Respiratory viral coinfections identified by a 10-plex real time polymerase chain reaction assay in patients hospitalised 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[3]; Meera Chhagan[3,5]; Sumayya Haffjee[4];Kathleen Kahn[6]; Adrian Puren[1]; Marietjie Venter[1, 7 *]

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

Contact details

Marthi A Pretorius[1]: marthin@nicd.ac.za

Dhamari Naidoo[1]: dhamarin@nicd.ac.za

Shabir A Madhi[1,2]: shabirm@nicd.ac.za

Michelle Groome[2]: groomem@rmpru.co.za

Amelia Buys[1]: ameliab@nicd.ac.za

Jocelyn Moyes[1]: jocelynm@nicd.ac.za

Sibongile Walaza[1]: sibongilew@nicd.ac.za

Halima Dawood[3]: Halima.Dawood@kznhealth.gov.za

Meera Chhagan[3, 5]: chhaganmeera@gmail.com

Sumayya Haffjee[4]: sumayya.haffejee@nhls.ac.za

Kathleen Kahn[6]: kathleen.kahn@wits.ac.za

Cheryl Cohen, M.D.[1]: cherylc@nicd.ac.za

Adrian Puren, M.D. Ph.D.[1]: adrianp@nicd.ac.za

Marietjie Venter, Ph.D.[1, 7,*]: marietjiev@nicd.ac.za

*Corresponding Author's address: Marietjie Venter, Respiratory Virus Unit, National Institute for Communicable Diseases, Private bag X 4 Sandringham, 2131 Johannesburg, South Africa. Tel: +27 11 555 0478, Cell: +2782 902 0412, Fax: +27 11 386 6455; email: marietjiev@nicd.ac.za

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Abstract

Background: Data about respiratory co-infections of Influenza A H1N1 during the pandemic in Africa are limited. We used an existing surveillance programme for severe acute respiratory illness (SARI) to evaluate a new multiplex real-time polymerase chain reaction assay and investigate the role of influenza and other respiratory viruses in pneumonia hospitalisations during and after the influenza pandemic in South Africa.

Method: The multiplex assay was developed to detect 10 respiratory viruses including Influenza (INF) A and B, Parainfluenza (PIV1-3), Respiratory Syncytial Virus (RSV), Enterovirus (EV), human metapneumovirus (hMPV), Adenovirus (AdV) and Rhinovirus (RV), followed by influenza subtyping. Nasopharyngeal and oropharyngeal specimens were collected from patients hospitalized with pneumonia at six hospitals during 2009–2010.

Results: Validation against external quality controls confirmed the high sensitivity (91%) and specificity (100%) and user-friendliness when compared to other PCR technologies. Of 8173 patients, 40% had single-infections, 17% co-infections and 43% remained negative. The most common viruses were: RV (25%), RSV (14%), AdV (13%), Influenza A (5%). Influenza, RSV, PIV3 and hMPV showed seasonal patterns.

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

Introduction

Pneumonia is a major cause of morbidity and mortality in children worldwide and causes 18% of all deaths in children less than 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 papers on specific viruses such as influenza and respiratory syncytial virus (RSV) in selected regions, data remain limited from sub-Saharan Africa [2-4]. In April 2009, Influenza A (H1N1) pdm09 ((H1N1)pdm09) emerged as a new pathogen. South Africa reported 12640 cases and 93 deaths during the first wave from June-October 2009, the most of any country in Africa [5].

Respiratory viruses traditionally associated with acute respiratory tract infection include influenza (INF) A and B; respiratory syncytial virus (RSV); parainfluenza virus (PIV) types 1, 2 and 3; adenovirus (AdV); enterovirus (EV); human metapneumovirus (hMPV) and rhinovirus (RV) [6, 7]. While a few studies have 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 a limited number of viruses, and little has been reported on viral co-infections. Few data are available about the contribution of other respiratory viruses to respiratory tract infections during the pandemic or their role in (H1N1)pdm09 infections in Africa, and limited data is available from elsewhere [12, 13].

Comparative studies have shown that the detection of respiratory viruses using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays is substantially more sensitive than using conventional methods such as viral culture and immunofluorescence assays (IFA) [14-16]. Furthermore, compared to 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 infection (SARI) surveillance programme which commenced in February 2009 in South Africa, we developed a two-step real-time multiplex reverse transcriptase PCR (rRT-PCR) assay that could detect ten different viruses (Influenza A and B, RSV, EV, hMPV, AdV, RV, PIV 1, 2 and 3) in order to investigate the role of the most common viral agents as aetiological agents in patients hospitalised with SARI in South Africa.

Materials and Methods

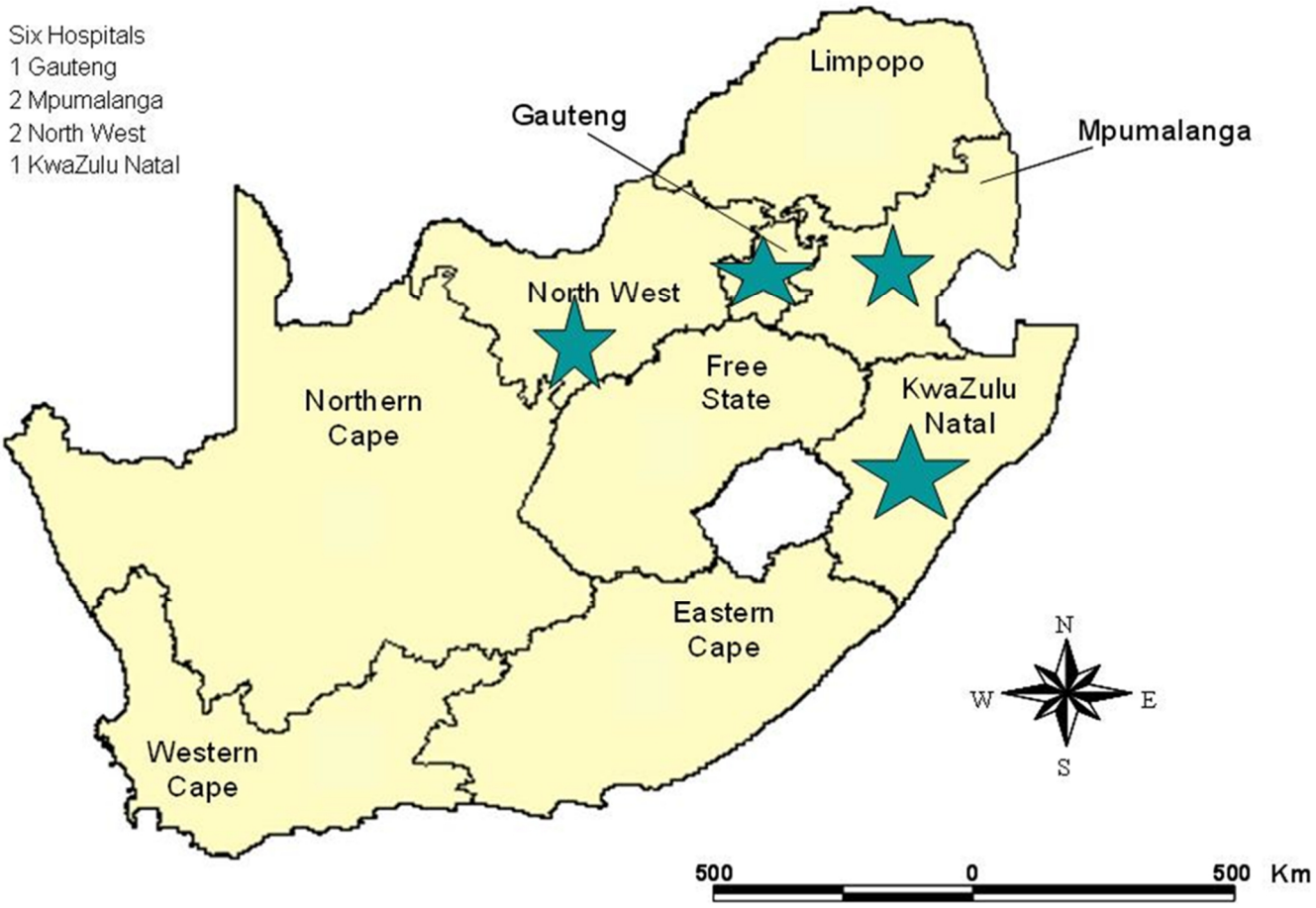
Setting

Specimens used in this study were obtained through routine surveillance for hospitalized SARI in six government hospitals around the country, including Chris Hani Baragwanath (2009-2010), an urban hospital in Gauteng province; Edendale (2009-2010), a semi-urban hospital in KwaZulu-Natal province; Matikwana and Mapulaneng (2009-2010), two rural hospitals in the Bushbuckridge district in Mpumalanga province; and Tshepong and Klerksdorp hospital complex (2010), semi-urban hospitals in the North-West province (Figure 1). Nasopharyngeal aspirates were collected from children <5 years old and nasopharyngeal as well as oral pharyngeal swabs were collected from patients >5 years old. Specimens were sent to the Respiratory Virus Unit (RVU) at the National Institute for Communicable Diseases (NICD) in Johannesburg within 72 hours of collection 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 children and adults ≥5 years old, hospitalized with onset of illness within 7 days of admission. We defined SARI in children 2 days through 2 months old as physician-diagnosed sepsis or lower respiratory tract infection (LRTI), and we defined SARI in children 3 months through 5 years old as physician-diagnosed acute LRTI. Surveillance officers administered a

Figure 1: Geographical Map of South Africa indicating the locations of the SARI surveillance sites.



questionnaire with basic demographic and clinical information and examined medical records to collect data on admitting diagnoses. Specimens were collected on the day of admission.

Validation and Optimization of Real-Time rRT-PCRMultiplex

A real-time multiplex PCR assay detecting ten different viruses (Influenza A and B, RSV, EV, hMPV, AdV, RV, PIV 1, 2 and 3) was established as two-step rRT-PCR with 5 separate reactions (Table 1). The assay was validated using conserved regions of the target viruses, to

Table 1: Primers and probes used in multiplex rRT-PCR run in five PCR mixtures as indicated.

Target	Gene	Oligonucleotide sequence (5'- 3')		REPORTER	PCR GROUP	Published
Parainfluenza PIV 1	HN gene	GTT GTC AAT GTC TTA ATT CGT ATC AAT AAT T	Forward			[26]
		GTA GCC TMC CTT CGG CAC CTA A	Reverse			
		TAG GCC AAA GAT TGT TGT CGA GAC TAT TCC AA	Probe	Cy 5-BBQ		
PIV 2	HN gene	GCA TTT CCA ATC TTC AGG ACT ATG A	Forward		A[§]	[26]
		ACC TCC TGG TAT AGC AGT GAC TGA AC	Reverse			
		CCA TTT ACC TAA GTG ATG GAA TCA ATC GCA AA	Probe	FAM-BHQ1		
PIV 3	HN gene	AGT CAT GTT CTC TAG CAC TCC TAA ATA CA	Forward			[26]
		ATT GAG CCA TCA TAA TTG ACA ATA TCA A	Reverse			
		AAC TCC CAA AGT TGA TGA AAG ATC AGA TTA TGC A	Probe	Red 610-BHQ2		
Respiratory Syncytial virus (RSV)	Matrix protein	GCA AAT ATG GAA ACA TAC GTG AAC A	Forward			[7]
		GCA CCC ATA TTG TWA GTG ATG CA	Reverse			
		CTT CAC GAA GGC TCC ACA TAC ACA GCW G	Probe	FAM-BHQ1	B[¥]	
Influenza B (INF B)	HA	AAA TAC GGT GGA TTA AAT AAA AGC AA	Forward			[27]
		CCA GCA ATA GCT CCG AAG AAA	Reverse			
		CAC CCA TAT TGG GCA ATT TCC TAT GGC	Probe	Red 610-BHQ2		
Enteroviruses (EV)	5' UTR	TCC TCC GGC CCC TGA	Forward			[28]
		RAT TGT CAC CAT AAG CAG CCA	Reverse			
		CGG AAC CGA CTA CTT TGG GTG WCC GT	Probe	Cy 5-BBQ	C[¥]	
human Meta pneumovirus (hMPV)	N protein	GAA GAR ATA GAC AAA GAR GCA AG	Forward			[6]
		TCC CAC TTC TAT KGT TGA TGC TAG	Reverse			
		TCA GCA CCA GAC ACA CC	Probe	Red 610-LNA		
Adenoviruses (AdV)	Hexon	GCC ACG GTG GGG TTT CTA AAC TT	Forward			[29]
		GCC CCA GTG GTC TTA CAT GCA CAT C	Reverse			
		TGC ACC AGA CCC GGG CTC AGG TAC TCC GA	Probe	Red 610-BHQ2	D[¥]	
Influenza A (INF A)	M1	GAC CRA TCC TGT CAC CTC TGA C	Forward			*[18]
		AGG GCA TTY TGG ACA AAK CGT CTA	Reverse			
		TGC AGT CCT CGC TCA CTG GGC ACG	Probe	FAM-BHQ1		
Rhinoviruses (RV)	5 ' UTR	GGT GTG AAG AGC CSC RTG TGC T	Forward			[7]
		GGT GTG AAG ACT CGC ATG TGC T	Forward			
		GGG TGY GAA GAG YCT ANT GTG CT	Reverse			
		GGA CAC CCA AAG TAG TYG GTY C	Reverse		E[¥]	
		CCG GCC CTG AAT GYG GCT AAY C	Probe	FAM-BHQ1		
Human sapiens ribonuclease RNP (IQC)	30kDa subunit (RPP30)	AGA TTT GGA CCT GCG AGC G	Forward			*[18]
		GAG CGG CTG TCT CCA CAA GT	Reverse			
		TTC TGA CCT GAA GGC TCT GCG CG	Probe	Cyan 500-BHQ1		

* Primers and Probes were obtained from the CDC co-operative agreement after the first case of Pandemic H1N1 was detected.

[¥]Multiplex was combined from pre-existing published primer and probe sets

[¥]Existing published multiplex

minimize the effect of genetic changes within each of the viruses. External quality control panels which included isolates of PIV 1, 2, 3 and 4; RSV A and B; EV; hMPV I and II; AdV; RV; and INF A and B viruses and specimens for the bacterial species *Chlmaydophila 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 using 200 µl sample and a final elution volume of 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 10 respiratory viruses (PIV 1, 2 and 3; RSV; EV; hMPV; AdV; RV and INF A and B viruses) were identified for the qualitative studies. All primers and probes (Table 1) were optimized in different combinations for this assay. DNAMAN was used (Lynnon Corporation, Québec, 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 primers recommended by the WHO Collaborating Center for Influenza, Centers for

Disease Control and Prevention (CDC), USA for the universal detection of Influenza A strains, which were updated to include the (H1N1)pdm09 [18].

Real-Time RT-PCR

cDNA was synthesized using the Transcriptor 1stStrand cDNA Kit (Roche Diagnostics, Mannheim Germany), according to manufacturer's instructions. Qualitative real-time polymerase chain reactions (PCR) using the LightCycler® 480 Probes Master kit (Roche Diagnostics, Mannheim Germany) and the LightCycler® 480 System (Roche Diagnostics, Mannheim Germany) were performed. Each real-time PCR reaction contained 15 µl of 2X Master Mix, 1 µM of each primer and 0.5 µM of each probe and 10 µl of cDNA reaction mixture as template for a final volume of 30 µl. PCR cycles was initiated at 95°C for 15 minutes to activate *Taq* DNA polymerase enzyme, followed by 45 cycles of 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 Ct=36 to Ct=40. The influenza positive specimens were subtyped using the CDC Real-time RTPCR (rRTPCR) Protocol for Detection and Characterization of Influenza, which was distributed to National Influenza Centres under a Material Transfer Agreement [18].

Statistical Analysis

We analysed the positive cases and seasonal patterns of the respiratory viruses included in the multiplex. Results were analysed according to virus proportion per month and subject age-group. The Chi-square test and Fischer's exact test were used for univariate analysis, P-values <0.05 were considered to be statistically significant. Analysis was performed using STATA 11, (Stata Corporation, Texas USA). Data for Kappa and Bland-Altman analysis were analysed

using Analyse-it® Method Evaluation Edition add-in software for Microsoft Excel 2007 (Analyse-it Software Ltd., Leeds, UK).

Ethical considerations

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

Results

Validation and Optimization of the Real-Time RT-PCR (rRT-PCR).

Using the external quality control panels provided by QCMD as the gold standard, the multiplex rRT-PCR assay had a high overall accuracy (98%) (*i.e.* the degree of closeness of measured or calculated quantity to its actual (true) value), negative predicative value (97%), positive predicative value (100%), sensitivity (91%) and specificity (100%) [19]. The rRT-PCR assay was compared to Immunofluorescence assays (IFA) and the rRT-PCR assay was more sensitive in all cases (data not shown). Use of QCMD panels established reproducibility, repeatability, as well as lower detection limits ranging from Ct values of 36 to 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 demographics

From February 2009 up to December 2010, we collected and tested specimens from 8173 SARI patients. 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 from children <1 year of age (3157 (38.6%), and 6098 (74.7%) were from patients admitted to 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 the rRT-PCR, 3240 (39.6%) had single infections, 1426 (17.4%) had co-infections with two or more viruses, and 3507 (42.9%) were negative for pathogens included in this assay. The most common respiratory viruses identified were RV (2034, 24.9%), RSV (1169, 14.3%), AdV (1083, 13.3%), and influenza (704, 8.7%; Table 2). Among the 3240 patients with single infections (Table 2, diagonal), RV was the most frequently detected virus, occurring in 1171 (36.1%) followed by RSV in 591 (18.2%) and AdV in 364 (11.2%). No difference was observed in the proportion of these viruses detected at each of the surveillance sites (data not shown).

Table 2: Contribution of respiratory viruses to SARI in hospitalized patients in South Africa as single (diagonal) or co-infection (matrix)

Total specimens received n=8173 Total Single infection n=3240(39.6%), Total co-infections n=1426 (17.4%)											
Total viruses detected	RV n=2034 (25%)	RSV n=1169 (14%)	AdV n=1083 (13%)	EV n=515 (6%)	PIV3 n=354 (4%)	hMPV n=303 (4%)	H3N2 n=276 (3%)	INF B n=223 (3%)	H1N1)pdm09 n=204 (3%)	PIV1 n=90 (1%)	PIV2 n=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					
H3N2	38	19	53	12	6	3	182				
INFB	32	10	38	6	6	5	1	144			
(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

The percent of specimens positive for influenza 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 mainly Influenza A was detected (354, 9.7%), which comprised mostly of H3N2 (194, 5.3%) and (H1N1)pdm09 (160, 4.4%); Influenza B was much less common (25, 0.7%). However, in 2010 mainly influenza B viruses were detected (198, 4.4%), followed by Influenza A (126, 2.8%)

Table 3: Distribution of Respiratory Virus infections and specimens received per age group, SARI surveillance, South Africa, 2009 and 2010

Age Groups		0-1	2-4	5-24	25-44	45-64	65+	Unknown
Specimens Received (n=)		3157	992	732	2086	961	227	18
RV	Total	985	399	186	318	119	20	7
	%	31.2%	40.2%	25.4%	15.2%	12.4%	8.8%	38.9%
	OR	-	1.48	0.76	0.39	0.31	0.21	
	p-values	-	0.001	0.003	0.001	0.001	0.001	
RSV	Total	845	189	33	73	26	2	1
	%	26.8%	19.1%	4.5%	3.5%	2.7%	0.9%	5.6%
	OR	-	0.64	0.13	0.09	0.07	0.02	
	p-values	-	0.001	0.001	0.001	0.001	0.001	
AdV	Total	513	315	88	110	47	6	4
	%	16.2%	31.8%	12.0%	5.3%	4.9%	2.6%	22.2%
	OR	-	2.41	0.71	0.28	0.26	0.14	
	p-values	-	0.001	0.001	0.001	0.001	0.001	
EV	Total	318	133	28	23	7	5	1
	%	10.1%	13.4%	3.8%	1.1%	0.7%	2.2%	5.6%
	OR	-	1.38	0.36	0.99	0.67	0.20	
	p-values	-	0.003	0.001	0.001	0.001	0.001	
PIV3	Total	214	44	17	59	15	5	0
	%	7%	4%	2%	3%	2%	2%	0%
	OR	-	0.64	0.33	0.40	0.22	0.31	
	p-values	-	0.008	0.001	0.001	0.001	0.010	
hMPV	Total	188	52	13	36	12	1	1
	%	6.0%	5.2%	1.8%	1.7%	1.2%	0.4%	5.6%
	OR	-	0.87	0.29	0.29	0.19	0.06	
	p-values	-	0.401	0.001	0.001	0.001	0.008	
H3N2	Total	112	60	28	48	19	7	2
	%	3.5%	6.0%	3.8%	2.3%	2.0%	3.1%	11.1%
	OR	-	1.75	1.08	0.64	0.54	0.86	
	p-values	-	0.001	0.717	0.011	0.017	0.714	
(H1N1)pdm09	Total	72	28	37	38	22	7	0
	%	2.3%	2.8%	5.1%	1.8%	2.3%	3.1%	0.0%
	OR	-	1.24	2.28	0.79	1.00	1.36	
	p-values	-	0.333	0.000	0.257	0.987	0.441	
INF B	Total	58	28	22	80	28	7	1
	%	1.8%	2.8%	3.0%	3.8%	2.9%	3.1%	5.6%
	OR	-	1.61	1.72	2.09	1.57	1.71	
	p-values	-	0.040	0.034	0.000	0.049	0.188	
PIV1	Total	47	30	4	4	3	1	1
	%	1.5%	3.0%	0.5%	0.2%	0.3%	0.4%	5.6%
	OR	-	2.06	0.37	0.13	0.21	0.29	
	p-values	-	0.002	0.055	0.000	0.008	0.225	
PIV2	Total	40	16	8	16	2	1	1
	%	1.3%	1.6%	1.1%	0.8%	0.2%	0.4%	5.6%
	OR	-	1.28	0.87	0.60	0.16	0.34	
	p-values	-	0.412	0.719	0.088	0.120	0.294	

The Chi-square test and Fischer's exact test were used for univariate analysis and Odds ratio calculated for each of the age group. Analysis was performed using STATA 11, (Stata Corporation, Texas USA). P-values <0.05 were considered to be statistically significant.

comprised of H3N2 (82, 1.8%) and (H1N1)pdm09 (44, 0.9%). During 2009 a total of 14/494 (2.8%) Influenza A specimens could not be subtyped due to too low concentrations. In the univariate analysis patients infected with (H1N1)pdm09 (OR=2.28, p=0.001), were more likely between 5-24 years, while patients infected with H3N2 (OR=1.75, p=0.001), were between 2-4 year old, however the patients infected with Influenza B (OR=2.09, p=0.001) were more likely between 25-44 years (Table 3). In addition no difference was observed between the two years with regards to the distribution and proportion of each of the other respiratory viruses.

The highest overall virus detection rate was in the 2-4 year old age group, where 833 (83.9%) of 992 specimens were positive for at least one virus, and lowest in persons ≥65 years old, where 55 (24.2%) of 227 specimens were positive for at least one virus. Compared to 2-4 year olds, the positivity rate for other age groups was significantly less [0-1 years (76.5% positive), 5-24

years (51.0% positive), 25-44 years (33.8% positive), and 45-64 years (28.7% positive; p<0.001)] for each compared with 2-4 year olds. In the 0-1 year old age group the most common virus was RV (985/3157 (31.2%)) followed by RSV, (845/3157 (26.7%)). In contrast, in other age groups, the more common pathogens were RV, and AdV (Table 3).

Respiratory viral co-infections

Among the 1426 patients with co-infections (Table 2, matrix), RV was detected most frequently [860 (60.3%)] followed by AdV in 719 (50.4%) and RSV in 578 (40.5%). Of the 51 patients with (H1N1)pdm09 co-infections, RV was detected in 19 (37.3%), followed by PIV-3 in 10 (19.6%). There were no co-infections with Influenza A (H1N1)pdm09 and H3N2 viruses. However there was 1 co-infection with Influenza B and H3N2 (Table 2). Of the 94 patients with Influenza A H3N2 co-infections, RV was detected in 38 (40.4%) followed by RSV in 19 (20.2%) and PIV 3 in 6 (6.4%).

Of the 1426 co-infections, the following combinations of viruses were detected most frequently in the same specimen: 356 cases of RV and AdV, 320 cases of RV and RSV, 212 cases of RSV and AdV and 142 cases of AdV and EV (Table 2, matrix).

Seasonality

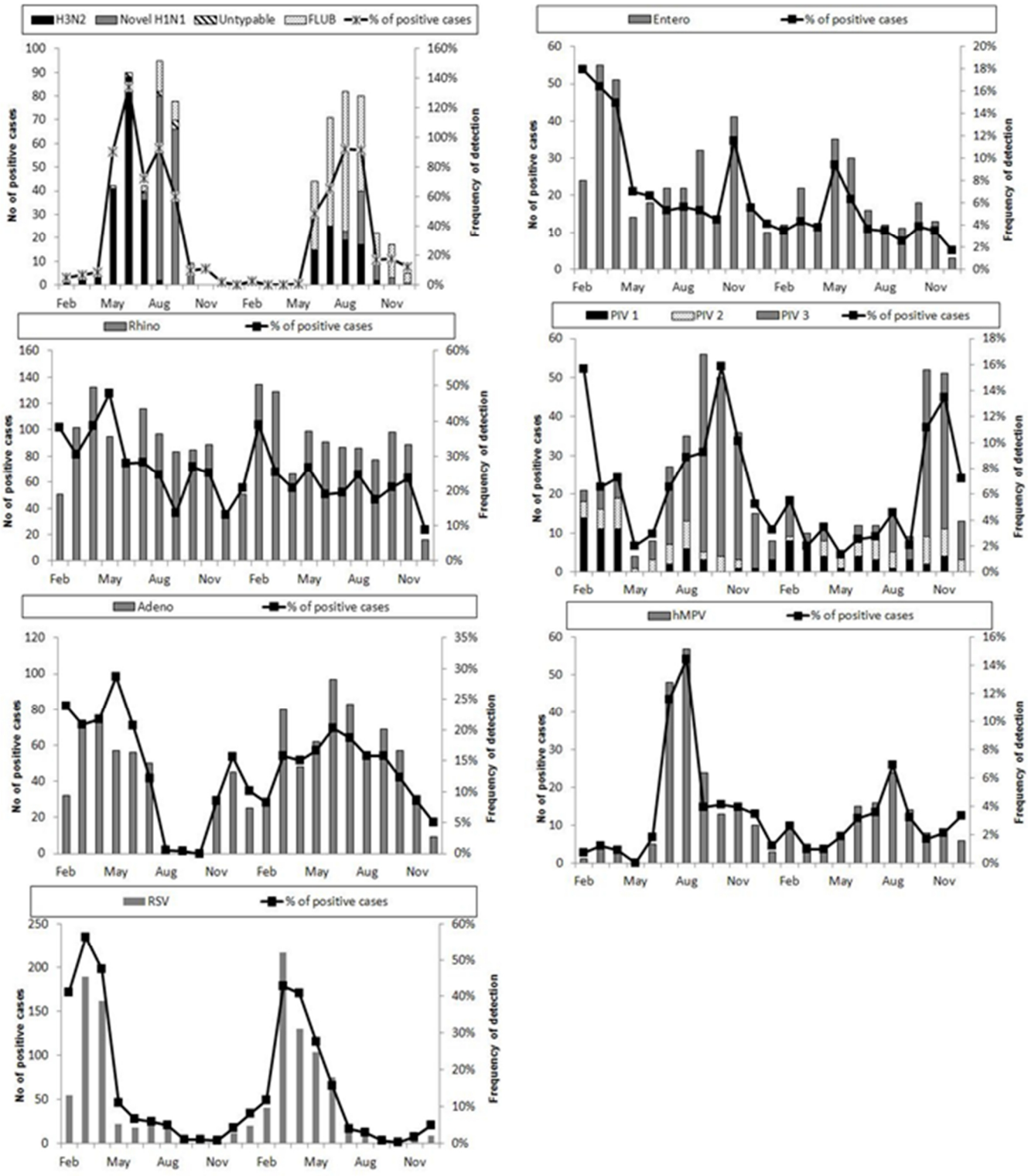
Seasonal patterns were visible for RSV, Influenza A and B, EV, hMPV and PIV3 in both 2009 and 2010. RSV occurred from February to June before the influenza season, which typically falls between May to September [20]. EV was detected throughout the year with peak activity between February and April and again between November and December. Peak activity for hMPV was observed between July and August and for PIV3 between September and November. Adenovirus and RV were detected throughout the two years without seasonal variability. PIV 1 and PIV 2 were detected sporadically throughout the two years (Figure 2).

The seasonal patterns of both Influenza A and B viruses during 2009 and 2010 were different. In 2009, Influenza A H3N2 and (H1N1)pdm09 occurred as two waves peaking between May to July and July to October, respectively, while Influenza B appeared briefly in August. During 2010 Influenza B season predominated from June to November, H3N2 circulated from June to September and (H1N1)pdm09 circulated between the last week of July and October.

Discussion

The validated assay was implemented for routine surveillance prior to the pandemic and enabled us to investigate the contribution of other respiratory viruses to SARI during the first two pandemic seasons. The assay also helped define the distribution and seasonality of these

Figure 2: Distribution of Respiratory Viruses detected during 2009 and 2010, showing specific seasonal trends and peak activity. (Please note that the scales on the y-axis differ for each graph.)



respiratory viruses in South Africa and the role of viral co-infections in hospitalized patients infected with (H1N1)pdm09 in South Africa during the 2009 and 2010 seasons. Using 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 IFA. The assay does not give any false positive results while the lower detection limit determined may give false negatives with specimens with very low viral load.

The majority of patients enrolled were infants less than 1 year of age, and the most commonly identified pathogens within this group were RSV and RV. While RV was detected throughout the year, RSV was detected in a distinct seasonal pattern with the peak months of detection occurring from February to June. Seasonal peaks were also identified for PIV3, hMPV and enterovirus. Year-round detection of Rhinovirus and Adenovirus make these two viruses the more likely to co-infect with all 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 two years prior to the 2009 pandemic in hospitals situated in South Africa, RSV was detected in a much higher rate in symptomatic infants with severe disease than in asymptomatic infants attending an immunization clinic in the same region[16]. Nevertheless, in a study conducted during 2006 and 2007 in South Africa, RV 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 for the potential of (H1N1)pdm09 reassorting with existing human influenza viruses giving 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

subtypes had co-circulated for overlapping periods both in 2009 and 2010, but peak months of detection was distinct for H3N2 and (H1N1)pdm09 in both years, while Influenza B was detected from June to November, overlapping with both H3N2 and pandemic H1N1 peaks. No seasonal 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 between 5–24 years of age with a detection rate of 5.1% [23].

Our study has some limitations. First, rRT-PCR assays are more sensitive for detecting respiratory viruses compared to viral culture, and with the increased detection of mixed viral infections, the clinical interpretation of positive PCR results have become more challenging. Although the viral nucleic acids detected here does 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 single and co-infections requires further investigation. Because of the low numbers of each specific co-infection combination, we did not report on clinical outcomes and how single and co-infections differed from each other clinically. This study also did not include bacterial testing and therefore gaps remain in our understanding of all the aetiologies of SARI in South Africa. Lastly, the study period of 2 years could also be a limitation since the circulation of viruses could change from year to year, however only two years of data is represented here, the surveillance study is on-going and changes in the seasonal circulation of the viruses will be detected. Understanding the contribution these viruses to severe respiratory disease will allow for informed decision making when selecting specific respiratory pathogens as part of sustainable respiratory disease surveillance. Currently the overall cost of

the assay from extraction to detection is USD 63, with a panel (consisting of two to three viruses) costing USD 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 co-infecting viruses in patients presenting with SARI, and highlights the important role of viral co-infection. Continued use of the rRT-PCR multiplex assay in conjunction with the SARI surveillance programme will enhance our ability to detect circulation of respiratory viruses in patients hospitalised for SARI and help to clarify the contribution of these respiratory viruses among patients with SARI in South Africa.

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