Concurrent Detection of Other Respiratory Viruses in Children Shedding Viable Human Respiratory Syncytial Virus

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Human respiratory syncytial virus (HRSV) is an important cause of respiratory disease. The majority of studies addressing the importance of virus co-infections to the HRSV-disease have been based on the detection of HRSV by RT-PCR, which may not distinguish current replication from prolonged shedding of remnant RNA from previous HRSV infections. To assess whether co-detections of other common respiratory viruses are associated with increased severity of HRSV illnesses from patients who were shedding viable-HRSV, nasopharyngeal aspirates from children younger than 5 years who sought medical care for respiratory infections in Ribeirão Preto (Brazil) were tested for HRSV by immunofluorescence, RT-PCR and virus isolation in cell culture. All samples with viable-HRSV were tested further by PCR for other respiratory viruses. HRSV-disease severity was assessed by a clinical score scale. A total of 266 samples from 247 children were collected and 111 (42%) were HRSV-positive. HRSV was isolated from 70 (63%), and 52 (74%) of them were positive for at least one additional virus. HRSV-positive diseases were more severe than HRSV-negative ones, but there was no difference in disease severity between patients with viable-HRSV and those HRSV-positives by RT-PCR. Co-detection of other viruses did not correlate with increased disease severity. HRSV isolation in cell culture does not seem to be superior to RT-PCR to distinguish infections associated with HRSV replication in studies of clinical impact of HRSV. A high rate of co-detection of other respiratory viruses was found in samples with viable-HRSV, but this was not associated with more severe HRSV infection. *J. Med. Virol.* **85:1852–1859, 2013.** © 2013 Wiley Periodicals, Inc.

INTRODUCTION

Human respiratory syncytial virus (HRSV) is a frequent cause of acute respiratory infections (ARI), especially in children. Upper HRSV infections spread to the lower respiratory tree to an extent generally considered proportional to the severity of the disease [Collins and Crowe, 2007]. With the advent of highly sensitive molecular diagnostic methods, reports of co-detection of multiple respiratory viruses in the same sample have become increasingly frequent [Greensill et al., 2003; Wolf et al., 2006; Paranhos-Baccalà et al., 2008; Gagliardi et al., 2009]. However, the implications of the simultaneous detection of other respiratory viruses in children with HRSV-attributable disease have not been fully clarified [Semple et al., 2005; García-García et al., 2006]. The vast majority of clinical studies concerning this question have attributed "HRSV-positivity" to nucleic acid

detection by RT-PCR, with no attempt to distinguish current HRSV replication from prolonged shedding of remnant RNA from previous HRSV infections [Semple et al., 2005; Martin et al., 2012]. The present study was done to try shedding light on whether co-detections of other common respiratory viruses were associated with increased severity of ARI episodes confirmed to be HRSV-related by the shedding of viable HRSV, recoverable from respiratory secretions in cell cultures.

METHODS

Sample Collection and HRSV Testing

This study was done with all nasopharyngeal aspirates submitted to the virology laboratory for HRSV screening in the year 2005. The samples were collected from children under 5 years of age with ARI seen at the University of São Paulo Hospital or at the Santa Lydia Community Hospital, both in the city of Ribeirão Preto, Brazil (21°10′S; 47°50′W), from January through December of 2005. Nasopharyngeal aspirates were obtained by previously described procedures [Cintra et al., 2001] and sent to the laboratory in less than 4 hr, to be screened for HRSV by indirect immunofluorescence (IF) and hemi-nested RT-PCR. In the virology laboratory the sample suction trap was washed with a small amount of PBS to complete a total volume of approximately 3 ml, and samples were treated with antibiotic/antimycotic (1 µg/ml of penicillin, 1 µg/ml of streptomycin, 0.0025 µg/ml of amphothericin B and 1 µg/ml of ciprofloxacin) for 1 hr at 4°C, and then distributed into two 250 µl aliquots mixed with 750 µl of TRIzol (Life Technologies, Carlsbad, CA), and another two 500 µl backup aliquots mixed 1:1 with 500 µl of VTM. VTM consists of minimal essential medium with Eagle's salts plus 20% fetal bovine serum (FBS), 15% glycerol, and 1% antibiotic-antimycotic solution (all from Life Technologies). All backup aliquots were stored at −70°C until being used. The remainders of NPAs were used to prepare glass slides for HRSV screening by IF using HRSV-specific monoclonal antibody (MAb 858-4; Millipore, Bilerica, MA) diluted 1:100 in PBS, followed by washing three times in PBS and detection with Alexa Fluor 488 Donkey Antimouse IgG (Life Technologies) 1:200 in PBS for 30 min.

To screen nasopharyngeal aspirates for HRSV by hemi-nested RT-PCR, total RNA was extracted from one of the TRIzol aliquots using the manufacturer's protocol. Reverse transcription was carried out with ImProm-II reverse transcriptase (Promega, Madison, WI), primed with random hexamers, following manufacturer's protocol. A first round of PCR was done with primers FV and GAB, and a second round was carried out in a hemi-nested format by adding primer F1AB (Table I). Both PCR rounds were done with *Taq* DNA-polymerase (Biotools, Madrid, Spain) using 35 cycles of 94°C (1 min), 55°C (1 min), 72°C (1 min),

and a final extension period of 10 min at 72°C. Amplicon sizes were 489 pb for RSV-A and 492 pb for RSV-B. All HRSV amplicons were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) on an ABI Prism 377 DNA sequencer.

HRSV Isolation

Prior to the sample inoculation, monolayers were incubated for 30 min with 500 µl of PBS pH 5 (Life Technologies) at 37°C. VTM backup aliquots were inoculated 200 µl/well in duplicate sub-confluent monolayers of HEp-2 cells in 24-well plates with MEM supplemented with 2% FBS, incubated for absorption for 1 hr at room temperature, then replenished with 800 µl of fresh MEM with 2% of FBS and incubated at 37°C in 5% CO_2. When cytopathic effect (CPE) developed, cells were removed by scraping and spotted onto glass slides that were fixed in cold acetone, to confirm HRSV isolation by IF. Cell cultures without discernible CPE were harvested on the 7th day of incubation and blind passaged once onto fresh monolayers of the same cell line, for an additional 7 days. All observed CPE was confirmed by IF for HRSV.

PCR for Other Respiratory Viruses

All samples which were positive for HRSV by isolation in cell culture were tested by PCR for human metapneumovirus (HMPV), picornaviruses (human rhinovirus—HRV, and enteroviruses—EV), human coronaviruses (HCoV) OC43 and 229E, human parainfluenzaviruses (HPIV) 1, 2, and 3, human influenza viruses (Flu) A and B, human adenovirus (HAdV), and human bocavirus (HBoV) (Table I). RNA was extracted as describe for the HRSV RT-PCR, and reverse transcription was carried out with ImProm-II Reverse Transcriptase (Promega) by manufacturer's protocol, primed with random hexamers, except for HCoVs, for which primers 229E-2 and OC43-2 were used (Table I). To test for DNA viruses, DNA-enriched fractions obtained by TRIzol extraction protocol were used for DNA purification using a DNA purification kit (Promega).

Individual PCR assays were done for HMPV, picornaviruses, HCoV-OC43 and -229E, HPIV-1 to -3, Flu A and B, HAdV, and HBoV, all with specific primers (Table I). Picornavirus PCR products were further tested with a probe (GGCCGCCAACG-CAGCC) specific for enteroviruses (EV) in a hybridization assay following previously published procedures [Pitkäranta et al., 1997]. Picornavirus amplicons found to be negative for EV by this assay were sequenced before confirming positivity for human rhinovirus (HRV). Negative and positive controls were included in all PCR assays and PCR for β-actin was done as internal control for the presence of extracted DNA in tested samples. After amplification, PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

TABLE I. Primers and Probes Used in PCR Assays for Respiratory Viruses

Virus	Name	Sequence (5′–3′)	Alvo	Refs.
HRSV	FV	GTTATGACACTGGTATACCAACC	G	Zheng et al. [1996]
	GAB	YCAYTTTGAAGTGTTCAACTT	F	Peret et al. [2000]
	F1AB	CAACTCCATTGTTATTTGCC	F	Peret et al. [1998]
Picornavirus	OL26	CGGACACCCAAAGTAG	5′UTR	Pitkäranta et al. [1997]
	OL27	Biot—CGGACACCCAAAGTAC	5′UTR	Pitkäranta et al. [1997]
Enterovirus	Probe	GGCCGCCAACGCAGCC		Pitkäranta et al. [1997]
HCoV (229E, OC43)	229E-1	Biot—GGTACTCCTAAGCCTTCTCG	N	Pitkäranta et al. [1997]
	229E-2	GACTATCAAACAGCATAGCAGC	N	Pitkäranta et al. [1997]
	OC43-1	Biot—AGGAAGGTCTGCTCCTAATTC	N	Pitkäranta et al. [1997]
	OC43-2	GCAAAGATGGGGAACTGTGG	N	Pitkäranta et al. [1997]
HMPV	FF1	GAGCAAATTGAAAATCCCAGACA	F	Falsey et al. [2003]
	FR1	GAAAACTGCCGCACAACATTTAG	F	Falsey et al. [2003]
HPIV (1, 2, 3)	Para1	Biot—CCTTAAATTCAGATATGT	HN	Echevarría et al. [1998]
	Pr1	GATAAATAATTATTGATACG	HN	Echevarría et al. [1998]
	Para2	Biot—AACAATCTGCTGCAGCAT	HN	Echevarría et al. [1998]
	Pr2	ATGTCAGACAATGGGCAAAT	HN	Echevarría et al. [1998]
	Para3	Biot—CTGTAAACTCAGACTTGG	HN	Echevarría et al. [1998]
	Pr3	TTTAAGCCCTTGTCAACAAC	HN	Echevarría et al. [1998]
HFlu (A, B)	AM149	CTCATGGAATGGCTAAAGACA	M	Ruest et al. [2003]
	AM501R	TGCTGGGAGTCAGCAATCTG	M	Ruest et al. [2003]
	BM26	TGTCGCTGTTTGGAGACACA	M	Ruest et al. [2003]
	BM470R	TGTGATGCTTGTTTTTCGCA	M	Ruest et al. [2003]
HBoV	188F	GAGCTCTGTAAGTACTATTAC	NP1	Allander et al. [2005]
	FR1	GAAAACTGCCGCACAACATTTAG	F	Allander et al. [2005]
HAdV	AV2	Biot—TACGCCAACTCCGCCCACGCGCT	Hexon	Hierholzer et al. [1993]
	AV1	GCCGAGAAGGGCGTGCGCAGGTA	Hexon	Hierholzer et al. [1993]

PCR preparations and product analysis were done in separate rooms, with segregated materials and instruments.

Clinical Information

Patient records were analyzed to check symptoms/signs and requirement for oxygen/respiratory support, which were compared to assess disease severity using an index of clinical severity (ICS), a numeric score adapted from Walsh et al. [1997], based on the presence of wheezing, requirement for hospitalization, oxygen therapy and requirement for positive airway pressure. ICS was obtained by attributing one point each for the presence of wheezing, requirement for hospitalization, length of hospital stay longer than 5 days, requirement for O_2, and use of O_2 for over 5 days, totalizing five points, and two additional points were given when the patient required ventilation support with positive airway pressure, thus resulting in a maximum ICS of 7 for the most severe diseases (Table II).

Statistical Analysis

Clinical data were analyzed by chi-square test looking for associations of qualitative and quantitative variables. Continuous variables were analyzed by Wilcoxon–Mann–Whitney test. When applicable, odds-ratios were estimated by logistic regression using the procedure "PROC LOGISTIC" of the SAS 9.0 software. Statistical significance was considered with P-value < 0.05.

RESULTS

HRSV Overall Frequency

A total of 266 nasopharyngeal aspirates were collected from 247 children (130 boys) with median age 5 months (range 8 days to 50 months), and screened for HRSV immediately by IF, and later by RT-PCR. Forty-six percent of the samples were from outpatients, and the remaining were from inpatients admitted to the pediatric general ward (38%), pediatric intensive care (10%), or pediatric infectious disease (6%) units. HRSV were detected in 111 (42%) aspirates, of which all 111 (100%) were positive by RT-PCR and 95 (86%) by indirect IF. HSRV positivity occurred from February to November, with peak activity between April and June, corresponding to the

TABLE II. Index of Clinical Severity (ICS) Definition

Symptoms	Note
Upper respiratory symptoms (URS) only	0
URS + wheezing	1
Hospitalization	1
Hospitalization longer than 5 days	1
Use of O_2	1
Use of O_2 for over 5 days	1
Requirement for ventilatory support	2
Total	0–4 = "moderate Illness" 5–7 = "severe Illness"

autumn (Fig. 1). The HRSV-positive samples came from 111 children (58 boys) with median age 3 months (range 8 days to 36 months), and the remaining 155 HRSV-negative samples were collected from 144 patients (75 boys), with median age 5 months (range 13 days to 50 months). HRSV-positive samples came from the pediatric general (47%) and infectious diseases (9%) wards, pediatric intensive care (6%) unit, and outpatient pediatric clinic (32%).

Clinical features were compared between 111 children with HRSV-positive samples and those negative for HRSV (Table III). The median ICS for HRSV-negative children was 2, as opposed to an ICS of 4 for the HRSV-positive group. In keeping with that, severe disease (ICS$\geq$5) occurred in 37% of HRVS-positive, as opposed to only 7% of HRSV-negative patients ($P < 0.001$). Also, HRSV-positive patients required oxygen ($P < 0.001$) and mechanical ventilation ($P < 0.005$) more often than the HRSV-negative ones (Table III). Sneezing ($P < 0.001$) and nasal obstruction ($P < 0.005$) occurred more frequently in HRSV-positive children (Table III). Therefore, the data confirm that HRSV-positive ARI episodes were clinically more severe than HRSV-negative ones.

Frequency of Isolation of Viable HRSV

In order to restrict the evaluation of clinical features only to ARI episodes associated with current HRSV replication, primary isolation of HRSV was attempted in all HRSV-positive nasopharyngeal aspirates from backup aliquots stored at $-70°C$. HRSV was isolated in Hep-2 cells from 70 of the 111 (63%)

HRSV-positive samples. Viable HRSV shedding was distributed from March to September, a period slightly narrower than that of overall HRSV circulation (Fig. 1). Gender distribution (38 boys; 54%) and median age (3 months; range 8 days to 36 months) were similar in patients shedding viable HRSV and those positive for HRSV without recovery of viable virus. Patients shedding viable HRSV were most frequently outpatients (60%), but included also inpatients from the general pediatric (23%), intensive care unit (16%), and infectious disease (1%) wards. Clinical and demographic features were not significantly different between patients shedding viable HRSV and those positive for HRSV without shedding of viable virus (Table III). In addition, distribution of patients positive for HRSV, or shedding viable HRSV, were not significantly different from the overall distribution of samples per origin, according to hospital wards.

Co-Detection of Other Respiratory Viruses in Patients Shedding Viable HRSV

To assess whether disease severity in patients shedding viable HRSV was different when other respiratory viruses were simultaneously detected by PCR, the 70 nasopharyngeal aspirates that yielded HRSV by isolation in Hep-2 cells were tested by PCR for the other respiratory viruses. Only 18 of 70 (26%) samples were negative for all the other tested viruses, whereas the other 52 (74%) yielded detection of at least one additional respiratory virus. These 52 samples with co-detected respiratory viruses followed
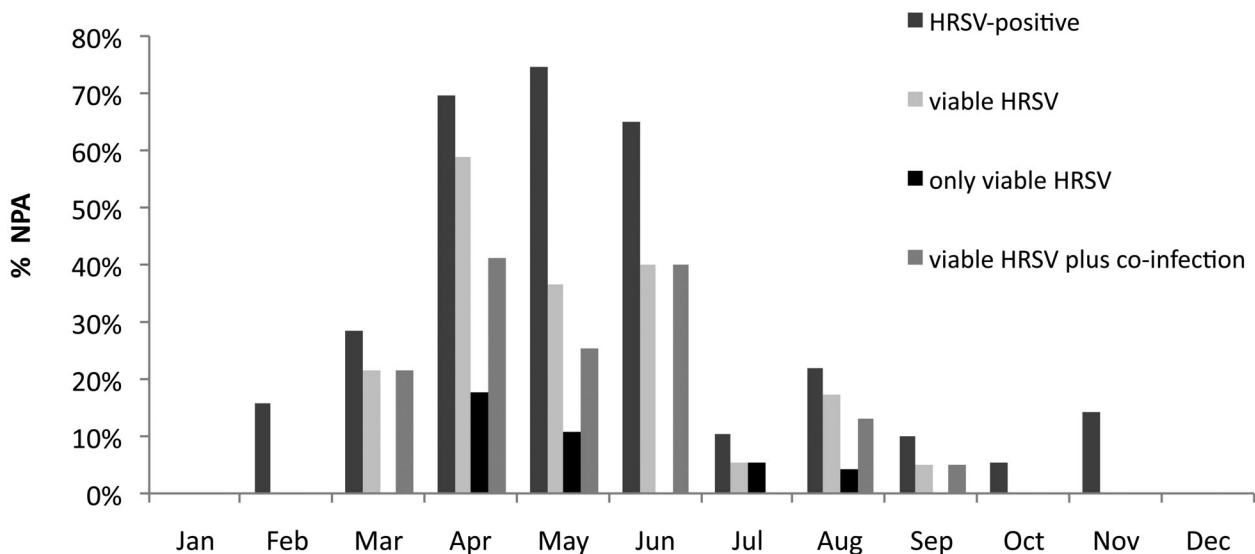

Fig. 1. HRSV seasonality in Ribeirão Preto, 2005. Of 266 NPAs, 111 (42%) were HRSV-positive and were collected from February to November, with a seasonal increase from April to June (autumn). HRSV was isolated from 70 (63%) NPAs, collected from March to September, with peak activity from April to May. In addition to viable HRSV, other respiratory viruses were detected by PCR in 52 NPAs (74%) collected from March to September. The 18 (26%) NPAs positive only for viable HRSV by cell culture were collected from April to August.

TABLE III. Clinical Features of Patients Shedding Viable HRSV

HRSV	NPA (n)	ICS (median)	Severe (%)	Boys (%)	Age (median)	Cough (%)	Coryza (%)	Sneezing (%)	Fever (%)	Wheezing (%)	Dyspnea (%)	Nasal obstruction (%)	O_2 therapy (%)	Mechanical support (%)	Median hospitalization (days)
Negative	155	2[a]	7	49	5[b]	87	30	6[c]	53	33	50	27[d]	35[e]	2[f]	7
Positive	111	4[a]	37	52	3[b]	93	53	22[c]	57	48	63	39[d]	71[e]	11[f]	8
Positive but not Viable	41	4	32	49	3	88	48	15	58	43	55	28	75	13	8
Viable	70	4	40	54	3	96	57	26	57	51	68	46	68	10	8
Viable + co-detection	52	4	38	63	3	96	61	22	59	53	67	45	69	10	8
Viable without co-detection	18	4	44	28	2	94	44	39	50	44	72	50	67	11	8

[a]Differences between negative and positive were significant ($P < 0.001$).
[b]Differences between negative and positive were significant ($P < 0.001$).
[c]Differences between negative and positive were significant ($P < 0.001$).
[d]Differences between negative and positive were significant ($P < 0.001$).
[e]Differences between negative and positive were significant ($P < 0.005$).
[f]Differences between negative and positive were significant ($P < 0.005$).

the same monthly distribution (March–September) of the samples positive only for HRSV (Fig. 1). The gender ratio (33 boys) and median age (3 months, range of 14 days to 36 months) were also similar to the overall HRSV-positive patients. Recovery of viable HRSV in association with detection of other virus nucleic acids was more frequent in secretions from outpatients (60%), followed by inpatients in the general (25%), intensive care (13%), and infectious diseases (2%) pediatric wards.

Of the 52 aspirates containing viable HRSV simultaneously with nucleic acids from other viruses, 27 (52%) were PCR-positive for one virus, 17 (33%) for two viruses, 7 (13%) for three viruses, and 1 (2%) for four viruses, in addition to HRSV. The respiratory viruses detected simultaneously with HRSV were 29 HAdV, 18 picornaviruses (9 EV and 9 HRV), 15 HBoV, 14 HMPV, and 12 HCoV-229E (Table IV).

No significant differences of demographic and clinical features were observed between patients shedding only viable HRSV (18 samples) and those with viable HRSV plus one (27 samples), two (17 samples), three (7 samples), or four (1 sample) viruses (data not shown). The only patient in whose secretions viable HRSV was detected along with nucleic acids from four additional respiratory viruses was a 2-month old girl with Wolf-Hirschorn syndrome, bronchiolitis obliterans and gastro-esophageal reflux, with an ICS of 6. She was hospitalized for 120 days and received O_2 and mechanical ventilation. Remarkably, a nasopharyngeal aspirate collected at admission had been negative for all tested viruses, and the sample that

TABLE IV. Results of Testing for Other Viruses in Samples From Patients Shedding Viable HRSV (N = 70)

Groups of NPAs (N;%)	Non-HRSV viruses	PCR-positive NPA (N)
Only viable HRSV (18;26%)	0	18
Viable HRSV + 1 virus (27;39%)	HMPV	2
	EV	3
	HRV	2
	HCoV	5
	HAdV	11
	HBoV	4
Viable HRSV + 2 viruses (17;24%)	HMPV + HAdV	4
	HMPV + HBoV	1
	EV + HAdV	4
	EV + HBoV	1
	HRV + HCoV	1
	HRV + HAdV	2
	HCoV + HAdV	2
	HAdV + HBoV	3
Viable HRSV + 3 viruses (7;10%)	HMPV + EV + HBoV	1
	HMPV + HRV + HBoV	1
	HMPV + HCoV + HAdV	1
	HMPV + HCoV + HBoV	1
	HMPV + HAdV + HBoV	1
	HRV + HCoV + HAdV	2
HRSV + 4 viruses (1;1%)	HMPV + HRV + HAdV + HBoV	1

yielded four viruses by PCR in addition to recovery of HRSV in cell culture was collected after 1 month of hospitalization, indicating nosocomial transmission of all detected viruses.

Clinical features were also compared among groups of patients who shed viable HRSV alone (18 samples), or in addition to HAdV (29 samples), picornaviruses (18 samples), HRV (9 samples), EV (9 samples), HBoV (15 samples), or HMPV (14 samples; Table V). HAdV was detected by PCR in association with the recovery of viable HRSV in 29 of 70 samples. Interestingly, there were no significant differences of clinical features between these 29 patients and the 41 patients with replicating HRSV in the absence of HAdV detection, nor when comparing with the 18 patients in whom exclusively viable HRSV was detected (Table V). Therefore, although co-detection of HAdV was frequent, it was not directly associated with obvious increase in disease severity in comparison with children with HRSV-attributable disease.

Picornavirus nucleic acid was detected by RT-PCR in 18 (9 HRV and 9 EV) of 70 nasopharyngeal aspirates from children excreting viable HRSV. Comparisons of clinical data between 18 patients shedding exclusively viable HRSV, 9 with and 61 without HRV co-detection, showed that co-detection of HRV was associated with higher frequency of coryza in patients with HRSV-attributable disease ($P < 0.05$; Table V). Patients with co-detection of EV had their clinical features compared with those excreting only viable HRSV, and with those with HRSV plus non-EV co-detections. No significant difference was observed between the clinical findings from patients with only viable HRSV and those with co-detection of EV. However, it is noteworthy that none of the patients with co-detection of EV required mechanical ventilation, as opposed to 22% of the HRV-positive ones (Table V).

The third most frequent virus detected by PCR in nasopharyngeal aspirates from patients with HRSV disease was HBoV, detected in 15 (21%) of them. Children positive for HBoV in addition to viable HRSV were older (median age 5 months) than those exclusively with viable HRSV (median age 2 months). Clinical features were not different among the groups of 18 patients with exclusively viable HRSV, 15 with HBoV in association with HRSV, and 55 without HBoV co-infection (Table V). The clinical findings were also not significantly different between patients with only viable HRSV and those with co-detection of HMPV (Table V).

DISCUSSION

With the advent of clinically applicable rapid antigen detection and RT-PCR-based assays for HRSV, time-consuming primary isolation of this

TABLE V. Clinical Features of Patients Shedding Viable HRSV Alone or With Other Viruses

Viruses	NPA (n)	ICS (median)	Severe disease (%)	Boys (%)	Age (median)	Cough (%)	Coryza (%)	Sneezing (%)	Fever (%)	Wheezing (%)	Dyspnea (%)	Nasal Obstruction (%)	O$_2$ (%)	Mechanical ventilation (%)	Median hospital stay (days)
Only HRSV	18	4	44	28	2	94	44	39	50	44	72	50	67	11	8
HRSV + HAdV	29	5	48	69	3	96	61	25	57	50	71	36	75	11	7
HRSV Without HAdV	41	4	48	44	3	95	54	27	56	51	66	54	63	10	8
HRSV + HRV	9	3	44	44	3	100	89*	11	44	33	56	56	78	22	9
HRSV without HRV	61	4	39	56	3	95	52*	28	58	53	70	45	67	8	7
HRSV + HEV	9	3	33	56	3	100	67	0	56	67	67	22	44	0	7
HRSV without HEV	61	4	39	54	3	92	54	30	56	48	66	49	69	10	8
HRSV + HBoV	15	3	27	67	5	93	47	20	53	47	67	33	53	13	9
HRSV without HBoV	55	4	44	51	3	95	58	27	56	51	67	49	71	9	7
HRSV + HMPV	14	4	36	57	2	100	50	14	43	57	57	36	71	7	7
HRSV without HMPV	56	4	41	54	3	93	57	29	59	48	70	48	66	11	8

*Difference between HRSV + HRV and HRSV without HRV was significant ($P < 0.005$).

agent in cell culture has become restricted to research laboratories. Detection of respiratory virus antigens or nucleic acids has facilitated studies of multiple agents all over the world confirming that HRSV is a very frequent agent, yet often detected simultaneously with other respiratory viruses [Collins and Crowe, 2007; Sly and Jones, 2011; Martin et al., 2012]. Several of those studies compared the severity of respiratory illnesses in patients whose secretions were positive by RT-PCR for HRSV-only, with those positive by PCR for other respiratory viruses concurrently with HRSV, and most of them have not reported significant differences [Canducci et al., 2008; Nascimento et al., 2010; De Paulis et al., 2011]. However, detection of HRSV nucleic acids by RT-PCR in respiratory secretions should not be regarded as firm evidence of current HRSV-associated illness, since that could result from the presence of a molecular remnant of previous infection [Franz et al., 2010]. Therefore, the present study was done to reassess the relevance of co-detecting other respiratory viruses in secretions collected from ARI patients currently harboring replication of HRSV, whose viability was confirmed by virus recovery in cell culture.

This study was done with samples collected in 2005 from children younger than 5 years, and HRSV-positive patients, detectable by any diagnostic methods, had disease significantly more severe than those negative for HRSV, thus reaffirming HRSV as an important pathogen. HRSV-positive patients had higher clinical severity scores and required more oxygen and mechanical respiratory support, thus reiterating that HRSV-related illnesses impact strongly on healthcare resources. But clinical features were not significantly different between patients excreting viable HRSV and those shedding only HRSV nucleic acid detectable by RT-PCR. This finding indicates that shedding of HRSV RNA is a practical marker of current replication of the agent, and supports its detection by RT-PCR in routine HRSV clinical diagnosis. The frequency of HRSV primary recovery in cell culture would probably have been higher if monolayers had been inoculated with freshly collected, rather than frozen specimens, due to reduced virion stability upon freeze-thawing. However, primary HRSV isolation in fresh samples is labor intensive and only rarely done on routine basis, especially in health care facilities of developing countries. Moreover, long turnaround times would likely make it impractical for acute situations when a more rapid test is crucial. Nevertheless, further prospective studies may be required to ascertain whether fresh specimen inoculation would be better as a marker of HRSV current infection.

It was remarkable that three quarters of patients who shed viable HRSV, and therefore likely had HRSV-caused illnesses, also shed nucleic acids of other respiratory viruses, and frequently more than two of them. This suggests that a significant propor-

tion of children with HRSV-caused illness also had true co-infections by other viruses. While highly sensitive RT-PCR assays enable the co-detection of trace amounts of viral nucleic acids, including possible vestiges of past infections, authentic co-infections between two replicating respiratory viruses may also occur with non-negligible frequency [Arruda et al., 1991]. Further studies based on testing for markers of active replication of other respiratory viruses will be required to establish the proportion of true viral co-infections in patients shedding viable HRSV.

In this study, the severity of respiratory illness was not significantly different among patients with viable HRSV who were co-infected by one, two, or three additional viruses. This is in agreement with other studies done with samples positive for HRSV just by RT-PCR [Canducci et al., 2008; Nascimento et al., 2010; De Paulis et al., 2011]. The effects of detecting HAdV, HRV, HEV, HBoV, and HMPV on disease severity phenotype were also evaluated. With the exception of a higher frequency of coryza found in patients who shed viable HRSV and also had HRV by RT-PCR, no other significant differences in disease phenotype were noted among HRSV patient groups co-infected with other specific viruses. Coryza is a frequent and bothersome symptom of HRV infection, and it was interesting that more coryza was noted in children shedding viable HRSV who also had HRV detected by RT-PCR. This suggests that the co-detection of HRV was associated with actual concurrent HRV replication in the upper respiratory tract of children with HRSV disease.

The concurrent detection of HAdV or HBoV DNA in patients shedding viable HRSV could be explained by the propensity of these viruses to be detected for prolonged periods [Kalu et al., 2010; Proenca-Modena et al., 2011]. However, co-detection of nucleic acids from other respiratory viruses in secretions, along with viable replicating HRSV, is not easy to ascribe to persistence without testing for markers of active replication for each specific virus, which was not attempted in the present study. Developing trustworthy virus strand-specific RT-PCR assays has been hampered by the RNA self-priming that yields cDNA originated from either RNA strands, even in the absence of exogenously added primers [Boncristiani et al., 2009].

Although most children whose respiratory samples were tested in this hospital-based study had community-acquired acute respiratory infection, some may have had nosocomial infections. One particular 2-month-old girl had a negative sample collected at admission and 1 month later another sample submitted for testing was positive for viable HRSV, as evidenced by isolation in cell culture, and for four additional respiratory viruses by PCR, a typical example of nosocomial infection by respiratory viruses, which are easily transmissible agents in the hospital environment [Mlinaric-Galinovic and Varda-Brkic, 2000].

This hospital-based study was done on respiratory samples from young children sent for routine HRSV screening in the clinical virology laboratory. The children had HRSV infections associated with shedding of viable HRSV, as opposed to HRSV detection only by RT-PCR or rapid antigen detection methods. The results indicate that children with true HRSV-attributed disease, shedding only HRSV, had clinical features indistinguishable from those who, in addition to viable HRSV, had other respiratory viruses detected by PCR. In addition to reiterating HRSV importance as a pathogen, by using viable HRSV recovery as evidence of current infection, the present study provides new evidence for the association of the majority of disease episodes attributable to HRSV by RT-PCR, with the actual replication of this important agent.

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