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REVIEW

Newly identified human rhinoviruses: molecular methods heat up the cold viruses

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SUMMARY

Human rhinovirus (HRV) infections cause at least 70% of virus-related wheezing exacerbations and cold and flu-like illnesses. They are associated with otitis media, sinusitis and pneumonia. Annually, the economic impact of HRV infections costs billions in healthcare and lost productivity. Since 1987, 100 officially recognised HRV serotypes reside in two genetically distinct species; HRV A and HRV B, within the genus *Enterovirus*, family *Picornaviridae*. Sequencing of their ~7kb genomes was finalised in 2009. Since 1999, many globally circulating, molecularly-defined 'strains', perhaps equivalent to novel serotypes, have been discovered but remain uncharacterised. Many of these currently unculturable strains have been assigned to a proposed new species, HRV C although confusion exists over the membership of the species. There has not been sufficient sampling to ensure the identification of all strains and no consensus criteria exist to define whether clinical HRV detections are best described as a distinct strain or a closely related variant of a previously identified strain (or serotype). We cannot yet robustly identify patterns in the circulation of newly identified HRVs (niHRVs) or the full range of associated illnesses and more data are required. Many questions arise from this new found diversity: what drives the development of so many distinct viruses compared to other species of RNA viruses? What role does recombination play in generating this diversity? Are there species- or strain-specific circulation patterns and clinical outcomes? Are divergent strains sensitive to existing capsid-binding antivirals? This update reviews the findings that trigger these and other questions arising during the current cycle of intense rhinovirus discovery. Copyright © 2010 John Wiley & Sons, Ltd.

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DEFINITIONS

We use the term 'strain' to indicate the molecular equivalent of a classical human rhinovirus (HRV) serotype or distinct newly identified HRV (niHRV) detected from a patient specimen. A 'variant' is a clinical detection of the same strain from a different individual, usually sharing 95–100% nucleotide or amino acid identity, depending on the analysed region. To simplify strain identification, we add the species identifier to the strain names e.g. HRVB-14 or HRVC-QPM.

When relevant, we refer to nucleotide-based strain typing targets using their encoding region name and viral protein (VP) name is used for the encoded product only. Thus, the 1A/1B region is the RT-PCR target which encodes VP4 and VP2.

An HRV C strain is defined in this review as a member of the proposed species consisting of HRV strains that cluster most closely with the first polyprotein sequence described, HRVC-QPM [1]. These include HRVC-NAT001, HRV-HRVC-NAT045, HRVC-C024, HRVC-C025, HRVC-C026, HRVC-NY074, HRVC-QCE, HRVC-N4 and HRV-N10. If the 5′ NTR is used for strain typing instead of the capsid sequence, the 'HRV C' membership

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Abbreviations used

ARTI, acute respiratory tract illness; CFLI, cold and flu-like illness; COPD, chronic obstructive pulmonary disease; cre, cis-acting replication element; HBoV, human bocavirus; HEV, human enterovirus; HMPV, human metapneumovirus; HPIV, parainfluenza viruses; HRSV, human respiratory syncytial virus; HRV, human rhinovirus; ICAM, intercellular adhesion molecule; ICTV, international committee on taxonomy of viruses; IFV, influenza viruses; LRT, lower respiratory tract; LRTI, lower respiratory tract illness; niHRV, newly identified human rhinovirus; NTR, non-translated region; rtPCR, real-time PCR; SARS-CoV, Severe acute respiratory syndrome coronavirus; URT, upper respiratory tract; VLDLR, very low density lipoprotein receptor; VP, viral protein

is swelled by some HRV A strains and is thus better described by the assignment, 'HRV AC'.

INTRODUCTION

In the 1950s and 1960s a flurry of research into colds and flu-like illnesses (CFLIs; [2]) and identification of 'common cold viruses' resulted in the first reports [3] of what became known as rhinoviruses [4,5]. By 1987, 100 serotypes had been amassed during three stages of submission and neutralising antibody studies [5]. HRVs are currently organised into two International Committee on Taxonomy of Viruses (ICTV)-approved species, *Human rhinovirus A* and *Human rhinovirus B* with a third, *Human rhinovirus C*, receiving initial approval by the Executive Committee and awaiting ratification by the ICTV [6] (Table 1).

HRVs infect the upper and lower respiratory tract (URT and LRT) [7,8] (Figure 1) and they cause more asthma [9–12] and chronic obstructive pulmonary disease (COPD) [13] exacerbations than any other factor identified to date, in addition to the majority of CFLIs [14,15]. The knowledge of viral involvement in asthma and wheeze has been percolating through the literature since at least 1957 [16,17], but the important role of HRVs in wheeze was made much more obvious once PCR became available [18,19]. Up to a quarter of children worldwide experience asthma symptoms, with prevalence plateauing in some countries while rising in other parts of the world [20]. In adults, COPD exacerbations are predicted to soon become the world's third leading cause of death [21] and HRVs play a central role in illness among adult transplant recipients [22]. HRVs have been the most common reason for prescribing antibiotics [23] and are associated with pneumonia [24], otitis media [25] and sinusitis [26]. It is interesting to note that, in contrast to other well-known respiratory viruses, the clinical symptoms of HRV infection are primarily caused by the host's immune response to infection rather than by viral cytopathicity [27–30]. Intriguingly, whether in the midst of an influenza pandemic or during an outbreak of an emergent virus such as the SARS-CoV, HRVs are the most common virus detected in patients meeting appropriate clinical criteria for presentation to hospitals or clinics [31,32]. The HRVs, therefore, create an enormous direct and indirect socio-economic burden across the developed and developing world [33,34].

Table 1. Members of the HRV species with complete polyprotein sequence residing on Genbank

Human rhinovirus A			*Human rhinovirus B*	*Human rhinovirus C*
1[M,B]	**34**[B]	**64**[B]	**3**[H,A]	QPM
2[M,B]	**36**[B]	**65**[B]	**4**[A]	QCE
7[H,B]	**38**[B]	**66**[B]	**5**[A]	NAT001
8[H,A]	**39**[B]	**67**[B]	**6**[H,A]	NAT045
9[H,B]	**40**[B]	**68**[B]	**14**[H,A]	C024
10[H,B]	**41**[B]	**71**[B]	**17**[H,A]	CO25
11[H,B]	**43**[B]	**73**[B]	**26**[H,A]	CO26
12[H,B]	44[B]	**74**[B]	**27**[H,B]	NY074
13[H,B]	**45**[B]	**75**[B]	**35**[A]	N4
15[H,B]	**46**[B]	**76**[B]	**37**[A]	N1O
16[H,B]	47[B]	**77**[B]	**42**[A]	
18[H,B]	49[B]	**78**[B]	**48**[A]	
19[H,B]	**50**[B]	**80**[B]	**52**[A]	
20[H,B]	**51**[B]	**81**[B]	**69**[A]	
21[H,B]	**53**[B]	**82**[B]	**70**[A]	
22[H,B]	**54**[B]	**85**[B]	**72**[A]	
23[H,B]	**55**[B]	**88**[B]	**79**[A]	
24[H,B]	**56**[B]	**89**[B]	**83**[A]	
25[H,B]	**57**[B]	**90**[B]	**84**[A]	
28[H,B]	**58**[B]	**94**[B]	**86**[A]	
29[M,B]	**59**[B]	**95**[A]	**91**[A]	
30[M,B]	**60**[B]	**96**[B]	**92**[A]	
31[M,B]	**61**[B]	**98**[B]	**93**[A]	
32[B]	62[B]	**100**[B]	**97**[A]	
33[B]	**63**[B]	N13	**99**[A]	

M and H indicate early cell tropism-based classification (monkey, human) abandoned in favour of a sequential numbering system [145]. HRV strains were later divided into the major and minor groups defined by receptor tropism [146,147]. Receptor-designated minor group HRV strains are underlined, major group are shown in bold. Antiviral groups (A and B) are labelled [148,149]. HRV-87 is not included as it has been previously defined as a variant of HEV-68 [150]. HRV-Hanks (not listed) and HRV-21, HRV-8 and HRV-95 are most likely the same serotype [119].

NEW RHINOVIRUS DIVERSITY: ACKNOWLEDGING THE ELEPHANT IN THE RESPIRATORY TRACT

For decades HRV testing data have correlated poorly with clinical outcomes or yielded untypeable strains, undermining HRV epidemiology [35,36] and reinforcing the 'common cold virus'

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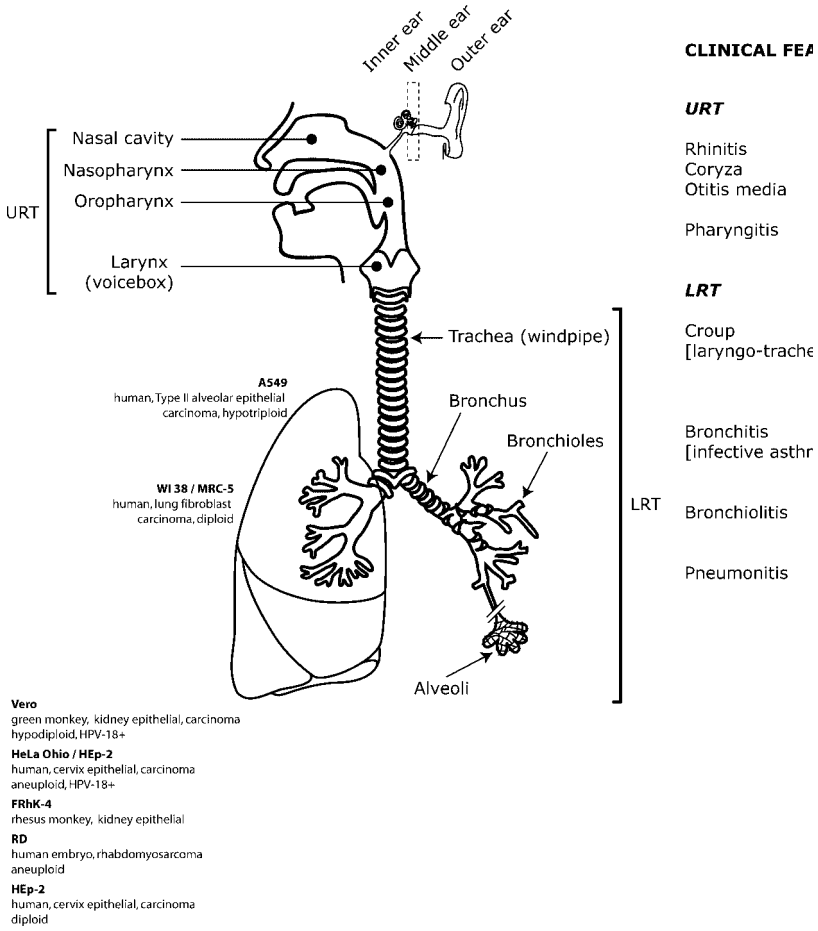

Figure 1. Acute illness and the respiratory tract. Upper and lower respiratory tract (URT/LRT), components of the ear and anatomical sites of interest are indicated. Beside the schematic are the approximate locations of URT and LRT illnesses associated with infection by respiratory viruses. Cell lines used to attempt HRV C isolations are listed. *Recurrent attacks of shortness of breath and wheezing caused by spasmodic contraction of the bronchi, attributed to infection. Adapted from Reference [165]

label that resulted in the underestimation of HRV impact. With the advent of diagnostic PCR, it became apparent that HRV infections are the most frequent of viral respiratory infections, even in hospitalised young children [23,37–39]. While the first classical HRV strain sequences were lodged onto GenBank in 1994 [40], it was not until 1999, using specimens collected in 1992–1995 [41], that the first hint of new HRV diversity appeared ($<98\%$ identity with classical strains in the 5′ non-translated region (NTR)). In 2006, using phylogeny of 1A/1B sequences from HRV positive specimens collected in 2003, we announced a distinct and previously undefined clade of HRVs [42] which was quickly followed by a report of related HRV strains among some cases of a 2004/2005 CFLI outbreak in New York [43]. Global identification of clade members ensued [15,36,37,43–60] and it now seems that the much overlooked HRVs have been brought in from the cold.

In our opinion, the current HRV discovery phase is ongoing, not, as was once suggested, largely complete [61]; there will be descriptions of many more novel strains in the years to come. The niHRV strains do not appear to be emerging viruses in that they have likely not arisen from a recent zoonotic event [62] but have instead been circulating for at least a decade [49,52] and probably much longer [51], without previous detection. Historically, HRV culture was fraught with unreliability and continues to fail in all attempts to isolate niHRVs [1,57]. Even when using a strain-typing PCR, HRV Cs have been missed if the HRV-positive patient specimens had first

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been passaged in culture [63]. By overcoming the insensitivity of culture, PCR with subsequent sequencing produced a large number of diverse viral discoveries from within the human respiratory tract [1,64–69]. Unfortunately, the inclusion of HRV screening has not yet become as accepted as it has become for other respiratory viruses including human respiratory syncytial virus (HRSV), influenza viruses (IFVs), parainfluenza viruses (HPIVs) and metapneumovirus (HMPV) [70–72].

PHYLOGENETIC TREES AND SHRUBS: DETECTING AND TYPING HRV STRAINS IN CLINICAL SPECIMENS

HRVs were one of the clinical virology laboratory's earliest targets for conventional PCR [73]. We have previously reviewed in detail the failings of culture-based HRV detection methods and listed many PCR-based methods. A review of the many recent papers reporting niHRVs reveals some general trends. HRV strains, both known and new, form a significant, although variable, proportion of respiratory virus detections (Table 2). It is not especially difficult to assign detections to one of the three species using sequence obtained from a variety of coding regions. Strain typing, however, is more challenging, as we discuss below. A combination of HRV As and Cs have the highest prevalence within the HRVs, although in different studies either the As or Cs may be much more frequently detected [37,47,50,51]. HRV Bs are consistently rare, constituting around 7% of HRV detections overall [50,54], thus smaller studies may fail to detect any [47,48,56]. Many different HRV strains may be detected within single studies, covering short periods of time [15,45], emphasizing the great diversity of this group.

Since 1988 [74] the transcriptionally important [75,76] 5′ NTR (Figure 2) has been the pre-eminent target for contemporary screening and is popular for strain typing (Table 3). Many subtly different assays target this area. It is relatively easy to amplify all HRV and human enterovirus (HEV) strains due to multiple, small, broadly conserved regions within its sequence [41,74,77]. It is more sensitive than the longer and more variable 1A/1B region [46,54,78] which is increasingly favoured for strain typing.

Today, several well-considered rtPCR or PCR-derived methods are popular for detecting HRVs in clinical specimens [51,57,79,80]. In particular, Lu *et al*. described a meticulously validated novel rtPCR assay which is capable of detecting HRV Cs (recently enhanced by a modified forward primer CPXGCCZGCGTGGY [Lu *et al*., personal communication]) in addition to the classical strains [79]. The assay detected 5×10^1 synthetic RNA copies per reaction and was $\geqslant 10^5$-fold less sensitive to HEVs [79], although HRV/HEV dual infections were identified [79]. A more recent rtPCR designed specifically to include the HRV Cs detected 10 synthetic RNA copies per reaction [80]. At present, viral loads of respiratory viruses in specimens, including HRV Cs, typically range between 10^4–10^7 copies/ml [46,81–85]. Other assays do not comprehensively detect niHRVs, possibly underestimating positive patients by more than two-fold [80]. Our experience with a conventional assay [86] uncovered very specific (equivalent amplicon size as from an HRV target) cross-amplification of human genomic DNA (accession number AL139807) which may result in over-estimation of HRV positives if strain typing is not used.

The trouble with typing and the resurgence of recombination

The niHRVs include an as-yet unquantified number of strains that seem to have arisen from, or contributed to, genetic recombination [45]. Recombination can thwart phylogeny based on the 5′ NTR by producing tree structures whose branching patterns cannot be compared with those from other regions because the evolution of the 5′ NTR may not always be congruent with that of the capsid coding regions [87] (Figure 3). Nucleotide sequences are particularly useful for recombination and evolution studies. However, because the virus immunogenically 'shows' the protein to its host, the translated capsid region may be a more clinically relevant sequence to examine for strain typing, antiviral design and for monitoring treatment efficacy, something not possible with the 5′ NTR. Among the capsid regions, VP4 shows the least variation in protein sequence among strains. A clinical detection may differ by $\geqslant 15\%$ from the nearest GenBank nucleotide match in 1A/1B but still be $\geqslant 98\%$ identical in amino acid sequence to a previously identified strain. This is likely due to the sequestration of VP4 and the amino terminus of VP2 within the capsid of HRVs, which affords

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Table 2. Virus positivity and viral co-detections

Sample numbers	Any virus (% of samples)	HRV RT-PCR-positive samples (% of all virus	HRV mono-detection (% of all HRVs) positive samples)	HRV species (proportion of all HRVs genotyped, %)/sole	Total co-detections (% of detections) detections	Study
406	224 (55)	53		A 22 (42) B 19 (36) C 19 (36)	142/224 (63)	[44]
827		64 (8) (66 strains)	42 (66)	A 27 (41)/19 () B 5 (8)/1 () C 34 (52)/22 ()		[45]
289		84 (87 strains)	54 (62%)	A 29 (33)/19 (22) B 8 (9)/5 (6) C 50 (58)/30 (34)		[15]
301	147 (49)	117 (80)	99 (84)	A 45 (53)[1] B 12 (14) C 28 (33)		[46]
44	27[2]	14 (32)		A 5 (36) 4/5 (80) B 0 C 9 (64) 8/9 (89)	6/27 (22)	[47]
203[3]		26		A 5 (19) B 0 C 21 (81)	NA	[48]
1052		167 (16)	140 (84)	A 64 (38)/49 (77) B 6 (4)/0 C 77 (46)/69 (90)		[49]
97	49 (51)	41/97 (42)	5/41 (12)	A 8 (20) B 3 (7) C 30 (73)	NA	[50]
326[5]		59/326 (18)		A 25 (42) B 11 (19) C 23 (39)		[51]
517	219 (42)	93/219 (42)	82/93 (88)	A 90 (97)[1,6] B 3 (3)	12/517 (2)	[52]
151		16/151 (11)	9/16 (56)	A 2 (15)[1] B 3 (23) C 8 (62)	NA	[43]
142	59 (42)	53/59 (90)		A 18 (62)[1] B 3 (10) C 8 (28)		[53]
447[7]		99	NA	A 39 (80)[1] B 2 (4) C 8 (16)	NA	[37]
NR		71	NA	A 33 (46)[1] B 2 (3) C 33 (46)	NA	[36]
728	638 (88)	240 (38)	112 (47)	A 131 (55) B 7 (3) C 62 (26)	140/728 (29)	[54]
82	53 (64)	37 (70)	37	A 16 (55) B 8 (28) C 5 (17)	2	[55]
43	29 (67)	21 (72)	11 (52)	A 5 (62) B 0 C 3 (38)	10	[56]

Continues

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Table 2. (Continued)

Sample numbers	Any virus (% of samples)	HRV RT-PCR-positive samples (% of all virus	HRV mono-detection (% of all HRVs) positive samples)	HRV species (proportion of all HRVs genotyped, %)/sole	Total co-detections (% of detections) detections	Study
181	159 (88)	108 (68)	80 (74)	A 60[8] B 4 C 17	41 (23)	[57]
54[4]	0		54	A 33 (61) B 4 (7) C 17 (31)	NA	[58]
258	NA	60	26 (43)	A 34 (57)/14 (23) B 12 (20)/6 (10) C 14 (23)/6 (10)	NA	[59]
315	211 (67)	140 (66)	96 C[9] 9 (23)	A 28 (70)[1] B 3 (8)	57 (18)	[42]

[1]Not all clinical HRV detections were/could be genotyped; [2]Specimens had previously tested negative by culture and direct immunofluorescence for IFV A and B, HRSV, HadVs; [3]83 specimens had been previously found positive for HboV, 120 were negative [151]; [4]Culture or PCR negative for other viruses including, but not limited to, HRSV and IFV [58,152]; [5]Small, mixed populations with a range of diagnostic investigations, screened for HRVs and collected from Africa, Asia, Australia, Europe and North America; [6]Not known as HRV C at the time but we believe this is currently the best label for these strains; [7]Specimens combined from categories including clinic-derived, hospital-based and asymptomatic; [8]We have used the authors numbers in this table however some of their 'A' strains align best with the International Committee on Taxonomy of Viruses proposed HRV C strains; [9]These strains were originally described as a sublineage of HRV A called HRV-A2 but have subsequently been renamed to HRV C [89] NA-not available; NR-not relevant to this study.

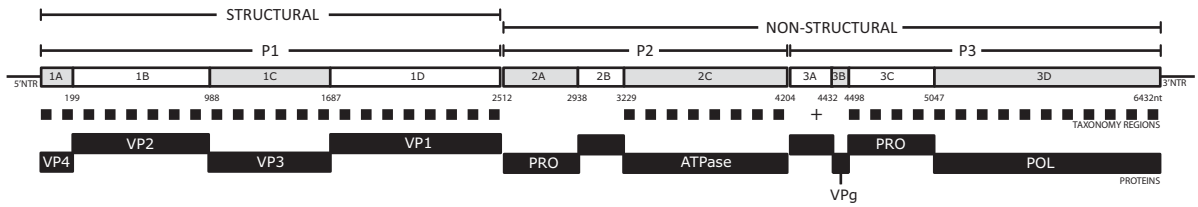

Figure 2. Schematic representations of the HRV genome. The example (HRVC-QPM, GenBank accession number EF186077) includes the nucleotide positions which divide the polyprotein into the precursory (P1-3) and matured proteins (filled boxes). The structural and non-structural regions encompass 11 proteins. The three regions contributing to ICTV species assignment criteria are underlined by dashed lines

some protection from immune pressure. Thus, examining only nucleotide sequences may overestimate the number of novel strains present but sole reliance on VP4/VP2 may underestimate strain diversity [63]. The 3′ portion of VP2, VP3 or VP1 sequences may provide better strain typing targets, although they have not been thoroughly evaluated. While sequencing other subgenomic regions of HRVs improves phylogenetic power [45,63], the deduction of complete polyprotein sequences yields the ultimate phylogenetic information [88].

In 2007, Lee *et al*. nominated a distinct clade of HRV strains be named HRV C based on pairwise nucleotide identity thresholds derived only from the classical strains' 5′ NTR region [57] (Madison clade; Figure 3). In the same month a separate proposal of a novel HRV C species was made by Lau *et al*. for the HRV-QPM-like strains. The latter was based on complete coding sequence analyses [48] which we subsequently confirmed with additional analyses and predictive modelling [89]. The Madison clade was subsequently noted by Kiang

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Table 3. Assays used to investigate HRV Cs

PCR primer target	Oligonucleotide origin	Genotyping target	Genotyping primer origin	Study
5′ NTR-1B	[63]	As for screening		[44]
5′ NTR-1B (snPCR)	[63,153]	5′ NTR, 1A/1B	This study	[45]
5′ NTR –1B (snPCR)	This study	1A	As for screening	[15]
5′ NTR	[79]	5′ NTR, 5′ NTR-1B	[63,90]	[46]
5′ NTR	[154]	5′ NTR-1B	[155]	[47,50,51]
5′ NTR-1B	[63]	As for screening		[48]
5′ NTR	[79]	5′ NTR-1B	[63]	[49]
5′ NTR	[156]	5′ NTR	[86]	[52]
5′ NTR	[32]	1D (nPCR)	This study	[53]
5′ NTR	[157]	5′ NTR	[86]	[37]
5′ NTR–1B	[63,158]	As for screening		[36]
5′ NTR	[158]	As for screening		[159]
5′ NTR	[79]	5′ NTR-1B	[63]	[54]
5′ NTR	This study	As for screening		[90]
5′ NTR	[160]^{A}	1A/1B	[63]	[55]
5′ NTR-1B	This study	As for screening		[63]
5′ NTR	[161]	5′ NTR	[90]	[56]
5′ NTR	[162]	5′ NTR (snPCR)	This study	[57]
5′ NTR	[120]	5′ NTR, 1D	[91]	[120]
5′ NTR	[57]	5′ NTR, 5′ NTR-1B	[57], This study.	[58]
5′ NTR-1B	[59]	As for screening		[59]
5′ NTR-1B	[163]	5′ NTR-1B	[163], This study	[42]
5′ NTR	[162]	5′ NTR (snPCR)	[57]	[164]
5′ NTR	[58], This study.	As for screening		[102]
5′ NTR	This study.	As for screening		[80]

nPCR–nested PCR; snPCR –semi-nested PCR; 1A and 1B – regions of the genome encoding the VP4 and VP2 polyprotein cleavage products, respectively; ^{A}-array-based detection of randomly amplified RNA.

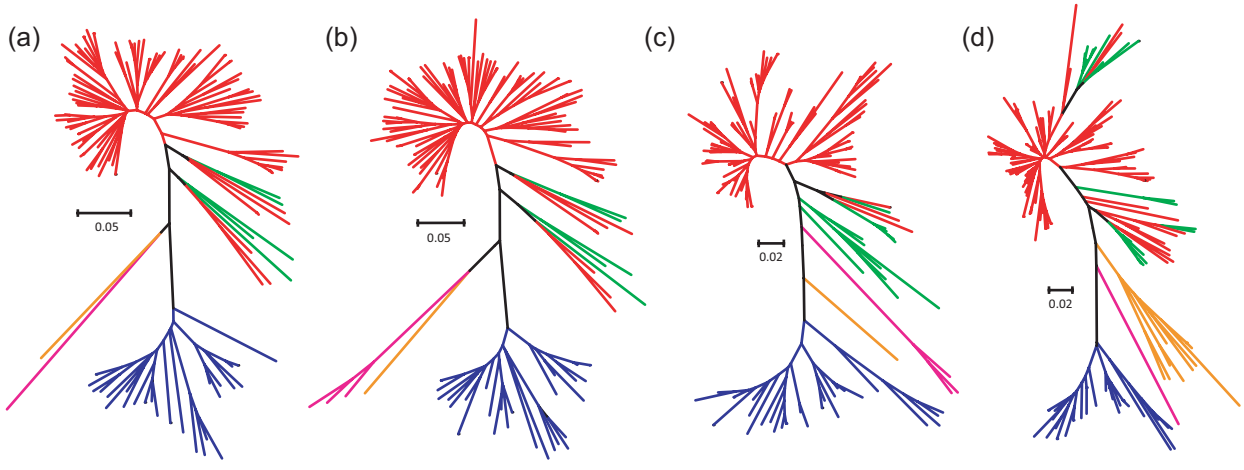

Figure 3. Phylogeny based on the 5′ NTR. A radiating tree representation of (a) complete 5′ NTR minus, (b) the first 350nt of the 5′ NTR, (c) the last ~300nt of the 5′ NTR and (d), (a) truncated to the length of, and including, the Madison clade sequences. Picornavirus species or clades of interest are identified by colour; orange-Madison clade; pink-HEVs; blue HRV Bs; red-HRV A or AC; green HRV C or AC. Sequences include the 5′ NTRs from all HRV strains with complete polyprotein sequences described by Palmenberg *et al.* [88], Huang *et al.* [45] and strains from the Wisconsin clade [57]. HRVC-QCE, HRVC-QPM, HRVC-NAT001 and HRVC-NAT045 were excluded from the alignments because of incomplete 5′ NTR sequences. This figure is available in colour online at www.interscience.wiley.com/journal/rmv

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et al. [90], called HRV C′ by Tapparel *et al* [80,91] and Cc by Huang *et al* [45] all using the 5′ NTR. However, examination of the 1A/1B region of a HRVC-N10 [45], the first complete polyprotein sequence of this clade, shows limited distinctiveness. The polyprotein of HRVC-N10 does not meet the ICTV sequence identity criteria within P1 and 2C+3CD ($\geqslant 70\%$ amino acid identity) exactly, however its falls within only a few percent of doing so for the average identities of the HRV C species. The significant point here is that reliable phylogeny depends on an appropriate choice of sequences. Use of too short a region, an inappropriate region (non-coding, too variable, too conserved) or insufficient reference sequences will stymie the resultant tree, no matter what program is used or how many bootstraps are performed.

An example of the problems experienced when using 5′ NTR phylogenetic trees that contain clinical strains for typing is the co-localisation of some HRV A sequences (usually HRV A-51, -65, -71 -12, -45 and -78) with HRVC-QPM-like sequences. Using this region makes rendering a clear definition of the two species almost impossible. For simplicity's sake, we call this newly defined clade HRV AC; the pattern is similar to the merging of HEV species when typing using the 5′ NTR. Kiang *et al*. [90] followed by Piralla *et al*. [46] have divided the HRV A group into nucleotide-based clades HRV A2, GAC-1 and GAC-2. Simmonds *et al*. defined additional clades too, this time using the 1A/1B region [60]. In doing so, they developed the first coding-region based objective threshold for defining inter- ($>10\%$ pairwise nucleotide difference) and intraspecies ($<10\%$ pairwise nucleotide distance) variation which is key to aiding the identification of new clinical detections as known or novel strains [60]. The clinical value of these genomically defined clades and cut-offs remains unknown. These clades are not apparent when the coding region is used for typing. We support the use of nucleotide-based coding region sequences for improved strain typing results [87].

It was once expected that HRVs were involved in frequent recombination events due to their similarity to the HEVs, some of which are well known for participation in genomic recombinations [63,92]. When Huang *et al*. compared clinical HRV strain typing results using the 5′ NTR with those from 1A/1B they noted two branching patterns which were the HRVC-QPM-like clade (which they called HRV Ca) and the Madison-like clade (HRV Cc) [45]. Further *in silico* investigation predicted recombination [45]. While these data suggest that the 5′ NTR shows HEV-like potential as a recombination 'hotspot' [45,88,91, 93], data for frequent recombination elsewhere in the genome are rare [91]. In addition, empirical demonstration of recombination among the HRVs is lacking. Data describing recombination in HRVs are derived only from *in silico* predictions of the likelihood that shared regions of sequence similarity between strains, identified through phylogeny, originate from a mixing between parental strains. Recombination seems to involve the classical strains [88] but our ability to detect potential recombination has been enhanced by the discovery of niHRVs [45]. At present we lack sufficient numbers of full HRV C genome sequences to perform reliable recombination analysis.

Contemporaneous detections (co-detections) and the rhinoviruses: patterns in the chaos

Previously co-detection was seen as a sign of weakness indicating that the respiratory virus, often an HRV, could not cause significant illness [94,95]. Some have noted more severe clinical outcomes among multiply positive patients [96–98]; others have not [99,100]. Strong correlation between detection of a specific virus and the co-occurrence of certain clinical signs or symptoms are often made when testing for the known respiratory viruses is incomplete [15,50,101,102]. The value of clinical data from patients lacking comprehensive testing is questionable although the caveat must be added that any association risks challenge while novel endemic respiratory viruses remain unidentified. Co-detections are influenced by the quality of the assays used to obtain the data. Previously the HRVs were often the forgotten diagnostic target, now, ironically they have become the focus of investigations in which other viruses are sometimes forgotten [36,50,55,56,103].

In HRV C studies so far, no clear clinical difference has been noted between patients with different numbers of pathogens detected in single specimens [48,49,56]. Of particular interest is that despite differences in study years and assays used, most HRV co-detections are with HRSV [15,44,45,49,52,59]. We recently reported that in a

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study of 1247 specimens, including 660 positive for at least one virus, HRVs were statistically the least likely virus of 17 examined to be associated with co-detections [104]. These co-detection data further prompt a rethink of the role of HRVs in driving respiratory illness rather than being passengers.

Several studies describing HRV Cs have identified multiple HRV strains from different HRV species in the same specimen [45,50,57] although not in a study of acute otitis media [36]. HRV/HRV co-detection suggests that strain typing studies need an intermediate amplicon cloning and analysis method to accurately represent every HRV strain present. It is interesting to note that at this point, only HRVs from different species have been co-detected. Further work is required to determine whether we are missing same-species co-detections because the sequences are too similar, or if there is some immune-mediated mechanism preventing same-species co-detections.

CONFIRMING VARIANTS AND IDENTIFYING NOVELTY

Why do we need to differentiate strains?

The amino acid similarity at key genomic regions (P1, 2C+3CD; Figure 2) aids assignation of an HRV strain to a species within the genus *Enterovirus*, but no equivalent molecular criteria exist to define an HRV detection as a known or novel HRV strain [89]. The viral genome is directly or indirectly responsible for a virus' antigenic and immunogenic potential, seasonal and epidemic circulation patterns, response to antiviral and vaccine interventions and clinical effects; factors which contribute to the impact of the virus. Genome deduction and characterisation and the development of strain-defining criteria are the first requirements for robust analysis and definition of respiratory viruses without which we cannot reduce the burden of infection. Strain-specific molecular epidemiology studies are rare for the HRV super-group [1] despite these being commonplace for the less populated respiratory virus species such as HMPV and HRSV. When Kistler *et al*. described the HRVs as being under 'purifying' selective pressure [105] (environmental forces that encourage each strain to remain intact, once formed) they described a very important feature of some, if not all members [106], of the heavily populated HRV group; their unexpected ability to remain largely unchanged over time. This can be seen during molecular epidemiology studies as high levels of conservation ($>96\%$ nucleotide identity) among variants of the same strain detected over the same time period. We saw this when studying the complete 1A, 1B and 1D regions of 17 variants of HRV-QPM detected throughout 2003 in Queensland, Australia in which we defined a 96% nucleotide identity threshold to identify HRVC-QPM variants. It should be noted that we used highly specific rtPCR assays which may have limited the diversity we saw within this strain. Conservation can also be seen among temporally disparate classical strains that we described in 2006 from the same specimen population; the close phylogenetic relationship between, for example, QPID03-007 (from 2003) and HRVA-80 (from 1967, [107]), which share 92% pairwise nucleotide and 100% amino acid identity in 1A (VP4) and QPID03-0027 and HRVB-48 (also from 1967, [107]) shared 88% and 100% amino acid identity. It is worth noting that despite low nucleotide identity, something found by others [49], they all shared 100% amino acid identity.

Practical contributions aiding strain identification: what does the host see?

A useful way to define what makes a strain different is to look at it from the host's perspective—what degree of sequence difference allows penetration of the host defenses by subsequent HRV challenges? In 1977 five co-infections were noted using culture; none contained the same HRV species in the pairing [108]. We can now show that each strain pairing shared $<51\%$ amino acid identity across the polyprotein sequence. In a more recent report two cases of recurrent CFLI with wheeze were reported, each yielding two HRV C strains, demonstrating that the same patient could be infected by genetically distinct strains (sharing 85% amino acid identity in VP4) within a period of 7 months [103]. In another report, two different (sharing 72% amino acid identify in VP4) HRV C strains were identified from recurrent human bocavirus (HBoV)-positive specimens collected two weeks apart [48]. These data may provide a practical threshold which are useful in molecularly defining the antigenic distinctiveness of strains. Future studies identifying and expanding upon the characteristics of consecutive HRV infections

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could significantly define this area of clinical rhinovirology and could contribute to understanding a role for nucleotide-based thresholds.

COMPARISON OF HRV GENOME SEQUENCE AND FEATURES

The HRV Cs are quite distinct from the As and Bs and they exhibit considerable intra-species diversity. At the time of writing there were 10 HRV C complete polyprotein coding sequences available for public analysis, not all with a complete 5′ NTR. HRVC-QCE and HRV-NY074 have the shortest polyproteins (2140aa), HRVC-C025 the longest (2152aa). Ten protease cleavage sites, common to all enteroviruses, are predicted to cleave each HRV C polyprotein into typical picornavirus structural (VP1-4) and non-structural proteins (2A-C and 3A-D, [109–111]). The VP1/2A junction of HRVC-N4 and NY074 VP1/2A junction consists of the more HRV A-like V/G scissile bond whereas the same HRVC-N10 junction consists of an L/D cleavage site, unique among the HRVs. HRVC-N10 also has an unusual Asp insertion at position 2 in VP1. A translation initiation site (MGAQVS) shared with all other HRVs and motifs (YGDD, YGL, TFLKR and SIRWT) including those crucial for RNA polymerase binding shared by HRV-As, HRV-Bs and HEVs [112,113] are present within most HRV C strains; HRVC-N4 and –N10 have SFLKR. HRV Cs differed in the amino acid sequence of two of three exposed VP1 motifs previously found to be conserved among HRV polyproteins [114]. Phylogeny inferred from a discontinuous alignment of 10 amino acids in VP3 and VP1, which comprise the predicted intercellular adhesion molecule (ICAM)-1 footprint [115] on major group HRV strains (Figure 4), placed the HRV Cs on a branch with HEV strains and, unexpectedly, HRV-1, but distinct from the known major (employing the ICAM-1 molecule as receptor) or minor (employing the very low density lipoprotein receptor, VLDLR) group HRV strains. Key residues on the VP1 BC loop involved in receptor contact with the VLDLR [116] are missing in strains from HRV C and there are differences in the HI loop compared to the classical strains. Antigenic site A [117] is absent in HRV C strains due to deletions within VP1 while the sequence of Site B is uniquely variable among the HRV C species. As with other HRV C strains identified to date [118], a conserved loop sequence motif constituting a cis-

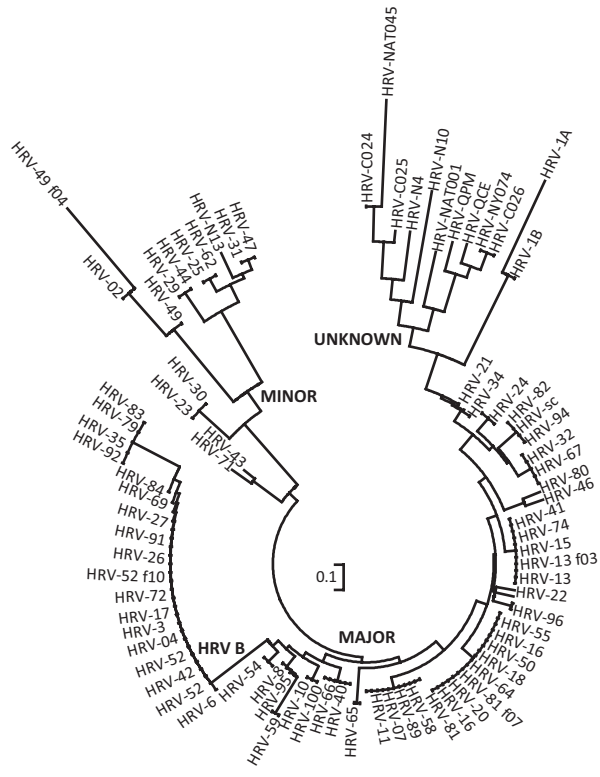

Figure 4. Phylogeny on the ICAM-1 Footprint. A circular representation of HRV and HEV strains (some strain titles removed for clarity) created from a set of 10 discontinuous VP3 and VP1 amino acids comprising the ICAM-1 footprint. Major and minor receptor groups are indicated. HRVA-N13, a niHRV strain appears to belong to the minor receptor group. Phylogeny was inferred using MEGA 4.1

acting replication element (cre), $R^1NNNA_1A_2$ $R^2NNNNNNR^3$ was identified in the 1B region of HRV C strains described by Piralla *et al*. in the context of an appropriate predicted RNA stem–loop structure. The secondary structure of the *cre* is essential for viral genome replication and its location differentiates all the species within the genus *Enterovirus* [118].

To date, no HRV C detection has been isolated in culture. Traditional methods have been attempted using cell types shown in Figure 1 [1,48,49,55] and even allowing for the variability of HRV isolation, it appears that different cell types, organ tissue or reverse engineered viruses will be required for success. We sought a molecular surrogate to replace some of the data important for taxonomic description and traditionally generated by culture [89].

Without an isolate or infectious clone to test, it is only possible to predict the likely interaction with

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capsid-binding anti-picornaviral drugs *in silico*. Their site of action has been mapped to a hydrophobic pocket beneath a canyon in the viral capsid [109] which encircles the union of five monomeric capsid units. Among the 25 key discontinuous contact residues of the 'footprint' of pleconaril (an anti-picornaviral drug), there are 12 HRV C-specific differences (INFQFLXIIMFPSVFITYMINMLAF) compared to the HRV A (INLQFSILYMYASVFLLYMTNMLHH) or HRV B (INLSFSYIAMYPSVFVVYCINMIHG) consensus sequences [119]. There are 11 differences between HRV A and B strains. Changes to two sites important for identifying naturally resistant strains were identified as Tyr_{152} (HRV B residue numbering from Reference [119]) to Phe_{152} in some HRV C strains and Val_{191} to Thr_{191} in all strains. The His_{245} position conserved in most other classical HRV strains (excluding HRV-8, -45 and -95 which contain Leu) was highly variable among the HRV Cs (Gly, Ala, Val or Ile instead). Other highly conserved HRV A and HRV B residues including Ile/Leu_{106} (Phe in HRV C) and Tyr/Phe/Ala_{150} (Ile or Val in HRV C), differed in the HRV C sequences. The empirical impact on HRV C sensitivity to pleconaril remains unknown.

CLINICAL OUTCOMES: ARE HRV CS THE COMMON WHEEZE VIRUSES?

The HRV Cs are found in patients with the same clinically indistinct, broad range of clinical outcomes as the HRV As and Bs and other respiratory viruses (Table 4). Symptoms of infection with HRVCs include CFLIs [1,48,102], pharyngitis [50], croup-like cough [89], wheeze [15,48,103], acute otitis media [36], febrile convulsion [89], bronchiolitis [1,15,102] and pneumonia [15,48, 120] among otherwise healthy children and adults as well as those with underlying conditions including asthma [1,53,55,103], immunocompromise [47,120], cystic fibrosis [89] or multiple sclerosis [121].

In our strain-specific study we identified that most sole detections of HRV-QPM were in patients with signs of mainly LRT illness related to wheeze [89]. Jin *et al*. reported that children with HRV C sole detections did not have wheezing, but were diagnosed with bronchopneumonia [44]. These diagnoses were not dissimilar to those linked to other respiratory virus detections and in fact HRVs, including Cs, are found in similar numbers to HRSV in children hospitalised due to CFLI [37].

Many recent studies do include data on the HRV As, Bs and Cs making comparisons between species possible. Species-specific illness is not always observed (2879} [37] but when found, the HRV Cs, more so than As or Bs, are seen as the major contributors to febrile wheeze in infants and toddlers, to asthma exacerbations in older children (who are able to be accurately diagnosed as asthmatic) and to illness in hospitalised children with asthma [48,49,53]. Not only did more children with an HRV C have a cough than those with an HRV A (who had more fever than the HRV C positive children) but HRV Cs were associated with more wheezing and supplemental oxygen than those positive for HRV A strains [49,54]. First time and recurrent wheezers with HRV C detected all responded well to bronchodilators (with or without steroids) [15]. HRV C strains accounted for half of all detections from middle ear fluids of children with acute otitis media [36].

The strain-specific severity data may be related to the higher average 'viral loads' (relative quantification based on comparative threshold cycles in the absence of normalisation) reported in HRV C positive specimens relative to positives with other HRV species [46]. Higher levels of RNA can equate with more severe illnesses, usually categorised with LRT rather than URT symptoms [46] although there may not be a definite correlation for asthmatics; the mere presence of HRV can induce illness that, while more severe in asthmatics, does not significantly differ either in virus load or duration of viral RNA detection [122]. HRV C-positive children have more pre-existing conditions and exhibit more LRT than URT illness than adults. HRV C strains are often detected in more serious clinical outcomes than HRV A or B [46] although hospitalisations may be fewer for HRV Cs than the other species [44].

To date, most studies have sought HRV C strains in samples collected from the respiratory tract (mostly the nasopharnyx) although extra-respiratory detections have been reported in the blood and pericardium [120,123–126]. HRV C studies have mostly examined patients from asthma or hospital-based populations often with a focus on inpatients (Table 4), which may characterise the HRV Cs as more severe pathogens than they really are. While differences between study popu-

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Table 4. Clinical features of patients positive for divergent HRVs detected in PCR-based studies

Study group features (Country)	Age of patients (median)	Specimen Number/type	Length of study, months (year of specimen origin)[4]	Commonly noted clinical features (% of HRV C positives)	Reference
Hospital-based[1], in/outpatient (Antwerp, Belgium)	Children	517/NPA	8 (Oct 1998–May 1999)	CFLI	[52]
Hospital-based, in/outpatient, retrospective, CFLI. (Brisbane, Australia)	Mixed (14 month)	315/NPA	NA (2003–2004)	CFLI	[42]
Community[1](USA)	Mixed (25 years)	Respiratory swab	8 (Oct 2004–May 2005)	ILI	[43]
Hospital-based[3], in/outpatient, retrospective, CFLI (Brisbane, Australia)	Mixed (16 month)	1244/NPA	12 (Jan 2003–Dec 2003)	Wheeze (53), persistent cough (29)	[1,89]
Birth cohort (Madison, USA)	Children, 0–12mth	181/NL	12 (1999–2001)	$\geq$5 SRI/yr	[57]
Hospital-based/inpatient, retrospective (Hong Kong SAR, China)	Children (1 year)	26/NPA[3]	12 (Nov 2004–Oct 2005)	Fever (100), wheeze (76).	[48]
Community-based, prospective, with/without asthma. (San Francisco, USA)	Adults	82/NL	NA (Nov 2001–Dec 2004)	CFLI	[55]
Hospital-based, inpatient, LRTI (Bad Kreuznach, Germany)	Children (10 month)	97[5]/NPA	36 (2003–2006)	Fever, cough, rhinitis Bronchitis, pneumonia	[50]
Hospital-based, inpatient, prospective (Beijing, China)	Children (10 month)	258/NPA	6 (July 2007–Dec 2007)	LRTI	[59]
Hospital-based, CFLI (Denver, USA)	Children	44/NPW	8 (Dec 2004–July 2005)	Rhinorrhoea (78}, cough {78), retractions (56), fever (44), wheezing (44).	[47]
Case-control, prospective, asthmatic (Atlanta, USA)	Children and adult, 18 year	29/NPS[7]	12 (March 2003–Feb 2003)	Asthma exacerbation. Lower FEV than with other HRVs	[53]
Hospital/Clinic based, in/outpatient (New Haven, USA)	Children $<$2 years	447/NPA	12 (Jan 2004–Dec2004)	Fever, cough, rhinorrhoea, wheezing	[37]
Hospital-based, inpatient, retrospective, Case study (Bangkok, Thailand)	Children	289/NPA	12 (Feb 2006–Feb 2007)	Fever, cough. Bronchiolitis, pneumonia	[15,103]

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Table 4. Continues

Study group features (Country)	Age of patients (median)	Specimen Number/type	Length of study, months (year of specimen origin)[4]	Commonly noted clinical features (% of HRV C positives)	Reference
Hospital-based, prospective, in/outpatient, CFLI (Lanzhou, China)	Children	406/NPA	12 (Dec 2006–Nov 2007)	Cough (82), runny nose (53), crackle (53), fever (37). URTI (53), bronchopneumonia (37)	[44]
Hospital-based, inpatient, prospective (Nashville & Rochester, USA)[2]	Children (6 month-HRV positive, 12 month-HRV negative)	1052/NTS	24 (Oct 2001–Sept 2003)	Cough (91), fever (60). Asthma	[49]
Hospital-based, inpatient, LRTI (Oakland, USA)	Children and adult, 18 year	Mixed	8 (Oct 2006–June 2007)	Cough, wheeze, fever Pneumonia	[56]
Hospital-based, inpatient, prospective, CFLI (Pavia, Italy)	Mixed	301/NPA	2 (Autumn; 2008)	NA	[46]
Hospital-based, outpatient, prospective, ARTI (Shanghai, China)	Children <14 years	827/NPS	24 (Oct 2006–Oct 2008)	Bronchitis (79), pneumonia (21)	[45]
Cohort study, outpatient, retrospective (Finland)	Children ≤ 2 years	MEF and NPA	33 (Dec 1995–Sept 1998)	Acute otitis media	[36]
Hospital-based, inpatient, prospective (Amman, Jordan)	Children (4.4 month)	728[6]/NTS	2 (Jan 2007–March 2007)	Nasal congestion, poor appetite Wheezing.	[54]
Hospital-based, case report (Geneva, Switzerland)	Child, 14 month	Mixed	NA (April 2008)	Fever, dyspnoea URTI, LRTI, pericarditis.	[120]
Hospital-based, inpatient, retrospective (Seoul, South Korea)	Children (14 month)	470/NPA	12 (Jan 2006–Dec 2006)	LRTI. Asthma exacerbation, bronchiolitis.	[58]
Laboratory-based analysis, retrospective (California, USA)	Mixed	24/Mixed	NA (2002–2007)	CFLI	[90]
Chart analysis, retrospective Hospital-based, inpatient, prospective (Turku, Finland)	Children Children ≥ 1 year	580/NPA 163/NS	240 (1987–2006) 2 Sept 2005–Nov 2005	NA NA	[85]
Hospital-based, inpatient, prospective (Madrid province, Spain)	Children <1 year	16[6]/NPA	49 (Nov 2004–Dec 2008)	Apnoea (67), cough (50), rhinorrhoea (50) Gastroesophageal reflux disease (83)	[102]
Hospital-based, inpatients, retrospective (Singapore)	Children <12 years	500/NPS	17 (Oct 2005–March 2007)	Asthma, bronchiolitis.	[164]

Children—includes neonates (0–1 month), infants (1–12 months), toddler (12–24 months) and children (2–14 years); CFLI-cold and flu-like illness; FEV-forced expiratory volume; ILI-influenza-like illness (nonspecific ARTI with fever $>38°C$, cough and/or pharyngitis [43]); LRT-lower respiratory tract; MEF-middle ear fluid; NA-not available; NL-nasal lavage; NPA-nasopharyngeal aspirate; NPS-nasopharyngeal swab; NPW-nasopharyngeal wash; NTS-nose/throat swabs; SRI-symptomatic respiratory illness; URTI-upper respiratory tract illness; ARTI-acute respiratory tract infection; [1]visiting health care provider; [2]some hospitals admit patients, particularly children, from the majority of the surrounding populated area and so some of these are more accurately described as 'population-based' studies; [3]strain-specific study; [4]unless specific, the period was inclusive of the months described in the reference; [5]IFV and HRSV PCR-negative [152]; [6]Patient numbers not specimen numbers; [7]only examines HRV-positive specimens.

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lations can be a problem, it is also important to investigate a wide range of illness groups, as they give an indication of the scope of clinical outcomes possible. Well considered, large community-based studies have not yet included HRV C investigations but are important to present the full clinical spectrum of clinical outcomes. Much longer study timeframes will also be required if strain diversity and circulation patterns are to be measured usefully and if HRV species-specific clinical outcomes are to be robustly investigated.

Detection among the asymptomatic

While the HRV Cs, like many other respiratory viruses, are found among some patients with none of the study-defined symptoms at the time of sampling, they are more often detected among sick children than asymptomatic controls [37]. Viruses detected in asymptomatic people can be perceived as playing a minor role in illness. However, for the definition of a symptom to be most useful, it should include any departure from normal function or feeling, not just the presence of one or more signs such as cough, fever above 38°C, rhinorrhoea or vomiting. Mild headaches or mood changes may not be as noticeable or describable but nonetheless probably reflect a minor, immune-managed symptom that could be correlated to PCR-related HRV detections. Negative mood and reduced alertness and reaction times have previously been identified in those suffering HRV infections [127] but do not register among typical clinical criteria.

Persistence

Reports of unusually long periods of HRV positivity (>2–3 weeks [128,129]) have increased in frequency since more sensitive PCR methods replaced cell culture for HRV detection. Identification of the same HRV serotype over a four-week period has been reported [130]. HRV RNA has been detected days before symptoms commence through to five or more weeks after they cease [129,131,132] although commonly strain typing is not done. Epidemiology that incorporates strain typing usually does not find chronic shedding [133]; strain typing indicates that HRV shedding normally ceases within 11–21 days [79,134]. Thus, the perception of persistence is more than likely the result of serial or overlapping infections by multiple untyped strains [130,135,136] and caution is required when describing persistent HRV infections [137] in the absence of strain typing investigations if the implication is that persistence is a feature of individual HRV strains. Few data are available [37] to address persistence since pre- and post-sampling clinical data are rarely described [138,139] and the definition of 'well' subjects prior to or at the time of sampling or inoculation is sometimes not clear especially for young children who cannot reliably report symptoms [129,133,140]. It is likely that any plan to link long-term HRV positivity in otherwise healthy individuals will require strain typing, regular and frequent sampling and, as with the previous section, identification of a truly asymptomatic, rather than adequately asymptomatic, state.

To date, true persistence, seen as ongoing detection of a single confirmed HRV strain, has been limited to individuals with underlying immunosuppression or immune dysfunction [141]. Piralla *et al*. reported that HRV C strains were detected more than three-times longer in immunocompromised young patients than in immunocompetent children, with a mean of 16 versus 53 days [46]. Multiple detection of the same strains (100% identical 1A sequence in each patient over time) extended to 4 months in haematopoietic stem cell transplant recipients.

Seasonality

During the 1960s, large epidemiology studies of HRVs in temperate climes identified the major epidemic peak during autumn with a smaller spring peak [61,142–144]. It can be assumed that the HRV C strains did not contribute to these findings which employed culture methods. HRV Cs and other HRV species have been identified across all months in tropical, subtropical and semi-arid regions [15,45,47]. In our initial study (subtropical [42]), HRV Cs were mainly detected in spring and in our single strain study we noted HRV-QPM variants had a bi-modal peak in which detection frequencies were 5.0% of all virus positives in August (late winter) and 4.2% in February (summer) [1]. A bimodal trend was also apparent when HRV Cs were studied in other subtropical and temperate areas [37,48,49] while single HRV C epidemic peaks were described in two studies in temperate climates [58,59]. No strong conclusions can be drawn from these data, beyond the need to obtain more data, since sample size and length of

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sampling differed between studies. It is clear that many HRV C strains circulate in a single year and while some studies indicate that the same strains can be detected in neighbouring years [63], we did not find this to be the case for the HRV-QPM strain in Brisbane [1].

CONCLUSIONS

With properly deployed PCR-based testing, it is no longer possible to overlook the central role HRVs play in human morbidity. The finding of the niHRVs has reinvigorated HRV research but raised many questions that need data on a scale that does justice to the size of the HRV supergroup.

Future respiratory virus research must include more powerful studies to confirm the trend towards HRV C dominance in serious HRV illness and will face a daunting array of questions needing attention. What makes an HRV distinct to the host and how do nucleotide clades inform our understanding of HRV diversity? What will community-based studies teach us about the clinical impact of niHRV? What is needed to isolate the niHRVs in the laboratory and what cellular receptor(s) do they use? Can we rely on *in silico*-derived structural predictions or do we need crystal structures?

Apart from the basic virology aspects of identifying and characterising the rhinovirome, we have some fascinating questions to answer about why there are so many stable strains of HRV compared to other respiratory viruses and what their role is in 'training' our naïve immune systems to better defend against viral insult. If their role is central, what would be the impact on the population of introducing a vaccine? What is the immunobiology of the niHRVs and do the few strains used in studies to date accurately reflect the pathogenesis of infection by any HRV strain?

The discovery of this newfound diversity has surprised some authors and perhaps re-defined the assumptions made by others. Hopefully the HRV Cs can also teach the importance of an open mind towards rhinoviruses and their role in human illness.

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