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ORIGINAL ARTICLE

Performance evaluation of two multiplex qualitative RT-PCR assays for detection of respiratory infection in paediatric population

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Abstract

Introduction: Acute respiratory infection (ARI) contributes to significant mortality and morbidity worldwide and is usually caused by a wide range of respiratory pathogens. This study aims to describe the performance of QIAstat-Dx® Respiratory Panel V2 (RP) and RespiFinder® 2SMART assays for respiratory pathogens detection. *Materials and Methods*: A total of 110 nasopharyngeal swabs (NPS) were collected from children aged one month to 12 years old who were admitted with ARI in UKMMC during a one-year period. The two qPCR assays were conducted in parallel. *Results*: Ninety-seven samples (88.2%) were positive by QIAstat-Dx RP and 86 (78.2%) by RespiFinder assay. The overall agreement on both assays was substantial (kappa value: 0.769) with excellent concordance rate of 96.95%. Using both assays, hRV/EV, INF A/H1N1 and RSV were the most common pathogens detected. Influenza A/H1N1 infection was significantly seen higher in older children (age group > 60 months old) (53.3%, p-value < 0.05). Meanwhile, RSV and hRV/EV infection were seen among below one-year-old children. Co-infections by two to four pathogens were detected in 17 (17.5%) samples by QIAstat-Dx RP and 12 (14%) samples by RespiFinder, mainly involving hRV/EV. Bacterial detection was observed only in 5 (4.5%) and 6 (5.4%) samples by QIAstat-Dx RP and RespiFinder, respectively, with *Mycoplasma pneumoniae* the most common detected. *Conclusion:* The overall performance of the two qPCR assays was comparable and showed excellent agreement. Both detected various clinically important respiratory pathogens in a single test with simultaneous multiple infection detection. The use of qPCR as a routine diagnostic test can improve diagnosis and management.

Keywords: Acute respiratory infection, multiplex real-time polymerase chain reaction, QIAstat-Dx, RespiFinder.

INTRODUCTION

Acute respiratory infections (ARI) have been among the significant cause of morbidity and mortality in young children worldwide.[1,2] Globally, estimated about 138 million ARI episodes with 0.9 million deaths occurred among children younger than 5 years old.[3] In Malaysia, pneumonia is one of the principal causes of death in children aged 0-14 years old, with an overall mortality rate of 4.8%.[4]

A variety of respiratory pathogens can cause ARI with respiratory viruses being the most common causing pathogens.[5] There are several new emerging respiratory viruses have been recognised including human rhinovirus (hRV), human bocavirus (hBoV), human metapneumovirus (hMPV), human coronavirus-NL63 and -HKU1 and they have important roles as respiratory viruses that cause severe pneumonia.[6] Most recently another novel coronavirus, severe acute respiratory syndrome coronavirus-2 (SARS-COV-2) has been newly identified to cause respiratory illness responsible for the COVID-19 pandemic.[7] In some cases, co-infections with two or more viruses or viruses and bacteria can occur.[8] In addition, atypical bacteria including *Mycoplasma pneumoniae and*

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Chlamydia pneumoniae are important respiratory pathogens causing ARI symptoms.[9]

The respiratory infections of these pathogens may have overlapping symptoms and difficult to distinguish bacterial from viral infections. This is usually the reason empiric antibiotics are often initiated based on disease severity, leading to over-prescription of antibiotics and additional diagnostic testing to exclude bacterial infection.[10] In March 2019, the World Health Organization launched the Global influenza strategy 2019-2030. One of the goals is to reduce the burden of seasonal influenza.[11] This is achieved by promoting research and innovation for improved and novel diagnostics, vaccines and treatments against influenza.

Respiratory viruses can be detected by virus cell culture, immunofluorescence (IF) staining, rapid antigen detection and molecular methods. The previous gold standard method for respiratory viral pathogen identification is the virus cell culture. However, the methods are time-consuming, labor-intensive, require specialised laboratory training, operator dependent, long turn-around time and are inefficient to detect co-infection. Only a limited range of viruses can be detected by them. It also requires specialised facilities in the laboratory. Rapid antigen detection methods, although can provide rapid results to physicians compared to virus cell culture and immunofluorescence (IF), has low sensitivity and specificity in comparison to molecular method.[12] The introduction of diagnostic molecular methods such as the real-time polymerase chain reaction (qPCR) has improved respiratory pathogen testing that cannot be detected by the conventional methods (e.g.: viral culture and IF) with more accurate and rapid results.[13] Therefore, conventional methods have been gradually replaced by molecular assays which are more sensitive and specific.

The rapid advancement of molecular methods has increased our ability to identify multiple respiratory pathogens in a single test with increased sensitivity and specificity.[13-16] It has been recognised to play an important role in diagnosing respiratory infections and has made laboratory diagnosis more efficient. Multiplex qPCR assays have shown their superiority in detecting a wide range of viruses and a high detection rate of respiratory viruses.[17,18] In a meta-analysis, molecular methods provide accurate results with a pooled sensitivity of 90.9% and a pooled specificity of 96.1%.[16]

In 2018, Infectious Diseases Society of America (IDSA) recommends using multiplex qPCR assays targeting respiratory pathogens in patients presenting with ARI.[19] Therefore, molecular based syndromic testing systems have become widely available and they can simultaneously detect up to 22 pathogens in a single tube.[20-24] This approach can simplify the laboratory workflow while improving sensitivity and reducing time to result. The syndromic respiratory panel approach contributed to prompt treatments and antimicrobial stewardship resulting in reduced antibiotic usage as well as admission duration.[25-27]

This study aimed to evaluate the performance of two qPCR assays, QIAstat-Dx® Respiratory Panel (RP) (QIAstat RP, Qiagen, Hilden, Germany) and RespiFinder 2SMART (PathoFinder) in detecting respiratory pathogens in respiratory samples received in University Kebangsaan Malaysia Medical Centre (UKMMC).

MATERIALS AND METHODS

Study design and population

This cross-sectional study was conducted from June 2019 and June 2020, involving children from one to 12 years old admitted with acute respiratory (ARI) in UKMMC. Repeated respiratory samples from the same patient during similar hospitalisation were excluded from the final analysis. To exclude nosocomial infection, patients who have been admitted into UKMMC for more than one week were also excluded. Demographic variables including age, gender and ethnic group were recorded from the laboratory information system (LIS) and medical record office. All potential subjects (including carers or parents) were briefed on the study and written informed consent was obtained by the paediatrician in-charge of the case. Ethical approval was obtained from the Research Ethics and Committee of National University of Malaysia (UKM) before starting the study.

Sample collection, transport and storage

A total of 110 nasopharyngeal swabs (NPS) were collected using flocked swabs (FLOQSwabs™ , Copan Brescia, Italy) placed in a universal transport medium (UTM, Copan Diagnostics) and transported to the microbiology laboratory in ice. All respiratory samples received were immediately tested by QIAstat-Dx RP and the residual NPS specimen in UTM was kept at 2 to 4°C (<48 hours) or frozen at -70°C until tested with the comparator qPCR assay (RespiFinder® 2SMART). The RespiFinder

assay was performed in multiple batches of respiratory samples. Samples that did not fulfil the requirements sample collection, transport and storage requirements were excluded.

Viral nucleic acid extraction

Nucleic acids were extracted from 200 µl of the UTM using the SpinStar™ Nucleic Acid Kit 1.0 according to the manufacturer's protocol and were eluted in a final volume of 60 µl as per the recommendation from RespiFinder.

Multiplex qPCR assays

QIAstat-Dx® Respiratory Panel V2 (RP)

QIAstat-Dx® Respiratory Panel V2 (RP) (Qiagen, Hilden Germany) is a new multiplexqPCR platform where all of the sample preparations including nucleic acid extraction, reverse transcription, PCR detection and analysis steps are performed automatically by the QIAstat-Dx® Analyzer 1.0. The QIAstat-Dx RP cartridge has two loading ports where it can be directly inoculated with a dry swab or with UTM. Internal control which is tittered MS2 bacteriophage was included in the cartridge. This Internal Control material verifies all steps of the analysis process. According to the manufacturer's instruction, 300 µL of UTM (liquid sample) containing nasopharyngeal swab (NPS) was transferred into the main port of the QIAstat-Dx RP cartridge using one of the provided transfer pipettes. The cartridge was then inserted into the analyser and starting the run. Turn-around time (TAT) is 69 minutes per sample. The QIAstat-Dx® Analyzer performs automated result analysis with each target reported as positive or negative and the results are displayed on the screen. Amplification curve and cycle threshold (Ct) values are provided for each target pathogen and internal control in the analyse data. Pathogens that can be detected by QIAstat-Dx RP as listed in Table 1. For influenza A assay QIAstat-Dx RP is designed to detect influenza A as well as Influenza A subtype H1N1/2009, Influenza A subtype H1 or Influenza A subtype H3.

RespiFinder® 2SMART

The RespiFinder® 2SMART assay (two-step Single tube Multiplex Amplification in Real Time) (PathoFinder BV, Maastricht, Netherlands) detects the 22 most common respiratory pathogens as listed in Table 1. The SmartFinder technology uses a specific probe which is ligated after hybridisation to their respective target gene. The assay starts with a pre-amplification step which combines reverse transcription with PCR amplification to amplify the target cDNA followed by a probe hybridisation, a probe ligation and a probe amplification step on a real-time PCR platform. This assay detects the amplified PCR product by melting curve analysis on Rotor-Gene Q (Qiagen, Hilden Germany). The data was generated and analysed on the FastFinder software version 3.5.5. The assay ran in less than 2.5 hours. A positive result is defined as the presence of pathogens detected from a respiratory sample.

Data collection and analysis

The information regarding socio-demographic data (age, race and gender) and clinical characteristics were obtained and recorded from patients' files. The results were analysed using SPSS IBM and Excel. The Cohen's Kappa statistics were calculated to measure the agreement between the results of the two multiplex PCR (<0 = poor, 0- 0.2 = slight, 0.21- 0.4 = fair, 0.41- 0.6 = moderate, 0.61- 0.8 = substantial, and 0.81- 1 = almost perfect) (Landis and Koch, 1977). Descriptive and categorical variables were summarised using frequency and percentage and analysed using Fisher Exact and Chi-Squared tests.

RESULTS

A total of 110 respiratory samples were collected and tested in this study. The majority of patients included were male (57.3%) and Malay (89.1%) with a median age of 14.5 months old. The age group between 7 to 12 months constituted the majority (28.2%) of the samples received. The summary of the demographic characteristics of patients is presented in Table 2.

Performance characteristics

Out of 110 samples tested, QIAstat-Dx RP and RespiFinder assays showed positive results in 97 (88.2%) and 86 (78.2%) samples respectively (Table 3). The overall positive detection rate was significantly higher in QIAstat-Dx RP than RespiFinder assay (88.2% vs 78.2%, p-value: 0.013) (Table 4). The overall level of agreement between QIAstat-Dx RP and RespiFinder assays was shown to be substantial (kappa value: 0.769) with an excellent concordance rate of 96.95%. The concordance rate for the individual target of respiratory pathogens was 100% for INF B, PIV-2, PIV-3, hMPV and *M. pneumoniae*. Concordance rate of more than ≥ 90% for RSV,

Table 1: Characteristic of multiplex qPCR assays

Assays	QIAstat-Dx RP	Respifinder
Target virus	Inf A Inf A H1 Inf A H3 Inf A H1N1/2009 Inf B RSV A/B hMPV A and B hRV/EV AdV hBoV PIV-1 PIV-2 PIV-3 PIV-4 hCoV-229E hCoV-HKU1 hCoV-NL63 hCoV-OC43 *Bordetella pertussis* *Legionella pneumophila* *Mycoplasma pneumoniae*	Inf A Inf A H1N1pdm09 Inf B RSV A RSV B hMPV hRV/EV AdV hBoV PIV-1 PIV-2 PIV-3 PIV-4 hCoV NL63/HKU1 hCoV NL63 hCoV OC43 *Bordetella pertussis* *Chlamydophila pneumoniae* *Mycoplasma pneumoniae* *Legionella pneumophila*
Technology	Real-time PCR	Melting curve analysis and real-time RT PCR
Samples per run	1	36 to 96
Automated result display	Yes	No
Method step	1	2
Turn-around time	69 minutes	2.5 hours (excluding extraction)

Abbreviations: AdV, adenovirus; hBoV, human bocavirus; hCoV, human coronavirus; hMPV, human metapneumovirus; inf, influenza; PIV, parainfluenza virus; hRV/EV, human rhinovirus/enterovirus; RSV, Respiratory syncytial virus.

INF A/H1N1, PIV-1, PIV-4, hBoV, AdV. Kappa values were excellent for all targets except for hRV/EV (0.454), hBoV (0.479), AdV (0.786) and PIV-4 (0.663). The moderate kappa value for hRV/EV and hBoV, whereas substantial kappa value for PIV-4 and AdV. The lowest agreement (kappa value: 0.454) was observed for hRV/EV. Performance characteristics for individual targets of respiratory pathogens for both assays are summarised in Table 5.

Discrepant results were found in three samples as presented in Table 6 and due to financial limitations, we were unable to do discrepant testing. Immunofluorescence (IF) test was done on those three samples but only RSV was detected in sample number 60. Thirty-four samples were identified as negative by RespiFinder but were detected positive by QIAstat-Dx RP for hRV/EV (n=23, Ct values: 24.8-37.9), RSV (n=6, Ct values: 19.1-26.1), AdV (n=3, Ct values: 20.9-34.3) and INF A (n=2, Ct values: 32.8-34.9). Regarding INF A virus detection, the two samples with pan-influenza A target detected by QIAstat-Dx RP were detected as subtypes INF A/H1N1 by RespiFinder. On the other hand, a total of 12 samples detected as positive by RespiFinder were identified as negative by QIAstat-Dx RP. Those samples were unable to be retested or proceed with discrepant testing by the third method.

Among the respiratory pathogens detected in both assays, hRV/EV, INF A/H1N1 and RSV are the three most common. The most frequent respiratory pathogen detected was hRV/EV (35.5 %) by QIAstat-Dx RP followed by INF A/H1N1 (21.8%), RSV (19.1), AdV (8.2), PIV-1 (6.4%), *M. pneumoniae* (4.5%), hMPV (3.6%), hBoV

Table 2: Characteristics of patients in the study

Characteristics	Frequency, n n = 110	Percentage (%)
Age, median (IQR), months.	14.5 (8-29)	-
0-6	20	18.2
7-12	31	28.2
13-24	26	23.6
25-60	18	16.4
> 60 months-12 years	15	13.6
Gender		
Female	47	42.7
Male	63	57.3
Race		
Malay	98	89.1
Chinese	6	5.5
Indian	2	1.8
Others	4	3.6
Comorbidities		
Asthma/reactive airway	19	17.3
Chronic lung disease	4	3.6
Congenital heart disease	7	6.4
Malignancy	1	0.9
Neurological disorder	3	2.7
Trisomy 21	2	1.8
Others	7	4.5
Premature birth	33	30
Clinical presentation;		
Symptoms duration, mean ± SD(days)	5.15 ± 3.34	-
Fever	98	89.1
Cough	101	91.8
Coryza	80	72.7
Rapid breathing	83	75.5
Chest recession	76	69.1
Wheezing	22	20
Vomiting	28	25.5
Diarrhoea	9	8.2
Seizure	6	5.5
Laboratory parameters,		
WBC, mean ± SD (normal range: 4-10 x10^9/L)	13.1 ± 5.7	-
Lymphocyte, mean ± SD (normal range: 20-40%)	36.0 ± 18.3	-
Neutrophils, mean ± SD (normal range: 40-80%)	53.6 (19.5)	-
CRP (mg/L), median (IQR) (normal range:<0.5mg/dl)	2.19 (0.27-2.82)	-
Treatment,		
Antibiotic	73	66.4
Oseltamivir	18	16.4
ICU admission	39	35.5
Mechanical ventilation	32	29.1
Death during hospitalisation	1	0.91
Length of hospital stay (days), median (IQR)	5 (3-8)	-

Abbreviations: IQR, interquartile; SD, standard deviation; CRP, C-reactive protein

(2.7%), IFV B (1.8%), PIV-3 (1.8%), INF A (1.8%), PIV-2 (0.9%) and PIV-4 (0.9%). Whereas for RespiFinder assay, the most common pathogen detected was INF A/H1N1 followed by hRV/EV (15.5%), RSV (13.6%), hBoV (8.2%), PIV-1 (7.3%), AdV (5.5%), *M. pneumoniae* (4.5%), hMPV (3.6%), IFV B (1.8%), PIV-3 (1.8%), PIV-4 (1.8%), PIV-2 (0.9%) and only one sample was positive for *L. pneumophila* (0.9%). Of all samples, QIAstat-Dx RP and RespiFinder only detect bacterial pathogens in 4.5% (n=5) and 5.4% (n=6) samples respectively with the most common bacteria being *M. pneumoniae*. No detection of coronaviruses, subtype INF A/H3, *Bordetella pertussis* and *Chlamydophila pneumoniae* during this study period. A summary of respiratory pathogens detection in both multiplex qPCR assays is shown in Table 3.

Co-infection

Overall, two or more co-detection were detected in 17.5% (17/97) and 14% (12/86) of the total positive samples by QIAstat-Dx RP and RespiFinder respectively. Dual detections were observed in 12.4% (12/97) samples, triple detection in 4.1% (4/97) samples and quadruple detection in only one sample by QIAstat-Dx RP assay. For RespiFinder assay, dual and triple detection were observed in 12.8% (11/86) and 1.2% (1/86) samples respectively. No quadruple detection by RespiFinder assay (Table 3). The most frequently detected pathogen in co-infections was hRV/EV by QIAstat-Dx RP (15.5%) and RespiFinder (6.9%) (Table 7). We found that hRV/EV was the most frequently identified in multiple infections and mostly was found concurrently with AdV (data not shown).

Table 3: Distribution of respiratory pathogens detected by each qPCR assays, n=110

Pathogens	QIAstat-Dx RP n (%)	RespiFinder n (%)
hRV/EV	39 (35.5)	17 (15.5)
RSV A/B	21 (19.1)	15 (13.6)
INF A	2 (1.8)	0 (0)
INF A/H1N1	24 (21.8)	27 (24.5)
INF B	2 (1.8)	2 (1.8)
PIV-1	7 (6.4)	8 (7.3)
PIV-2	1 (0.9)	1 (0.9)
PIV-3	2 (1.8)	2 (1.8)
PIV-4	1 (0.9)	2 (1.8)
hMPV	4 (3.6)	4 (3.6)
hBoV	3 (2.7)	9 (8.2)
AdV	9 (8.2)	6 (5.5)
M. pneumoniae	5 (4.5)	5 (4.5)
L. pneumophila	0 (0)	1 (0.9)
Single infection	80 (82.5)	74 (78.3)
2 pathogens	12 (12.4)	11 (12.8)
3 pathogens	4 (4.1)	1 (1.2)
4 pathogens	1 (1.0)	0 (0.0)
Total positive sample	97 (88.2)	86 (78.2)
Total negative sample	13 (11.8)	24 (21.8)

Abbreviations: AdV, adenovirus; hBoV, human bocavirus; hCoV; hMPV, human metapneumovirus; inf, influenza; PIV, parainfluenza virus; hRV/EV, human rhinovirus/enterovirus; RSV, respiratory syncytial virus; *M. pneumoniae, Mycoplasma pneumoniae; L pneumophila, Legionella pneumophila*.

Table 4. Positive detection rates between QIAstat-Dx RP and RespiFinder analysis, n=110

		No. (%) of RespiFinder result	
		Positive	**Negative**
No. (%) of QIAstat RP result	Positive	83 (75.5)	14 (12.7)
	Negative	3 (2.7)	10 (9.1)

p-value = 0.013 by McNemar's test

Clinical characteristic

The most frequent symptoms were cough (91.8%), fever (89.1%), rapid breathing (75.5%), coryza (72.7%), chest recession (69.1%), vomiting (25.5%), wheezing (20%), diarrhoea (8.2%) and seizures (5.5%). The average day of presentation was on day five of symptoms. The blood parameter showed raised total white cell count (mean 13.1 ± 5.7 x 10^9/L) with neutrophilic predominance. A total of 43 (39.1%) children had underlying comorbidity, which constituted asthma/reactive airway (17.3%), congenital heart disease (6.4%), neurological disorder (2.7%) and underlying trisomy 21 (1.8%). Other comorbidities including chronic hemolytic anaemia, Hirschsprung's disease, thalassaemia, hydroxymethylglutaryl-CoA (HMG-CoA) synthase deficiency, congenital hypothyroidism,

Table 5: Agreement between QIAstat-Dx RP and RespiFinder for each pathogen

Pathogens	**Sample results QIAstat RP/RespiFinder**				**Kappa value**	**Concordance rate (%)**
	+/+	**+/-**	**-/+**	**-/-**		
hRV/EV	16	23	1	70	0.454	78.18
RSV	15	6	0	89	0.802	94.55
INF A	0	2	0	108	NA*	98.18
INF A/H1N1	24	0	3	83	0.924	97.27
IFV B	2	0	0	108	1.000	100.00
PIV-1	7	0	1	102	0.929	99.09
PIV-2	1	0	0	109	1.000	100.00
PIV-3	2	0	0	108	1.000	100.00
PIV-4	1	0	1	108	0.663	99.09
hMPV	4	0	0	106	1.000	100.00
hBoV	3	0	6	101	0.479	94.55
AdV	6	3	0	101	0.786	97.27
M. pneumoniae	5	0	0	105	1.000	100.00
L. pneumophila	0	0	1	109	NA*	99.09
Total	86	34	13	1407	0.769	96.95

Abbreviations: NA, non-applicable; AdV, adenovirus; hBoV, human bocavirus; hCoV; hMPV, human metapneumovirus; inf, influenza; PIV, parainfluenza virus; hRV/EV, human rhinovirus/enterovirus; RSV, respiratory syncytial virus; *M. pneumoniae, Mycoplasma pneumoniae; L pneumophila, Legionella pneumophila*.
All concordance rates were above 78.18% and Kappa values were above 0.454.
*Unable to calculate kappa
Interpretations (Koch and Landis, 1977);
0.00 - 0.20 -> Slight
0.21 - 0.40 -> Fair
0.41 - 0.60 -> Moderate
0.61 - 0.80 -> Substantial
0.81 - 1.00 -> Almost perfect

Table 6: Discordant results between QIAstat-Dx RP and RespiFinder

Sample No.	QIAstat-Dx RP	RespiFinder	Clinical Diagnosis
25	hRV/EV (C_T 28)	PIV-4	Acute bronchiolitis
60	RSV (C_T 25.2)	hBoV	Bronchopneumonia
101	RSV (C_T 24.9) + hRV/EV (C_T 28.9)	PIV-1	Bronchopneumonia

Abbreviations: hRV/EV, human rhinovirus/enterovirus; RSV, respiratory syncytial virus; PIV, parainfluenza virus; hBoV, human bocavirus.

pelvic ureter junction obstruction constituted 4.5% of the patients. Thirty-nine patients (35.5%) were admitted to the intensive care unit (ICU) with 29.1% of them requiring mechanical ventilation and only one child died with the diagnosis of parainfluenza 1 virus infection. He was born prematurely with underlying complex cyanotic heart disease. Meanwhile, most of the patients were discharged home with an average length of hospitalisation of five days (median 3 to 8 days) (Table 2). Influenza A/H1N1 infection was significantly seen higher in older children (age group > 60 months old) for both QIAstat-Dx RP and RespiFinder assays (53.3%, p-value < 0.05). Meanwhile, we found that the majority of RSV and hRV/EV infections were among the younger patients, below one-year-old (Table 7).

DISCUSSION

The QIAstat-Dx RP and RespiFinder assays enable simultaneous testing for 21 respiratory pathogens and can be used to aid diagnostic testing for acute respiratory infection (ARI). The broad range of respiratory pathogen coverage resulted in a significant higher detection rate of pathogens by both assays. The high positivity rate in this study was quite comparable in other studies using other multiplex respiratory panels.[15,18,20,21,28,29]

In this study, we described the performance of QIAstat-Dx RP and RespiFinder assays by measuring their result agreement. In general, the performance between the two assays was excellent at 96.7% (kappa value 0.778) with more pathogens detected by QIAstat-Dx RP (88.2% vs 78.2%). The recently published reports on QIAstat-Dx RP performance were found to be similar to our findings.[23,24] Our study showed high concordant results (ranged between 94.55% and 100%, kappa value: >0.80) for most pathogens including RSV, IFV A/H1N1, IFV-B, PIV-1, PIV-2, PIV-3, hMPV, and *M. pneumoniae*. Although the almost perfect agreement was observed for IFV-B, PIV-1, PIV-2, PIV-3, hMPV and *M. pneumoniae*, only a small number of positive samples were detected with those viruses. Possibly due to low levels of those respiratory pathogens circulating during this study period. Therefore, further evaluation is required to establish the significance of these findings.

The least agreement between these two assays was observed for hRV/EV and hBoV (kappa value: 0.454 vs 0.479 respectively). The QIAstat-Dx RP detected 23 samples of positive hRV/EV which were not detected by RespiFinder (QIAstat-Dx RP+/RespiFinder-), presumably the additional detection of hRV/EV in the respiratory sample could be due to the increased sensitivity of QIAstat-Dx RP. It also could possibly be explained by new hRV/EV strains which may not be detected or below the detection limit of the RespiFinder. However, cycle threshold (C_T) values for hRV/EV were within the range of 26.6 to 37.9 (mean C_T value: 31) which may represent a significant amount of virus present. Considering that these were residual samples with moderate levels of viral load, the integrity of viral nucleic acid present in these samples may have been affected during the freeze-thawing cycle. This action may compromise the hRV/EV detection. Meanwhile, RespiFinder detected six samples with hBoV but were negative by QIAstat-Dx RP (QIAstat-Dx RP-/RespiFinder+). They are usually co-infection with other viruses. In these samples, it is difficult to determine the viral load level for hBoV because RespiFinder does not provide a C_T value to determine the relative level of the virus. In RespiFinder assay, the potential of carry-over contaminations of the PCR reaction may present due to additional manipulation steps during sample preparation (extraction) and amplicon products. Unlike QIAstat-Dx RP, samples are tested in batches using RespiFinder assay. The large number of samples tested simultaneously may result in cross-contamination between the samples. As a consequence, false positive results of hBoV may occur due to carry-over contaminants from aerosolisation, contaminated work area, pipette or gloves.

Table 7: Distribution of pathogens detected according to age group by each qPCR assays N=110

Pathogen	Overall frequency, n (%)		Co-detection occurrence rate†, n (%)		0-6 months N=20 n (%)		7-12 months N=31 n (%)		13-24 months N=26 n (%)		25-60 months N=18 n (%)		>60 months N=15 n (%)		p-value*	
	QIA	RespiF	QIA	RespiF	QIA	RespiF	QIA	RespiF	QIA	RespiF	QIA	RespiF	QIA	RespiF	QIA	RespiF
hRV/EV	39 (35.5)	17 (15.5)	15 (15.5)	6 (6.9)	10 (50)	3 (15.0)	8 (25.8)	6 (19.4)	11 (42.3)	4 (15.4)	5 (27.8)	1 (5.6)	5 (33.3)	3 (20.0)	0.388#	0.749
RSV A/B	21 (19.1)	15 (13.6)	3 (3.1)	0 (0.0)	7 (35.0)	5 (25.0)	8 (25.8)	6 (19.4)	4 (15.4)	2 (7.7)	1 (5.6)	1 (5.6)	1 (6.7)	1 (6.7)	0.105	0.310
INF A/H1N1	24 (21.8)	27 (24.5)	4 (4.1)	4 (4.6)	2 (10.0)	2 (10.0)	5 (16.1)	5 (16.1)	4 (15.4)	6 (23.1)	5 (27.8)	6 (33.3)	8 (53.3)	8 (53.3)	0.029	0.035
INF B	2 (1.8)	2 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.2)	1 (3.2)	0 (0.0)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	0 (0.0)	0.762	0.762
PIV-1	7 (6.4)	8 (7.3)	2 (2.1)	0 (0.0)	2 (10.0)	3 (15.0)	2 (6.5)	2 (6.5)	3 (11.5)	3 (11.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.516	0.323
PIV-2	1 (0.9)	1 (0.9)	1 (1.0)	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.7)	1 (6.7)	0.136	0.136
PIV-3	2 (1.8)	2 (1.8)	1 (1.0)	2 (2.3)	0 (0.0)	0 (0.0)	2 (6.5)	2 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.504	0.504
PIV-4	1 (0.9)	2 (1.8)	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	0 (0.0)	0.300	0.762
hMPV	4 (3.6)	4 (3.6)	0 (0.0)	0 (0.0)	1 (5.0)	1 (5.0)	2 (6.5)	2 (6.5)	0 (0.0)	0 (0.0)	1 (5.6)	1 (5.6)	0 (0.0)	0 (0.0)	0.693	0.693
hBoV	3 (2.7)	9 (8.2)	2 (2.1)	5 (5.8)	2 (10.0)	2 (10.0)	1 (3.2)	2 (6.5)	0 (0.0)	3 (11.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (13.3)	0.309	0.582
AdV	9 (8.2)	6 (5.5)	7 (7.2)	2 (2.3)	2 (10.0)	1 (5.0)	2 (6.5)	1 (3.2)	3 (11.5)	3 (11.5)	1 (5.6)	1 (5.6)	1 (6.7)	0 (0.0)	0.942	0.680
M. pneumoniae	5 (4.5)	5 (4.5)	4 (4.1)	3 (3.5)	1 (5.0)	1 (5.0)	1 (3.2)	1 (3.2)	3 (11.5)	3 (11.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.492	0.492
L. pneumophila	0 (0)	1 (0.9)	0 (0.0)	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)	1.000

Fisher Exact test used to compare age groups; #Chi-Squared test; p-value < 0.05 consider significant.†Occurrence detection rate: number of occurrence (n)/number of total positive samples x 100%

Abbreviations: AdV, adenovirus; hBoV, human bocavirus; hCoV, human coronavirus; hMPV, human metapneumovirus; inf, influenza; PIV, parainfluenza virus; hRV/EV, human rhinovirus/enterovirus; RSV, Respiratory syncytial virus; *M. pneumoniae, Mycoplasma pneumoniae; L pneumophila, Legionella pneumophila*; RespiF, RespiFinder; QIA, QIAstat-Dx RP.

Influenza viruses, RSV and hRV/EV were the common cause of ARI worldwide, which was also seen in our study population.[17,29] Respiratory syncytial virus (RSV) and hRV have been reported as the most detected virus associated with ARI in Malaysia.[30,31] We found that the majority of the RSV infection detected among younger children was consistent with previously published reports. Respiratory syncytial virus (RSV) usually causes ARI (e.g.: bronchiolitis) among children below 5 years old.[29,32] One third of our patients are with underlying comorbidities such as asthma, chronic lung disease, prematurity and congenital heart disease and they are more likely to have severe respiratory infections such as severe RSV bronchiolitis.[8] Asthma or reactive airway disease is also a well-known risk factor in children requiring hospitalisation for ARI.[33]

Only a small number of bacterial detections were found in this study, which were *M. pneumoniae* (n=5, 4.5%) and *L. pneumophila* (n=1, 0.9%). All the bacterial detection in this study was co-detected with the other viral pathogens. This finding was lower than the detection rate reported in another study.[34] It could be due to many reasons such as small sample size, timing of sampling and type of sample. *Mycoplasma pneumoniae* is an atypical bacteria and is usually in a lower respiratory tract than the upper respiratory tract. Therefore, the collection of NPS samples in this study might be suboptimal for *M. pneumoniae* detection. Cho *et al*. reported that samples from the lower respiratory tract such as sputum and bronchoalveolar lavage (BAL) have higher diagnostic yield for atypical bacteria.[35] In view of QIAstat-Dx RP has been CE-IVD cleared for NPS transported in UTM only, validation of other respiratory samples such as sputum, BAL and throat swab is necessary.

In this study, there were only a small number of co-infections detected by both assays. We found that hRV/EV has the highest co-infection rate which was comparable to previous studies.[23,28,36] There was evidence that human rhinovirus/ enterovirus is more than just a common cold and it has been identified as the most common pathogen in causing acute exacerbation of asthma, viral pneumonia and bronchiolitis among children.[37] Study by Nolan *et al*. showed that co-infection was quite common among children presenting with community-acquired pneumonia and it caused more serious disease than a single infection.[8] However, other studies did not find any association between disease severity with co-infection.[36,38] Therefore, multiple pathogens detection in this study may reflect the true co-infection or asymptomatic carrier due to prolonged virus shedding particularly in children.[39] Moreover, these assays cannot distinguish between primary pathogens or not. The role of co-infections in the ARI is unclear and the true causative agent is impossible to establish. In view of the low number of co-infections detected in this study, it is not possible to draw any conclusions and more studies are needed to find out the association of co-infection with disease severity.

The two assays in this study were using different technology. QIAstat-Dx RP assay offers a different workflow compared to RespiFinder. QIAstat-Dx RP is an automated system with minimal hands-on time and a shorter time to result (approximately 69 minutes), clearly suitable for point-of-care testing. Despite the short hands-on time, only one sample can be processed per run which is not suitable during an epidemic when many samples need to be processed simultaneously. QIAstat-Dx RP assay can involve direct dry swab processing or one pipetting step of UTM into the cartridge, which is easier and quicker. Carry-over contamination or error in pipetting can be avoided. Meanwhile, RespiFinder assay has higher sample throughput (up to 96 samples per run). The RespiFinder assay will fit routine daily laboratory testing when the procedure is run in batches.

The QIAstat-Dx RP assay allows visualisation of the amplification curve with C_T value and helps with the result interpretations. RespiFinder assay does not provide C_T values for each pathogen that it detects or the Internal control (IC) used, but an internal amplification control (IAC) is included to exclude PCR inhibition within RespiFinder assay.[40] Moreover, a viral load that represents by C_T value may be useful to predict the significance of the pathogen presence particularly in the six discordant samples, with hBoV detection by RespiFinder that were not detected by QIAstat-Dx RP (QIAstat-Dx RP - / RespiFinder +). In terms of target detection, both assays detect similar respiratory pathogens, except that the RespiFinder assay contains additional bacterial respiratory targets which is *C. pneumoniae* and is able to distinguish between RSV subtypes (RSV A and B).

STUDY LIMITATION

There are limitations to our study. Firstly, a small number of samples were included in this study and mainly consisted of children with a median

age of 14.5 months old. Additional studies are needed for the adult population. The low number of detections for a few respiratory pathogens may cause the performance of both assays for the particular pathogens to be inconclusive. Second, the three discrepant results were unable to proceed further with repeat testing or discrepant testing by a third method due to financial limitations. There are no precise reference methods available for each pathogen tested in this study and the comparator assay used may have variations in their sensitivity and specificity that may affect the accuracy of our data. Fourth, the RespiFinder assay was performed on frozen archived samples. The storage or freeze-thaw cycle could have led to nucleic acid degradation causing a false negative result in the RespiFinder assay. Lastly, the study was conducted on nasopharyngeal swab only. Other respiratory samples including throat swab, nasopharyngeal aspirate and bronchoalveolar lavage should be included in future studies.

CONCLUSION

The QIAstat-Dx RP and RespiFinder assays are the real-time qPCRs with syndromic testing systems which have standard panels consisting of common respiratory pathogens. The overall performance of the two assays was comparable with excellent agreement between each other. They offer minimal hands-on time, rapid turn-around time to results and more respiratory pathogens detection compared to conventional methods. In addition, both assays also can detect co-infections however the clinical significance needs to be further evaluated. Thus, the qPCR method which is highly sensitive and specific can be incorporated into the current diagnostic workflow for ARI. The use of qPCR as a routine diagnostic service in hospital laboratories can improve ARI diagnosis, infection control measures and patient care.

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REFERENCES

1. Liu L, Oza S, Hogan D, *et al*. Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. The Lancet. 2016; 388(10063):3027–35.
2. Wang X, Li Y, O'Brien KL, *et al*. Global burden of respiratory infections associated with seasonal influenza in children under 5 years in 2018: a systematic review and modelling study. Lancet Glob Health. 2020; 8(4):e497-e510.
3. McAllister DA, Liu L, Shi T, *et al*. Global, regional, and national estimates of pneumonia morbidity and mortality in children younger than 5 years between 2000 and 2015: a systematic analysis. Lancet Glob Health. 2019; 7(1):e47–e57.
4. Department of Statistics, Malaysia. (2019). Statistics On Causes Of Death. Putrajaya: Department of Statistics, Malaysia.
5. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes clinical symptoms, virology, and immunology. Clin Microbiol Rev. 2010; 23:73-98.
6. Dunn JJ, Miller MB. Emerging respiratory viruses other than influenza. Clin Lab Med. 2014; 34:409-30.
7. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. N Engl J Med. 2002; 382: 1199–207.
8. Nolan VG, Arnold SR, Bramley AM, et al. Etiology and impact of coinfections in children hospitalized with community-acquired pneumonia. J. Infect. Dis. 2018; 2:179-88.
9. Zhang X, Chen Z, Gu W, *et al*. Viral and bacterial co-infection in hospitalised children with refractory Mycoplasma pneumoniae pneumonia. Epidemiol Infect. 2018; 146(11):1384-8.
10. Havers FP, Hicks LA, Chung JR, *et al*. Outpatient Antibiotic Prescribing for Acute Respiratory Infections During Influenza Seasons. JAMA Netw Open. 2018; 1(2): e180243.
11. World Health Organization. Global influenza strategy 2019-2030. Geneva: World Health Organization. Available from https://apps.who.int/iris/bitstream/handle/10665/311184/9789241515320-eng.pdf?ua=1
12. Bruning AHL, Leeflang MMG, Vos JMBW, *et al*. Rapid Tests for Influenza, Respiratory Syncytial Virus, and Other Respiratory Viruses: A Systemic Review and Meta-analysis. Clin Infect Dis. 2017; 65(6):1026-32.
13. Mahony JB, Petrich A, Smieja M. Molecular diagnosis of respiratory virus infections. Crit Rev Clin Lab Sci. 2011; 48(5-6):217-49.

14. Anderson TP, Werno AM, Barrat K, Mahagamasekera P, Murdoch DR, Jennings, LC. Comparison of four multiplex PCR assays for the detection of viral pathogens in respiratory specimens. J Virol Meth. 2013; 191:118-21.
15. Huang HS, Tsai CL, Chang J, Hsu TC, Lin S, Lee CC. Multiplex PCR system for the rapid diagnosis of respiratory virus infection: systemic review and meta-analysis. Clin Microbiol Infect. 2018; 24(10):1055-63.
16. Vos LM, Bruning AHL, Reitsma JB, *et al*. Rapid molecular Tests for Influenza, Respiratory Syncytial Virus, and Other Respiratory Viruses: A Systemic Review of Diagnostic Accuracy and Clinical Impact Studies. Clin Infect Dis. 2019; 69: 1243–53.
17. Gharabaghi F, Hawanm A, Drewsm SJ, Richardson SE. Evaluation of multiple commercial molecular and conventional diagnostic assays for the detection of respiratory viruses in children. Clin Microbiol Infect. 2011; 17(12):1900-6.
18. Kuan CS, Yew SM, Hooi PS, Lee LM, Ng KP. Detection of respiratory viruses from ARTI patients by xTAG RVP Fast v2 Assay and conventional methods. Malays J Med Sci. 2017; 24(5):33- 43.
19. Uyeki TM, Bernstein HH, Bradley JS, *et al*. Clinical practice guidelines by the Infectious Diseases Society of America: 2018 update on diagnosis, treatment, chemoprophylaxis, and institutional outbreak management of seasonal influenza. Clin Infect Dis. 2019; 68: e1–47.
20. Raymaekers M, de Rijke B, Pauli I, Van den Abeele AM, Cartuyvels R. Timely diagnosis of respiratory tract infections: Evaluation of the performance of the Respifinder assay compared to the xTAG Respiratory Viral Panel assay. J Clin Virol. 2011; 52(4): 314-6.
21. Dabisch-Ruthe M, Vollmer T, Adams O, Knabbe C, Dreier J. Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG respiratory virus panel fast assay, RespiFinder 19 assay and RespiFInder SMART 22 assay. BMC Infect Dis. 2012; 12: 163.
22. Boers SA, Melchers WJG, Peters CJA, *et al*. Multicenter evaluation of QIAstat-Dx Respiratory Panel V2 for detection of viral and bacterial respiratory pathogens. J Clin Microbiol. 2020; 58: e01793-19.
23. Leber AL, Lisby JG, Hansen G, *et al*. Multicenter evaluation of the QIAstat-Dx Respiratory Panel for the Detection of Viruses and Bacteria in Nasopharyngeal Swab Specimens. J Clin Microbiol. 2020; 58: e00155-20.
24. Parčina M, Schneider UV, Visseaux B, Jozić R, Hannet I, Lisby JG. Multicenter evaluation of the QIAstat Respiratory Panel—A new rapid highly multiplexed PCR based assay for diagnosis of acute respiratory tract infections. PLoS One. 2020; 15(3): e0230183.
25. Rappo U, Schuetz AN, Jenkins SG, *et al*. Impact of early detection of respiratory viruses by multiplex PCR assay on clinical outcomes in adult patients. J Clin Microbiol; 2016, 54: 2096-103.
26. Lee BR, Hassan F, Jackson MA, Selvarangan R. Impact of multiplex molecular assay turn-around-time on antibiotic utilization and clinical management of hospitalized children with acute respiratory tract infections. J Clin Virol. 2019; 110: 11e6.
27. Kitano T, Nishikawa HM, Suzuki R, *et al*. The impact analysis of a multiplex PCR respiratory panel for hospitalized pediatric respiratory infections in Japan. J Infect Chemother. 2020; 26: 82- 5.
28. Beckmann C, Hirsch HH. Comparing Luminex NxTAG-Respiratory Pathogen Panel and RespiFinder-22 for Multiplex Detection of Respiratory Pathogens. J Med Virol. 2016; 88: 1319–24.
29. Appak Ö, Duman, M, Belet N, Sayiner A. Viral respiratory infections diagnosed by multiplex polymerase chain reaction in paediatric patients. J Med Virol. 2018; 91(5): 731-37.
30. Yew SM, Tan KL, Yeo SK, Ng K, Kuan CS. Molecular epidemiology of respiratory viruses among Malaysian Young children with a confirmed respiratory infection during 2014-2015. J Thorac Dis. 2019; 11(11): 4626-33.
31. Etemadi MR, Jalilian FA, Othman N, *et al*. Diversity of respiratory viruses detected among hospitalized children with acute lower respiratory tract infections at Hospital Serdang, Malaysia. J Virol Methods. 2019; 269: 1-6.
32. Jain S, William DJ, Arnold SR, *et al*. Community-acquired pneumonia requiring hospitalization among U.S children. N Engl J Med. 2015; 372: 835-45.
33. Centers for Disease Control and Prevention. Weekly U.S. influenza surveillance report (FluView). Available from: https://gis.cdc.gov/grasp/fluview/FluHospChars.html/.
34. Zhao MC, Li G, Zhang D, *et al*. Clinical evaluation of a new single-tube multiplex reverse transcription PCR assay for simultaneous detection of eleven respiratory viruses, Mycoplasma pneumoniae and Chlamydia in hospitalized children with acute respiratory infections. Diagn Microbiol Infect Dis. 2017; 88(2):115-9.
35. Cho MC, Kim H, An D, *et al*. Comparison of sputum and nasopharyngeal swab specimens for molecular diagnosis of Mycoplasma pneumoniae, Chlamydophila pneumoniae, and Legionella pneumophila. Ann Lab Med. 2012; 32(2): 133-8.
36. Cebey-López M, Herberg J, Pardo-Seco J, *et al*. Viral Co-Infections in Pediatric Patients Hospitalized with Lower Tract Acute Respiratory Infections. PLoS One. 2015; 10(9): 1-11
37. Iwane MK, Prill MM, Lu X, *et al*. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. J Infect Dis. 2011; 204: 1702–10
38. Asner SA, Science ME, Tran D, Smieja M, Merglen A, Mertz, D. Clinical disease severity of respiratory viral co-infection versus single viral infection: A systematic review and meta-analysis. PloS One. 2014; 9(6): e99392.
39. To KKW, Chan KH, Li I, *et al*. Viral load in patients infected with pandemic H1N1 2009 influenza A virus. J Med Virol. 2010; 82(1): 1–7.

40. Reijans M, Dingemans G, Klaassen CH, *et al*. RespiFinder a New Multiparameter Test To Differentially Identify Fifteen Respiratory Viruses. J Clin Microbiol. 2008; 46(4): 1232-40.