SUPPLEMENT ARTICLE

Respiratory Viral Coinfections Identified by a 10-Plex Real-Time Reverse-Transcription Polymerase Chain Reaction Assay in Patients Hospitalized With Severe Acute Respiratory Illness—South Africa, 2009–2010

Marthi A. Pretorius,[1] Shabir A. Madhi,[1,2] Cheryl Cohen,[1] Dhamari Naidoo,[1] Michelle Groome,[2] Jocelyn Moyes,[1] Amelia Buys,[1] Sibongile Walaza,[1] Halima Dawood,[4] Meera Chhagan,[4,6] Sumayya Haffjee,[5] Kathleen Kahn,[3] Adrian Puren,[1] and Marietjie Venter[1,7]

[1]National Institute for Communicable Diseases, National Health Laboratory Service, and [2]Department of Science and Technology/National Research Foundation: Vaccine-Preventable Diseases, and [3]MRC/Wits Rural Public Health and Health Transitions Research Unit (Agincourt), School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg; [4]Pietermaritzburg Metropolitan Hospital Complex; and [5]Pietermaritzburg Metropolitan Hospital/NHLS Complex, Edendale; [6]Maternal and Child Health Unit, University of KwaZulu-Natal, Durban; and [7]Department Medical Virology, University of Pretoria, South Africa

Background. Data about respiratory coinfections with 2009 pandemic influenza A virus subtype H1N1 during the 2009–2010 influenza pandemic in Africa are limited. We used an existing surveillance program for severe acute respiratory illness to evaluate a new multiplex real-time polymerase chain reaction assay and investigate the role of influenza virus and other respiratory viruses in pneumonia hospitalizations during and after the influenza pandemic in South Africa.

Methods. The multiplex assay was developed to detect 10 respiratory viruses, including influenza A and B viruses, parainfluenza virus types 1–3, respiratory syncytial virus (RSV), enterovirus, human metapneumovirus (hMPV), adenovirus (AdV), and rhinovirus (RV), followed by influenza virus subtyping. Nasopharyngeal and oropharyngeal specimens were collected from patients hospitalized with pneumonia at 6 hospitals during 2009–2010.

Results. Validation against external quality controls confirmed the high sensitivity (91%) and specificity (100%) and user-friendliness, compared with other PCR technologies. Of 8173 patients, 40% had single-virus infections, 17% had coinfections, and 43% remained negative. The most common viruses were RV (25%), RSV (14%), AdV (13%), and influenza A virus (5%). Influenza virus, RSV, PIV type 3, and hMPV showed seasonal patterns.

Conclusion. The data provide a better understanding of the viral etiology of hospitalized cases of pneumonia and demonstrate the usefulness of this multiplex assay in respiratory disease surveillance in South Africa.

Pneumonia is a major cause of morbidity and mortality among children worldwide and causes 18% of all deaths among children <5 years of age [1]. Viral infections have been shown to play a major role in acute respiratory infections in the developed world, but apart from a few articles on specific viruses such as influenza virus and respiratory syncytial virus (RSV) in selected regions, data remain limited from sub-Saharan Africa [2–4]. In April 2009, pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) emerged as a new pathogen. South Africa reported 12 640 cases and 93 deaths during the first wave, from June through October 2009, the most of any country in Africa [5].

Respiratory viruses traditionally associated with acute respiratory tract infection include influenza A and B viruses, RSV, parainfluenza virus (PIV) types 1–3, adenovirus, enterovirus, human metapneumovirus (hMPV), and rhinovirus [6, 7]. While a few studies have

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Correspondence: Marietjie Venter, PhD, Respiratory Virus Unit, National Institute for Communicable Diseases, Private bag X 4 Sandringham, 2131 Johannesburg, South Africa (marietjiev@nicd.ac.za).

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determined the frequency of respiratory viruses in patients with acute lower respiratory tract illness in Africa [8–11], these studies have mainly been limited to single sites and to a limited number of viruses, and little has been reported on viral coinfections. Few data are available about the contribution of other respiratory viruses to respiratory tract infections during the recent influenza pandemic or their role in A(H1N1)pdm09 infections in Africa, and limited data are available from elsewhere [12, 13].

Comparative studies have shown that real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays are substantially more sensitive than conventional methods, such as viral culture and immunofluorescence assays, for detecting respiratory viruses [14–16]. Furthermore, compared with conventional PCR and other real-time methods, multiplex rRT-PCR has a significant advantage as it permits simultaneous amplification of several viruses in a single reaction [4, 15, 16]. This facilitates cost-effective diagnosis, enabling the detection of multiple viruses in a single clinical specimen.

As part of a severe acute respiratory illness (SARI) surveillance program that commenced in February 2009 in South Africa, we developed a 2-step multiplex rRT-PCR assay that could detect 10 different viruses (influenza A and B viruses, RSV, enterovirus, hMPV, adenovirus, rhinovirus, and PIV types 1–3) to investigate the role of the most common respiratory viruses as etiological agents in patients hospitalized with SARI in South Africa.

MATERIALS AND METHODS

Setting

Specimens used in this study were obtained through routine surveillance for hospitalized SARI in 6 government hospitals around the country, including Chris Hani Baragwanath (2009–2010), an urban hospital in Gauteng province; Edendale (2009–2010), a semiurban hospital in KwaZulu-Natal province; Matikwana and Mapulaneng (2009–2010), 2 rural hospitals in the Bushbuckridge district in Mpumalanga province; and Tshepong and Klerksdorp hospital complex (2010), semiurban hospitals in the North-West province (Figure 1). Nasopharyngeal aspirates were collected from children <5 years old and nasopharyngeal and oral pharyngeal swab specimens were collected from patients >5 years old. Specimens were sent within 72 hours of collection to the Respiratory Virus Unit at the National Institute for Communicable Diseases in Johannesburg for processing and storage at −70°C.

Case Definition

We defined a case of SARI according to a previously suggested WHO case definition [17] for all hospitalized children and adults ≥5 years old in whom onset of illness occurred within 7 days of admission. We defined SARI in children aged 2 days through 2 months as physician-diagnosed sepsis or lower respiratory tract infection (LRTI), and we defined SARI in children aged 3 months through 5 years as physician-diagnosed acute LRTI. Surveillance officers administered a questionnaire with basic demographic and clinical information and examined medical records to collect data on diagnoses at admission. Specimens were collected on the day of admission.

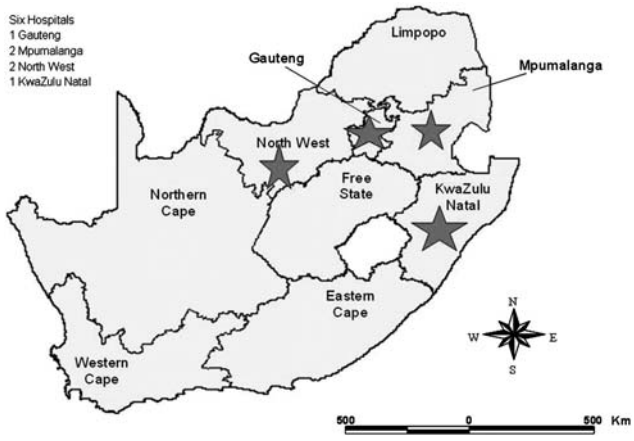

Figure 1. Geographical map of South Africa, indicating the locations of the severe acute respiratory illness surveillance sites.

Validation and Optimization of Multiplex rRT-PCR

A multiplex rRT-PCR assay detecting 10 different viruses (influenza A and B viruses, RSV, enterovirus, hMPV, adenovirus, rhinovirus, and PIV types 1–3) was established as a 2-step rRT-PCR with 5 separate reactions (Table 1). The assay was validated using conserved regions of the target viruses, to minimize the effect of genetic changes within each of the viruses. External quality control panels that included isolates of PIV types 1–4, RSV A and B, enterovirus, hMPV I and II, adenovirus, rhinovirus, and influenza A and B viruses and specimens of the bacterial species *Chlamydophila pneumoniae, Legionella pneumophila,* and *Mycoplasma pneumoniae* from Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) were used to optimize and validate the multiplex assays. Optimal primer annealing temperatures and primer and probe concentrations were calculated by experimentation. The QCMD panels were used to test all primers and probes for possible competitive interactions. The cross-reactivity of the assay was assessed in triplicate, to ensure repeatability, reproducibility, sensitivity, and specificity. TaqMan technology was selected for the multiplex rRT-PCR assay to ensure adaptability to different real-time platforms.

Nucleic Acid Extraction

The MagNA Pure LC Total Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) was used according to manufacturer's instructions, with a 200-μL sample and a final elution volume of

Table 1. Contribution of Respiratory Viruses to Severe Acute Respiratory Illness Among Hospitalized Patients in South Africa as Monoinfection (Diagonal) or Coinfection (Matrix)

Virus Detected	RV	RSV	AdV	EV	PIV3	hMPV	A(H3N2)	B	A(H1N1)pdm09	PIV1	PIV2
Overall[a]	2034 (25)	1169 (14)	1083 (13)	515 (6)	354 (4)	303 (4)	276 (3)	223 (3)	204 (3)	90 (1)	84 (1)
RV	1171	…	…	…	…	…	…	…	…	…	…
RSV	320	591	…	…	…	…	…	…	…	…	…
AdV	356	212	364	…	…	…	…	…	…	…	…
EV	0	107	142	219	…	…	…	…	…	…	…
PIV3	87	20	31	27	198	…	…	…	…	…	…
hMPV	73	18	52	9	14	155	…	…	…	…	…
A(H3N2)	38	19	53	12	6	3	182	…	…	…	…
B	32	10	38	6	6	5	1	144	…	…	…
A(H1N1)pdm09	19	3	6	3	10	7	0	0	153	…	…
PIV1	28	21	23	10	4	3	0	2	0	34	…
PIV2	28	16	18	12	5	2	3	3	1	2	23

Data are no. of specimens positive for virus monoinfection or coinfection, unless otherwise indicated. A total of 8173 specimens were evaluated, with monoinfection detected in 3240 (39.6%) and coinfection detected in 1426 (17.4%).

Abbreviations: AdV, adenovirus; A(H1N1)pdm09, 2009 pandemic influenza A virus subtype H1N1; A(H3N2), influenza A virus subtype H3N2; B, influenza B virus; EV, enterovirus; hMPV, human metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus.

[a] Data are no. (%) of tested specimens that were positive for virus.

50 μL; excess extracted nucleic acids were stored at –70°C. A negative and positive biological control was used in each extraction.

Primer and Probe Multiplexing

Primers and probes for the 10 respiratory viruses were identified for the qualitative studies. All primers and probes (Supplementary Table 1) were optimized in different combinations for this assay. DNAMAN was used (Lynnon, Quebec, Canada) to ensure primer complementary and primer dimers did not exist between different PCR groups and to select primer set candidates per multiplex. We used the influenza A virus primers recommended by the WHO Collaborating Center for Influenza, Centers for Disease Control and Prevention (CDC; Atlanta, GA), for the universal detection of influenza A virus strains, which were updated to include A(H1N1)pdm09 [18].

rRT-PCR

Complementary DNA (cDNA) was synthesized using the Transcriptor 1st Strand cDNA Kit (Roche Diagnostics), according to manufacturer's instructions. Qualitative rRT-PCR assays using the LightCycler 480 Probes Master kit (Roche Diagnostics) and the LightCycler 480 System (Roche Diagnostics) were performed. Each rRT-PCR reaction contained 15 μL of 2X Master Mix, 1 μM of each primer, 0.5 μM of each probe, and 10 μL of cDNA reaction mixture as template, for a final volume of 30 μL. PCR cycles were initiated at 95°C for 15 minutes to activate *Taq* DNA polymerase enzyme, followed by 45 cycles at 94°C for 15 seconds, 60°C for 20 seconds, and 72°C for 10 seconds. Specimens were considered positive when the Ct value was equal or above the Ct value of the lower limit of detection of the corresponding virus, which ranged between 36 and 40. The influenza virus–positive specimens were subtyped in accordance with a rRT-PCR protocol from the CDC [18], which was distributed to National Influenza Centers under a Material Transfer Agreement.

Statistical Analysis

We analyzed the positive cases and seasonal patterns of the respiratory viruses included in the multiplex. Results were analyzed according to the proportion of viruses detected per month and subject age group. The χ^2 test and Fischer exact test were used for univariate analysis, and P values of <.05 were considered to be statistically significant. Analysis was performed using Stata 11 (Stata Corporation, College Station, TX). Data for κ and Bland-Altman analysis were analyzed using Analyse-it Method Evaluation Edition add-in software for Microsoft Excel 2007 (Analyse-it Software, Leeds, United Kingdom).

Ethical Considerations

The protocol was reviewed and approved by the University of the Witwatersrand Human Research Ethics Committee and the University of KwaZulu-Natal Human Biomedical Research Ethics Committee (protocol numbers M081042 and BF157/08, respectively).

RESULTS

Validation and Optimization of the rRT-PCR

With the external quality control panels provided by QCMD serving as the gold standard, the multiplex rRT-PCR assay

had a high overall accuracy (98%; defined as the degree of closeness of the measured or calculated quantity to its actual [true] value), negative predictive value (97%), positive predictive value (100%), sensitivity (91%), and specificity (100%) [19]. The rRT-PCR assay was compared to immunofluorescence assays, and the rRT-PCR assay was more sensitive in all cases (data not shown). Use of QCMD panels established reproducibility, repeatability, and lower detection limits, ranging from Ct values of 36–40, depending on the different viruses. The coefficient of variation calculated from the QCMD panels for the 5 reactions ranged from 0.2% to 0.7%.

Study Group Demographic Characteristics

From February 2009 through December 2010, we collected and tested specimens from 8173 patients with SARI. The median age of patients was 3 years (range, 0–99 years). Half of patients (3974 [51.1%]) were male. The largest age group was children <1 year of age (3157 [38.6%]), and 6098 patients (74.7%) were from Chris Hani Baragwanath Hospital in Soweto.

Application of the rRT-PCR for Screening of Surveillance Specimens From Patients With SARI

Of the 8173 patients tested with rRT-PCR, 3240 (39.6%) had single infections, 1426 (17.4%) had coinfections with ≥2 viruses, and 3507 (42.9%) were negative for pathogens included in this assay. The most common respiratory viruses identified were rhinovirus (2034 [24.9%]), RSV (1169 [14.3%]), adenovirus (1083 [13.3%]), and influenza virus (704 [8.7%]; Table 1). Among the 3240 patients with single infections (Table 1), rhinovirus was the most frequently detected virus, occurring in 1171 patients (36.1%), followed by RSV in 591 (18.2%) and adenovirus in 364 (11.2%). No difference was observed in the proportion of these viruses detected at each of the surveillance sites (data not shown).

The percentage of specimens positive for influenza virus was similar in both years (393 [10.7%] in 2009 vs 324 [7.2%] in 2010), but there were differences in influenza types and subtypes. In 2009, influenza A virus was detected most often (354 [9.7%]), with most specimens yielding subtype H3N2 (A [H3N2]; 194 [5.3%]) or A(H1N1)pdm09 (160 [4.4%]); influenza B virus was much less common (25 [0.7%]). However, in 2010 influenza B virus was detected most often (198 [4.4%]); influenza A virus was detected in 126 specimens (2.8%), with A(H3N2) found in 82 (1.8%) and A(H1N1)pdm09 found in 44 (0.9%). During 2009, 14 of 494 specimens (2.8%) positive for influenza A virus could not be subtyped because concentrations were too low. In the univariate analysis, which used patients aged ≤1 year as the reference group, patients aged 5–24 years were more likely to be infected with A(H1N1)pdm09 (odds ratio [OR], 2.28; $P = .001$), while patients aged 2–4 years were more likely to be infected A(H3N2) (OR, 1.75; $P = .001$) (Supplementary Table 2). However, patients aged 25–44 years were more likely to be infected with influenza B virus (OR, 2.09; $P = .001$). In addition, no difference was observed between the 2 study years with regards to the distribution and proportion of each of the other respiratory viruses.

The overall rate of virus detection was highest among patients aged 2–4 years, for whom 833 of 992 specimens (83.9%) were positive for at least 1 virus, and lowest among persons aged ≥65 years, for whom 55 of 227 specimens (24.2%) were positive for at least 1 virus. Compared with patients aged 2–4 years, the rate of positivity in other age groups was significantly less (76.5% for ages 0–1 years, 51.0% for ages 5–24 years, 33.8% for ages 25–44 years, and 28.7% for ages 45–64 years; $P < .001$ for each comparison). Among patients aged ≤1 year, the most common virus was rhinovirus (985 of 3157 [31.2%]), followed by RSV (845 of 3157 [26.7%]). In contrast, in other age groups, the more common pathogens were rhinovirus and adenovirus (Supplementary Table 2).

Respiratory Viral Coinfections

Among the 1426 patients with coinfections (Table 1), rhinovirus was detected most frequently (860 [60.3%]), followed by adenovirus (719 [50.4%]) and RSV (578 [40.5%]). Of the 51 patients with A(H1N1)pdm09 coinfection, rhinovirus was detected in 19 (37.3%), followed by PIV type 3 in 10 (19.6%). There were no coinfections with A(H1N1)pdm09 and A (H3N2). However, there was 1 coinfection with influenza B virus and A(H3N2) (Table 1). Of the 94 patients with A (H3N2) coinfection, rhinovirus was detected in 38 (40.4%), followed by RSV in 19 (20.2%) and PIV type 3 in 6 (6.4%).

Of the 1426 coinfections, the following combinations of viruses were detected most frequently in the same specimen: 356 cases of rhinovirus and adenovirus, 320 cases of rhinovirus and RSV, 212 cases of RSV and adenovirus, and 142 cases of adenovirus and enterovirus (Table 1).

Seasonality

Seasonal patterns were visible for RSV, influenza A and B viruses, enterovirus, hMPV, and PIV3 in both 2009 and 2010. RSV was active from February through June, before the influenza season, which typically occurs from May through September [20]. Enterovirus was detected throughout the year, with peak activity between February and April and again between November and December. Peak activity for hMPV was observed between July and August, and peak activity for PIV type 3 occurred between September and November. Adenovirus and rhinovirus were detected throughout the 2 years, without seasonal variability. PIV types 1 and 2 were detected sporadically throughout the 2 years (Figure 2).

The seasonal patterns of both influenza A and B viruses during 2009 and 2010 were different. In 2009, A(H3N2) and A(H1N1)pdm09 activity occurred in 2 waves, peaking between May and July and between July and October,

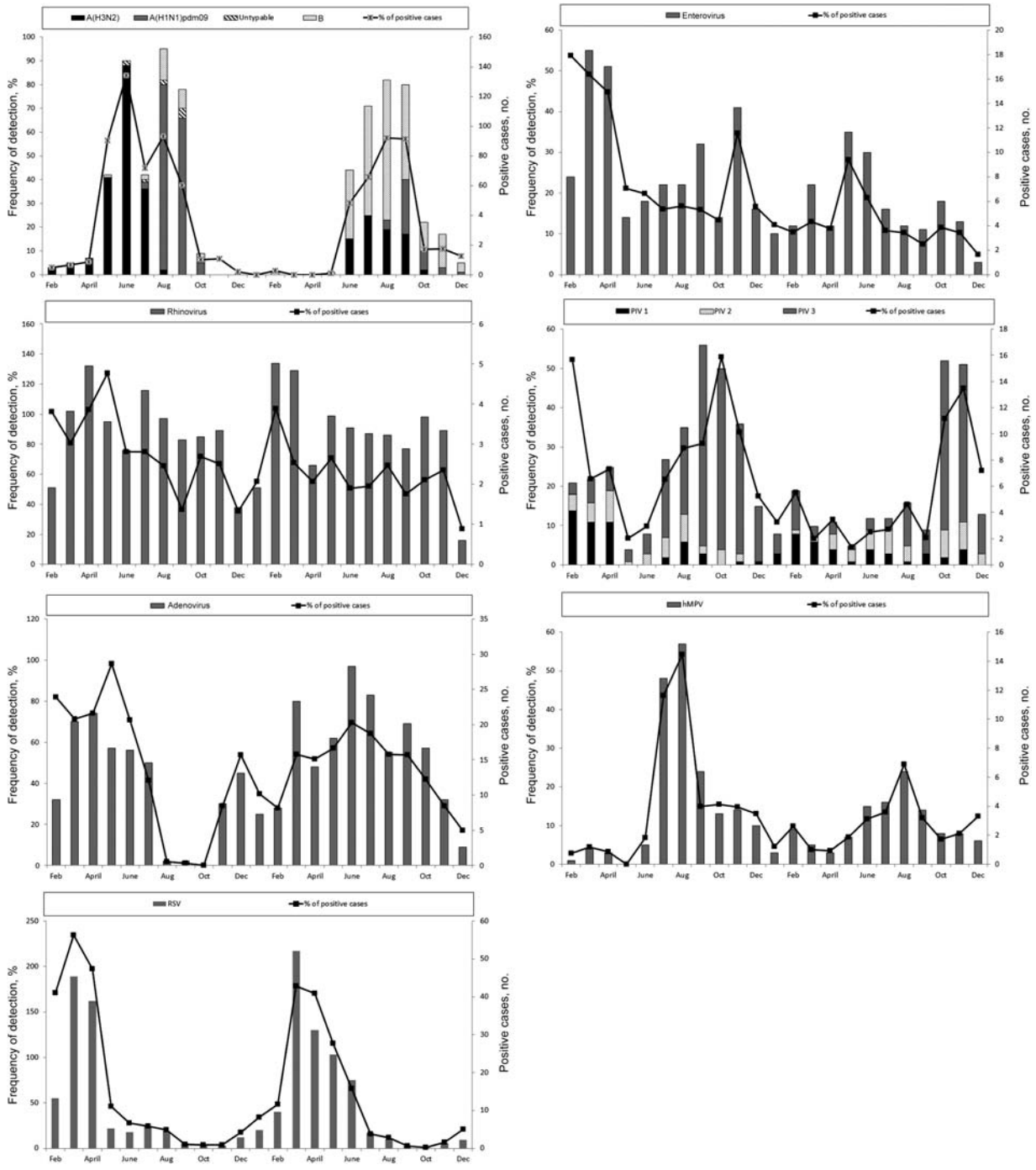

Figure 2. Distribution of respiratory viruses detected during 2009 and 2010, showing specific seasonal trends and peak activity. Abbreviations: A (H1N1)pdm09, 2009 pandemic influenza A virus subtype H1N1; A(H3N2), influenza A virus subtype H3N2; B, influenza B virus; hMPV, human metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

respectively, while influenza B virus appeared briefly in August. During 2010, influenza B season predominated from June through November, A(H3N2) circulated from June through September, and A(H1N1)pdm09 circulated between the last week of July and October.

DISCUSSION

The validated assay was implemented for routine surveillance prior to the 2009–2010 influenza pandemic and enabled us to investigate the contribution of other respiratory viruses to

SARI during the first 2 pandemic seasons. The assay also helped define the distribution and seasonality of these respiratory viruses in South Africa and the role of viral coinfections in hospitalized patients infected with A(H1N1)pdm09 in South Africa during the 2009 and 2010 seasons. By use of the validated rRT-PCR multiplex assay, viral agents were detected in 57% of cases identified through South Africa's SARI surveillance network, which is consistent with other studies using rRT-PCR multiplex assays for the detection of respiratory viruses [2, 4, 16]. Validation of the rRT-PCR multiplex assay suggested that it is as specific but more sensitive than immunofluorescence assays. The assay does not give any false-positive results, while its lower limit of detection may give false-negative results for specimens with a very low viral load.

The majority of patients enrolled were infants ≤1 year of age, and the most commonly identified pathogens within this group were RSV and rhinovirus. While rhinovirus was detected throughout the year, RSV was detected in a distinct seasonal pattern, with the peak months of detection occurring from February through June. Seasonal peaks were also identified for PIV type 3, hMPV, and enterovirus. Year-round detection of rhinovirus and adenovirus make these 2 viruses more likely to coinfect with each of the other viruses.

Although rhinovirus was the most commonly identified pathogen in this study, further studies are needed to determine how much rhinovirus contributes to disease severity [4, 9, 21]. In a study conducted 2 years prior to the 2009 pandemic in hospitals situated in South Africa, RSV was detected at a much higher rate in symptomatic infants with severe disease than in asymptomatic infants attending a vaccination clinic in the same region [16]. Nevertheless, in a study conducted during 2006 and 2007 in South Africa, rhinovirus was present in 18% of asymptomatic children and in >30% of children hospitalized with SARI, which suggests a possible role in disease severity [4].

Although there are growing concerns about the potential for A(H1N1)pdm09 to reassort with existing human influenza viruses and give rise to a highly transmissible or pathogenic virus [22], no mixed infections were detected with either subtypes in patients with SARI in this study. The influenza virus subtypes had cocirculated for overlapping periods both in 2009 and 2010, but peak months of detection were distinct for A(H3N2) and A(H1N1)pdm09 in both years, while influenza B virus was detected from June through November, overlapping with both A(H3N2) and A(H1N1)pdm09 peaks. No seasonal influenza virus A subtype H1N1 was observed during the 2009 and 2010 influenza season. In the present study, we found pandemic cases mostly in older children and young adults, which is similar to surveillance reports of the 2009 pandemic in other parts of the world, which have shown that up to 57% of cases occurred among people aged 5–24 years, with a detection rate of 5.1% [23].

Our study has some limitations. First, rRT-PCR assays are more sensitive than viral culture at detecting respiratory viruses, and with the increased detection of mixed viral infections, the clinical interpretation of positive PCR results has become more challenging. Although the viral nucleic acids detected here do not necessarily indicate the presence of viable virus, several studies have documented few persistent or recurrent PCR-positive respiratory specimens in patients after acute illness has resolved, suggesting a likely association with the diseased state [24, 25]. The relevance of the high frequency detected of respiratory viruses such as rhinovirus in monoinfections and coinfections requires further investigation. Because of the low numbers of each specific coinfection combination, we did not report on clinical outcomes and how single and coinfections differed from each other clinically. This study also did not include bacterial testing, and therefore gaps remain in our understanding of all the etiologies of SARI in South Africa. Last, the study period of 2 years could also be a limitation, since the circulation of viruses could change from year to year. However, only 2 years of data are represented here, the surveillance study is ongoing, and changes in the seasonal circulation of the viruses will be detected. Understanding the contribution of these viruses to severe respiratory disease will allow for informed decision making when selecting specific respiratory pathogens for inclusion in a sustainable respiratory disease surveillance program. Currently, the overall cost of the assay from extraction to detection is US$63, with a panel (consisting of 2–3 viruses) costing US$12. However, by selecting only the major contributors to SARI, the cost of running the assay could be reduced in future.

In conclusion, this study indicates a contributing role for coinfecting viruses in patients presenting with SARI and highlights the important role of viral coinfection. Continued use of the rRT-PCR multiplex assay in conjunction with the SARI surveillance program will enhance our ability to detect circulation of respiratory viruses in patients hospitalized for SARI and help clarify the contribution of these respiratory viruses among patients with SARI in South Africa.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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