infection is a major cause of wheezing and respiratory illness in young children, and rates of rhinovirus infection are significantly higher among sick children than among asymptomatic controls.

Specific Aims:

1. To define the epidemiology of HRV infection among children $\leq$ 2 years old in New Haven, CT
2. To determine whether HRV are a cause of wheezing and other respiratory tract disease in this population
3. To determine the molecular epidemiology of HRV strains circulating in New Haven, CT in 2004
4. To determine the clinical characteristics of HRV infection among children with HRV-positive respiratory specimens submitted to a diagnostic laboratory

III. METHODS

Specimens

Nasopharyngeal aspirates were collected from children < 2 years old between January and December 2004. Respiratory specimens from all groups were stored at –20^{O}C after addition of an equal volume of “viral freezing media” (2X Dulbecco’s modified Eagle medium, 200 mmol/L $MgSO_4$ and 100 mmol/L HEPES [pH 7.5]). Study subjects originated from four clinical groups, as summarized in table 1. Groups 1-3 were patients identified prospectively in the Yale Primary Care Center (PCC). Group 1 included children who had evidence of upper and/or lower respiratory tract infection without wheezing (PCC non-wheezing); Group 2 included children who had evidence of wheezing (PCC wheezing); Group 3 included asymptomatic children who were seen for routine well-child visits and did not have any evidence of either upper or lower respiratory tract infection (Asymptomatic). Group 4 included children from whom a respiratory specimen was submitted to the Yale-New Haven Hospital Clinical Virology Laboratory (Clinical Virology) for testing for respiratory viruses. Specimens from patients in Group 4 were submitted at the discretion of the medical team from the Emergency Department, inpatient wards, intensive care units and hospital-affiliated outpatient urgent care clinics. Patients were included in Group 4 only if the results of the tests on the specimens were negative for adenovirus, parainfluenza virus 1-3, influenza A and B and RSV by direct

immunofluorescence assay (DFA). Furthermore, all specimens from each group were screen for the human bocavirus (HBoV) by PCR as previously described [48].

RNA extraction, RT-PCR

RNA was extracted using a commercially-available nucleic acid purification kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Reverse transcriptase (RT) was performed using random hexamer primers and MuMLV RT (New England Biolabs, Beverly, MA), according to the manufacturer's specifications. PCR amplification was performed using the PICO-F3 and PICO-R3 primers [49] which amplify a 115-bp region of the *picornavirus* 5'-untranslated region (5'-UTR). For sequencing purposes, a larger (394 bp) portion of the *picornavirus* 5'-UTR was amplified using the OL-26 and OL-27 primers [50] PCR amplification was performed using HotStar *Taq* polymerase (Qiagen) according to the manufacturer's instructions. PCR amplification cycles were performed as follows: 95 ^{O}C for 15 min, followed by 40 cycles of 94 ^{O}C for 1 min, 58 ^{O}C for 1 min, and 72 ^{O}C for 1 min, and completed by a final extension cycle at 72 ^{O}C for 10 minutes. Each set of PCR included appropriate positive and negative controls. All primers used in this study were synthesized by the Oligonucleotide Laboratory, Department of Pathology, Yale University School of Medicine.

Sequencing and Phylogenetic Analysis:

All amplicons were sequenced using an Applied Biosystems 3730 XL DNA Analyzer at the W. M. Keck Biotechnology Resource Laboratory, Yale University School of Medicine. Alignments and phylogenetic analysis were performed using Lasergene MegAlign Software (version 5.05; DNAstar) with use of the clustal W alignment method.

Clinical data

The clinical data for patients in Groups 1-3 was collected on enrollment. The medical records of all HRV-positive children from Group 4 (Clinical Virology) were reviewed. Demographic and clinical characteristics were recorded on a standardized collection form. Specimen collection and collection of clinical data were approved by the Yale University Human Investigation Committee and collection of data was compliant with HIPAA regulations.

Statistical analysis

Fisher's exact tests were used to determine whether the differences in percentage of HRV-positive specimens among different groups were statistically significant.

IV. RESULTS

Overall, 447 symptomatic children (Groups 1, 2 and 4) and 93 asymptomatic controls (Group 3) were screened for HRV (Table 2, Figure 2). Of the symptomatic children screened, 245 (55%) were seen in the PCC with symptoms of respiratory tract disease and 202 (45%) had specimens submitted to the Clinical Virology Laboratory. A total of 102 isolates tested positive for a picornavirus by initial PCR screening with the PICO-F3 and PICO-R3 primers. Three of these isolates were non-HRV and were not included in the analyses (see below).

In all, 28 of 165 children with respiratory symptoms without wheezing (17.0%) (Group 1), 21 of 80 children with wheezing (26.3%) (Group 2), 47 of 202 samples obtained from the Clinical Virology Laboratory (23.3%) (Group 4) and 3 of 93 asymptomatic children (3.2%) (Group 3) had HRV identified in the sample. The differences between each of the three groups of symptomatic children (1, 2, 4) and the asymptomatic controls (Group 3) were statistically significant ($p \leq 0.01$). The difference in the proportion with HRV between the wheezing (Group 2) and non-wheezing (Group 1) children seen at the Primary Care Center (26.3% vs. 17.0) was not statistically significant ($p \leq 0.09$).

The monthly distribution of HRV-positive specimens is shown in figure 3. HRV-positive specimens were detected throughout the year with the exception of July. HRV-positive specimens were most frequently identified in the months of

October-December and April-May. The three cases of asymptomatic HRV-infection occurred in March and April.

The distribution of HRV-positive specimens by age is shown in figure 4. All children enrolled in the study were < 2 years old. The youngest child from whom HRV was identified was 15 days old.

The clinical features of 40 of the 47 HRV-positive children the children in the Clinical Virology group (Group 4) are shown in Table 3. Of the 7 children who were not included in the analyses, 2 were co-infected with HBoV and the medical records of the remaining 5 patients were not available for review. Overall, 22 (55%) of 40 of HRV-positive patients in this group were male. The most common features observed at or around the time of specimen collection included fever (31/40 patients, 77.5%), cough (24/40 patients, 60.0%) and rhinorrhea (24/40 patients, 60.0%). Wheezing was noted in 19 of 40 (47.2%) patients, and chest retractions were observed in 18 of 40 (45%) patients. Only 6 (15.4%) of 39 patients had a prior documented history of wheezing or of asthma. Of the 25 patients for whom chest radiographs were obtained, 5 (20%) patients had abnormal findings. Abnormalities included hyperinflation, peribronchial cuffing and atelectasis. Hypoxia was not a common feature of HRV infection, noted in only 6 (15.0%) of 40 patients in this population. Nine of the 40 HRV-positive patients had comorbid conditions, which included prematurity, pulmonary abnormalities, congenital heart abnormalities and metabolic disorder.

Of the 40 HRV-positive children in the Clinical Virology Group, 22 (55%) were hospitalized. We compared this rate of hospitalization to that of children

infected with another common respiratory virus, RSV. We reviewed the medical records of every 4th child < 2 years old who had a respiratory specimen submitted to the same diagnostic laboratory during the same study period and who tested positive for RSV. Of the 93 children in the RSV-infected group, 49 (52.7%) were hospitalized. The percentage of HRV-positive children who were hospitalized (55%) and the percentage of RSV-infected children who were hospitalized (52.7%) were not statistically significantly different (p=0.85).

A subset (87 isolates) of the 102 picornavirus-positive specimens was sequenced to confirm HRV infection. Among these 87 isolates, 84 (96.6%) had sequences that matched known HRV sequences and 3 (3.4%) most closely resembled non-HRV picornaviruses: the sequence of 2 of these isolates were consistent with a human Coxsackie virus and the sequence of one isolate resembled a human enterovirus. As stated above, these 3 specimens were excluded. Of the 15 picornavirus-positive specimens which were not sequenced, it is likely that 3.4%, or about (less than) one isolate, was a non-HRV picornavirus. Therefore, these 15 specimens were considered to represent HRV infection and were included in further analyses. While it is possible, albeit unlikely, that one or more of these 15 isolates were non-HRV, we performed the statistical analyses with these 15 isolates omitted from the data set (data not shown). The results of statistical comparisons were unchanged.

Phylogenetic analysis of HRV-positive specimens indicated that a wide variety of HRV genotypes circulated in New Haven throughout 2004 (figure 5). The data suggests that as many as eleven different genotypes of HRV may have

been circulating in Connecticut during the study period. Some identified sequences showed close identity to previously described HRV serotypes and genotypes, though some of the identified sequences showed as little as 82% identity with previously described HRV strains. No association could be identified between particular genotypes and either specific clinical groups or clinical features within children from Group 4. Specifically, genotypes were not clustered among patients from the wheezing group or among patients with clinical features of wheezing in the clinical virology group. Likewise, there appears to be no association between particular genotypes and the month or season of specimen collection (data not shown).

V. DISCUSSION

We found a high rate of HRV infection (17-26%) among sick children in New Haven, CT compared with asymptomatic control patients (3%). The data support recent findings that HRV are a major cause of morbidity among young children and suggest that the detection of these viruses does not simply represent asymptomatic infection.

We detected HRV in the respiratory specimens of 22.3% of children who had samples submitted to the Clinical Virology laboratory and who were negative for RSV, parainfluenza virus 1-3, influenza A and B, adenovirus and HBoV. This rate of infection is higher than the 8% rate of human metapneumovirus (hMPV) infection previously detected in a similar population in New Haven [51]. Other researchers have found HRV to have a prevalence of 26% in sick children, higher than the prevalence RSV, influenza, parainfluenza and enteroviruses [47]. This study and ours strongly suggest that HRV may be a more common pathogen than other viruses traditionally associated with lower respiratory tract infection.

Furthermore, 55% of children with specimens submitted to the Clinical Virology laboratory who were infected only with an HRV were hospitalized at the time of specimen collection. This suggests that HRV infection may be a major cause of hospitalization among young children. Indeed, the percentage of HRV-infected children who were hospitalized was not significantly different from the percentage of comparable RSV-infected children who were hospitalized. The

high prevalence of HRV, along with considerable morbidity as evidenced by high rates of hospitalizations, suggests that these viruses are of major clinical significance and that a rapid sensitive assay capable of detecting most rhinovirus strains may be an important diagnostic tool.

Our study suggests that only a small percentage (3.3%) of isolates detected using picornavirus-specific primers represent non-rhinovirus picornavirus infection. In this study, we detected two cases of infection with Coxsackie virus and one case of enterovirus infection. Likewise, Miller et al identified enterovirus infection in 2% of their study population [47]. These data confirm that, within the picornavirus family, HRV are overwhelmingly the most common pathogen of the respiratory tract in young children with respiratory symptoms.

Few other studies have included sequence data and phylogenetic analyses of HRV strains. Our phylogenetic analysis confirms the broad genetic diversity of HRV circulating in Connecticut over a one-year period. These viruses show a large number of nucleotide polymorphisms, suggesting that our current understanding of the diversity within this genus may underestimate the true genetic diversity of HRV strains in circulation.

Based on our data, no particular HRV strains are more likely to cause wheezing and asthma exacerbation than others. Likewise, it does not appear that certain isolates are more likely to cause severe disease and hospitalization than others at least among those found in 2004. The previously-described seasonality of HRV infection, with peaks in spring and fall months[16], is not supported by our

phylogenetic analysis. There appears to be no association between particular isolates and the seasonal distribution of this virus. While most cases of HRV infection occurred during spring and fall-winter months, there does not appear to have been any specific HRV isolates with peak activity during this period, suggesting that these viruses do not have a true seasonal distribution.

VI. CONCLUSIONS

We conclude that HRV are important pathogens among young children. The clinical significance of HRV infection may have previously been underestimated, as HRV appear to be more prevalent than other common respiratory viruses, such as hMPV, in children seeking medical care. Rapid diagnostic assays with a high degree of sensitivity for detecting this genotypically diverse group of viruses may prove to have an important role in the diagnosis of respiratory tract infection and of the cause of asthma exacerbations among young children.

VII. FIGURES

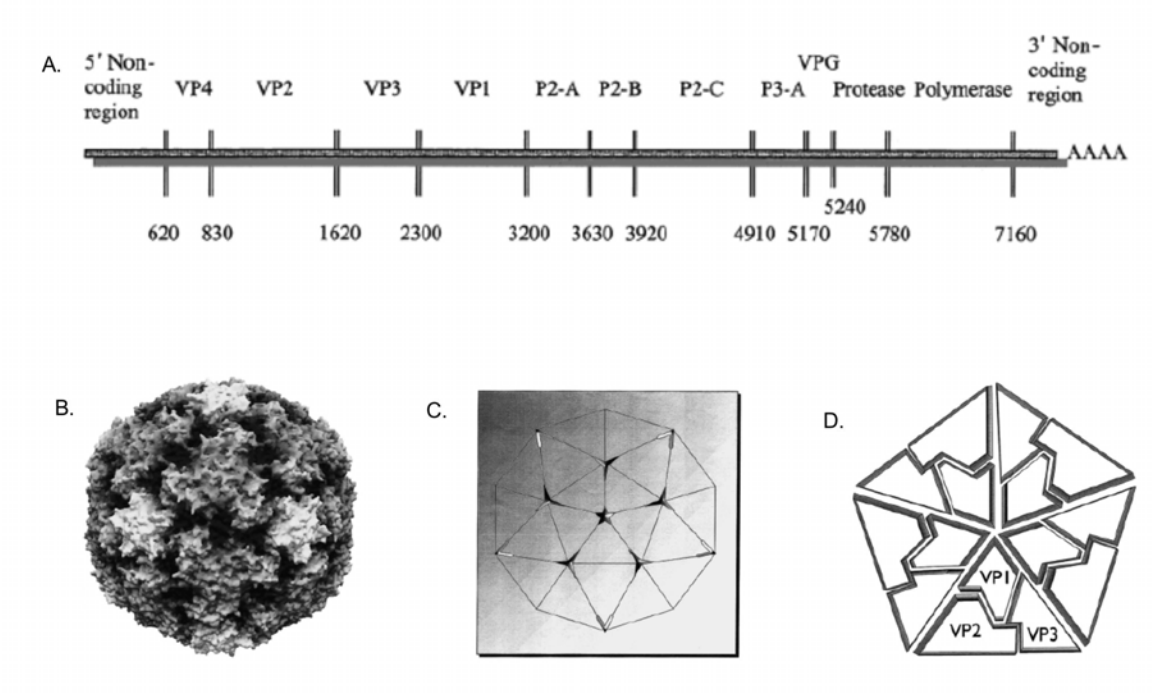


Figure 1, **General structure of the rhiovirus genome and capsid.**

A, Schematic representation of the rhinovirus genome. B, 3-dimensional computer-enhanced electron micrography of HRV14. C, Icosahedral symmetry of rhinovirus capsid. D, Arrangement of rhinovirus structural proteins VP1-3 on capsid surface.

Adapted from Papadopoulos, NG and Johnston, SL. 2000. Rhinoviruses. In *Principles and Practice of Clinical Virology.* AJ Zuckerman, JE Banatavala and JR Pattison, editors. John Wiley & Sons. 329-343.

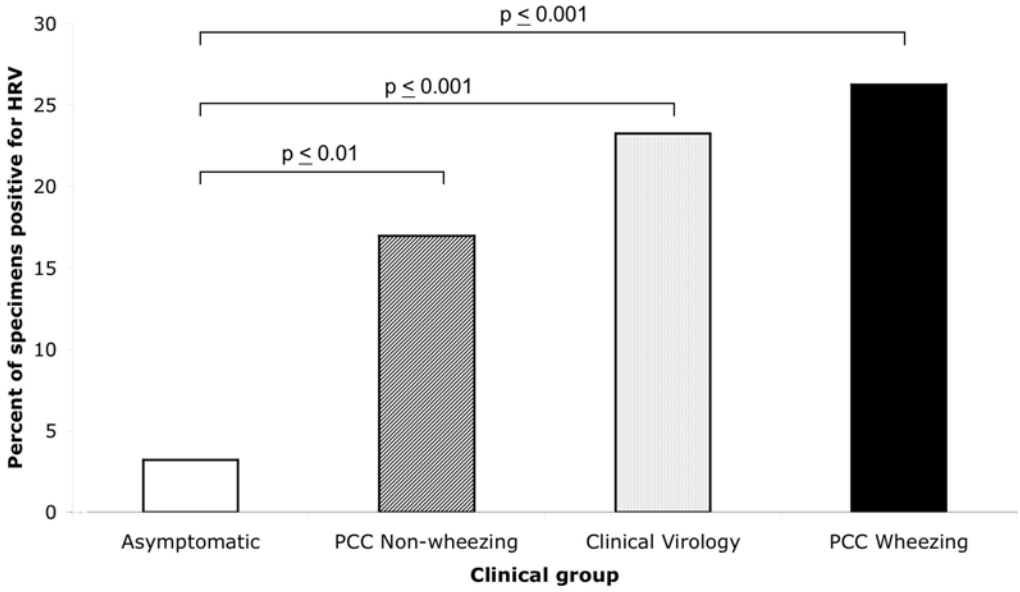


Figure 2, **Results of RT-PCR screening for HRV by group.**

Percent of HRV(+) specimens in each group is indicated. P-values versus asymptomatic control are indicated for each group. Chi-square test was used for each pair-wise comparison.

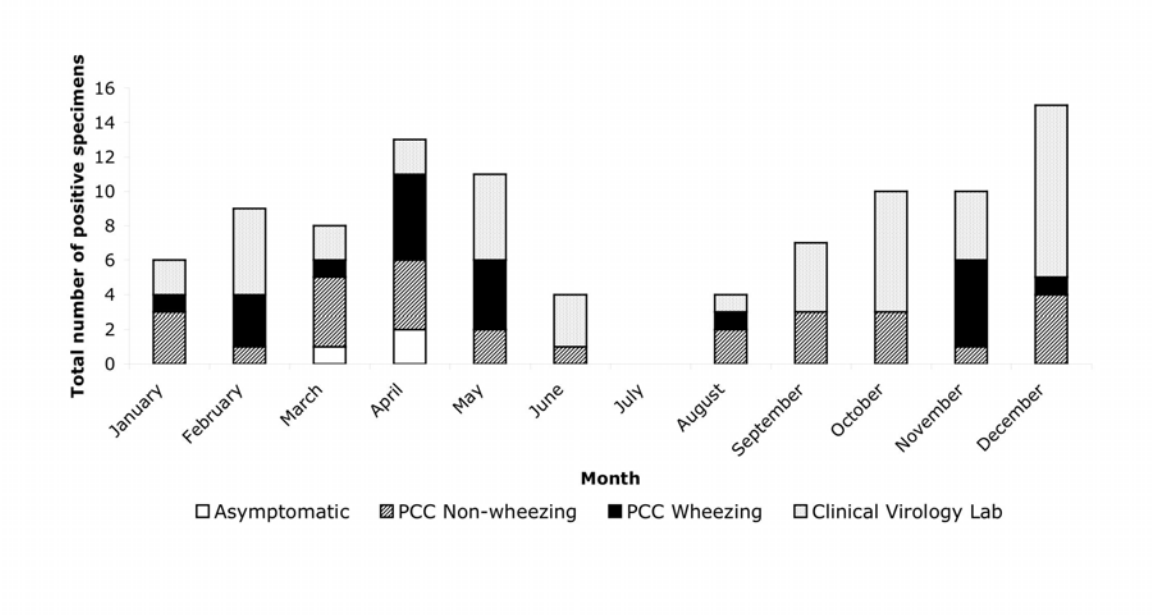


Figure 3, **Monthly distribution of HRV(+) specimens.**

The total number of HRV(+) specimens per month is indicated. The breakdown by group is indicated for each month.

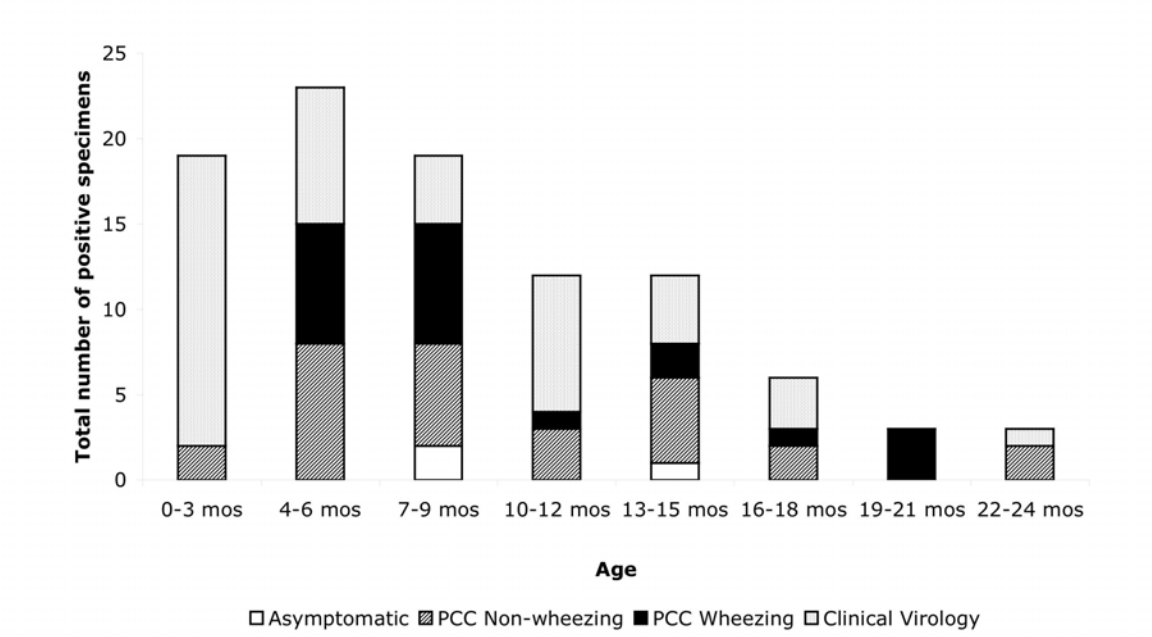
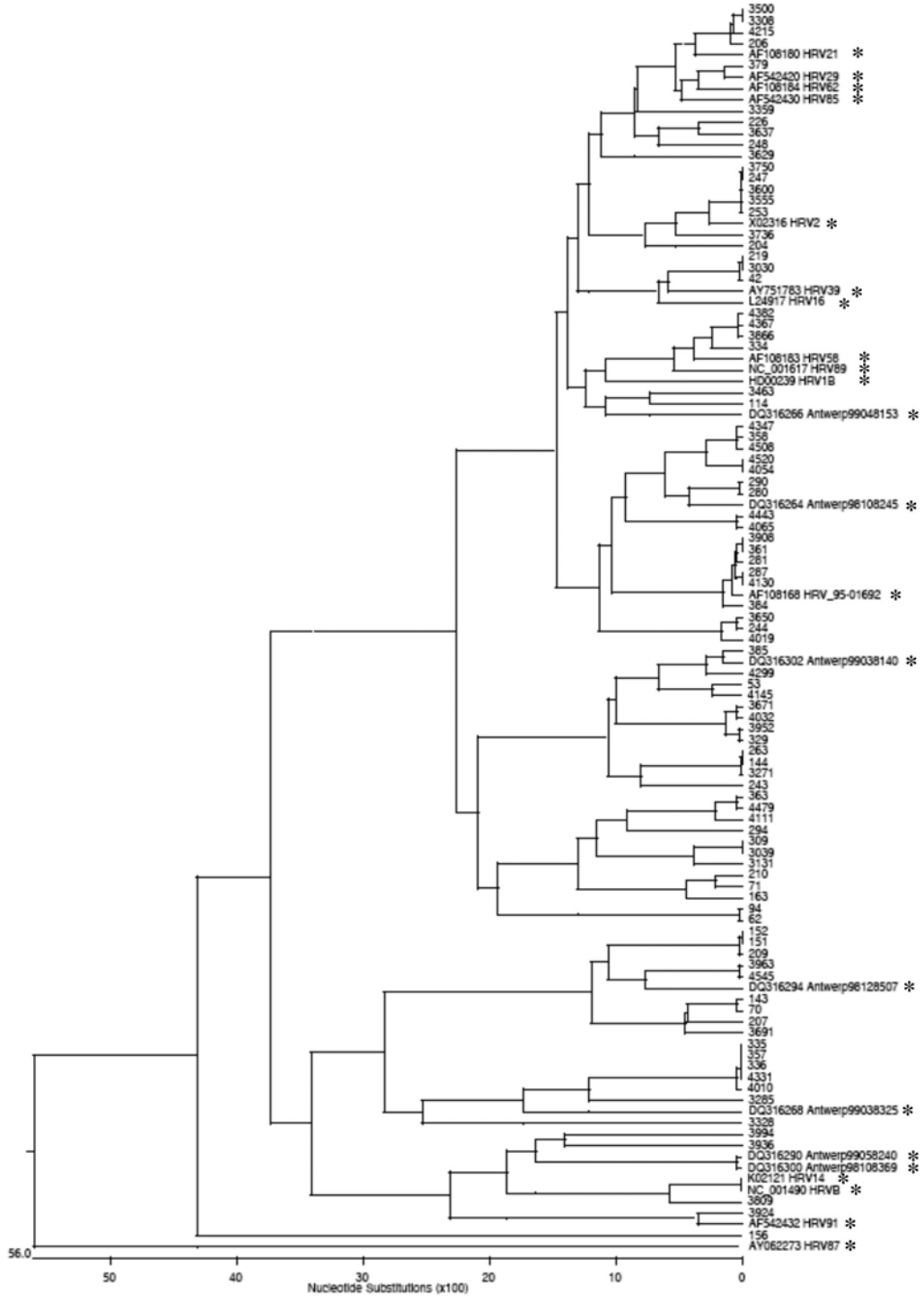


Figure 4, **Distribution of HRV(+) individuals by age at time of specimen collection.**

All patients screened were ≤ 2 years old. The total number of positive specimens is indicated for each age group. The breakdown of each bar by study group is indicated.


3500
3308
4215
206
AF108180 HRV21 *
379
AF542420 HRV29 *
AF108184 HRV62 *
AF542430 HRV85 *
3359
226
3637
248
3629
3750
247
3600
3555
253
X02316 HRV2 *
3736
204
219
3030
42
AY751783 HRV39 *
L24917 HRV16 *
4382
4367
3866
334
AF108183 HRV58 *
NC_001617 HRV89 *
HD00239 HRV1B *
3463
114
DQ316266 Antwerp99048153 *
4347
358
4508
4520
4054
290
280
DQ316264 Antwerp98108245 *
4443
4065
3908
361
281
287
4130
AF108168 HRV_95-01692 *
384
3650
244
4019
365
DQ316302 Antwerp99038140 *
4299
53
4145
3671
4032
3952
329
263
144
3271
243
363
4479
4111
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3131
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152
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DQ316294 Antwerp98128507 *
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207
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3285
DQ316268 Antwerp99038325 *
3328
3994
3936
DQ316290 Antwerp99058240 *
DQ316300 Antwerp98108369 *
K02121 HRV14 *
NC_001490 HRV8 *
3609
3924
AF542432 HRV91 *
156
AY062273 HRV87 *
56.0
50
40
30
20
10
0
Nucleotide Substitutions (x100)

Figure 5, **Phylogenetic analysis of HRV isolates.**

A 394 bp fragment of the 5’ untranslated region of the HRV genome was amplified for sequence analysis. The phylogenetic tree was constructed using sequence data from New Haven isolates in conjunction with reference sequences obtained from GenBank (reference strains denoted by an asterisk, *) Reference strains are identified by GenBank accession number and strain name. New Haven isolates are identified by Kahn lab ID number.

VIII. TABLES

Table 1: Study Groups

Group	Name	Description
1	Non-Wheezing (PCC)	Children < 2 years old evaluated in the PCC with evidence of upper/lower respiratory tract infection without wheezing.
2	Wheezing (PCC)	Children < 2 years old evaluated in the PCC with evidence of upper/lower respiratory tract infection including wheezing.
3	Asymptomatic	Children < 2 years old visiting the Yale Primary Care Center (PCC) for routine well-child visits with no evidence of respiratory tract disease
4	Clinical Virology	Children < 2 years old who had specimens submitted to the Clinical Virology Laboratory that tested negative for RSV, influenza A and B, human parainfluenza viruses 1-3 and adenoviruses

Table 2: Screening of children < 2 years of age for HRV, by Group

Group	Name	HRV-positive specimens/ Total no. of specimens screened (%)
1	Non-Wheezing (PCC)	28/165 (17.0)
2	Wheezing (PCC)	21/80 (26.3)
3	Asymptomatic	3/93 (3.2)
4	Clinical Virology	47/202 (23.3)

Table 3: Clinical features of HRV infection in the Clinical Virology group

Clinical Feature	Patients, no. (%)[a]
Fever[b]	31 (77.5)
Cough	24 (60.0)
Rhinorrhea	24 (60.0)
Wheezing	19 (47.5)
Retractions	18 (45.0)
Prior hx wheeze/asthma	6 (15.0)
Hypoxia[c]	6 (15.0)
Abnormal CXR (n=25)[d]	5 (20.0)
Comorbidity[e]	9 (22.5)
Hospitalized	22 (55.0)
Male	22 (55.0)

a. Percent of total (40 patients) *b.* Temperature > 38.0°C (Range 38.0°C-40.3°C, mean 38.7°C). *c.* O2 sat <90%. *d.* Chest X-Ray abnormalities included hyperinflation, infiltrates, peribronchial cuffing and atelectasis. *e.* Comorbidities included prematurity, pulmonary abnormalities (hypoplastic left lung, primary pulmonary hypertension, bronchopulmonary dysplasia) tracheostomy, congenital heart abnormalities (double aortic arch, aortic stenosis, patent foramen ovale, patent ductus arteriosis), DiGeorge syndrome, Very Long Chain Acyl CoA Dehydrogenase Deficiency, and anoxic brain injury/seizure disorder.

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