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Comparison of Anyplex II RV16 assay with conventional methods for detection of respiratory viruses

Kuan, C.S.[a], Hooi, P.S.[a], Lee, L.M.[a], Sam, I.C.[a] and Ng, K.P.[a*]
[a]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[*]Corresponding author e-mail: kpng@um.edu.my
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Abstract. Early detection of viral etiologies of acute respiratory tract infections of patients affects management and disease control in pediatric patients. In this study, the performance of Anyplex II RV16 assay (Seegene, Seoul, Korea) was evaluated by comparing with viral culture and direct immunofluorescence staining of clinical specimens for detection of respiratory viruses in patients. A total of 168 respiratory specimens were collected from 122 patients from November 2012 to May 2013 at the time of admission to the University of Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. The Anyplex II RV16 assay, viral culture, and direct immunofluorescence staining were positive in 74.4%, 18.5% and 14.9% of the specimens, respectively. HRV was the predominant virus detected by the Anyplex II RV16 assay. In 47 cases, two or more respiratory viruses were detected by the Anyplex II RV16 assay, which were missed by conventional methods. The performance of the Anyplex II RV16 assay was better than viral culture and direct immunofluorescence staining of clinical specimens for the detection of respiratory viruses. The implementation of the Anyplex II RV16 assay in hospital laboratories will provide rapid diagnosis of major viral infections of the respiratory tract.

INTRODUCTION

Acute respiratory tract infections (ARTIs) are a leading cause of morbidity and mortality in young children (Regamey *et al.*, 2008) and particularly dangerous for elderly adults as well as immunocompromised patients (Falsey & Walsh, 2000; Keller *et al.*, 2007). ARTIs are caused principally by viruses (Regamey *et al.*, 2008). ARTIs commonly occur in children between the ages of 2 and 24 months, with the highest incidence of infection occurring between the ages of 3 and 6 months (Bush & Thomson, 2007). Most of the ARTI patients are treated symptomatically as outpatients as viruses could not be easily detected due to the similarity in clinical presentation. A previous report showed that approximately 1.9 million children died from ARTIs worldwide, in which 70% of them is occurring in Africa and Southeast Asia (William *et al.*, 2002).

Respiratory viral diagnostics conventionally rely on the isolation of viral pathogens by viral culture and detection of viral antigens by direct immunofluorescence (IF). However, these conventional methods are not efficient for detection of co-infection caused by two or more respiratory viruses. Additionally, new respiratory viruses such as human metapneumovirus (HMPV), coronavirus (HCoV) HKU1, human bocavirus (HBoV) and influenza A (H1N1) were not able to be detected using viral isolation and direct IF (Khor *et al.*, 2012). Thus, the development of highly sensitive and specific multiplex RT-PCR assays is on the cusp of a paradigm shift to a new diagnostic test that provides cost-effective diagnosis while improves clinical management.

The Anyplex II RV16 assay is a qualitative multiplex molecular diagnostic test based on Tagging Oligonucleotide Cleavage and Extension (TOCE) technology (Seegene, Seoul, Korea). This advanced assay was developed to detect simultaneously 16 respiratory viruses, including respiratory syncytial virus (RSV) type A and B, influenza virus (IFV) type A and B, parainfluenza virus (PIV) types 1-4, adenovirus (AdV), human enterovirus (HEV), HBoV, HCoV OC43, 229E and NL63, HMPV and human rhinovirus (HRV). The aim of the study was to evaluate and compare the performance of the Anyplex II RV16 assay with the viral culture and direct IF for simultaneous detection of respiratory viruses in 168 specimens collected in Kuala Lumpur, Malaysia.

MATERIALS AND METHODS

Ethic statement

The authors did not interact with the donor respiratory specimens, nor did they have access to identify any information about the donor. Since our institute is a teaching hospital, the respiratory specimens were obtained as part of routine diagnostic tests in the virology laboratory and such cases are exempt from ethical approval (http://www.ummc.edu.my/view/content.php?ID=VGxSWlBRPT0=).

Subjects and respiratory specimens

From November 25, 2012 to May 2, 2013, a total of 168 respiratory specimens (nasopharyngeal secretions, sputum, tracheal secretions and bronchoalveolar lavages) were collected prospectively from 122 patients with ARTIs at the University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. All respiratory specimens were transported in 3.0 mL of viral transport medium (VTM) in a cold chain.

Direct IF and viral isolation

Direct IF and viral isolation were carried out as described by Khor *et al.* (2012). Common respiratory viruses (IFV type A and B, PIV type 1, 2, and 3, RSV, HMPV, and AdV) was performed using D^3 Ultra DFA Respiratory Virus Screening & ID Kit (Diagnostic Hybrids, Ohio, USA) according to the manufacturer's instructions. Viral isolation was performed by inoculating the respiratory specimens in VTM into into Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero), rhesus monkey kidney (LLC-MK2), human lung adenocarcinoma (A549) and human epithelial type 2 (HEp-2) cells, and incubated at 32ºC in a humidified atmosphere of 5% (v/v) CO_2 for 10 days. Cells showing cytopathic effects (CPE) were harvested and examined using direct IF. For HEV detection, the cells were tested by indirect IF assay using Light Diagnostics Enterovirus Screening Set Kit (Millipore, MA, USA) according to the manufacturer's instructions.

Anyplex II RV16 assay

The Anyplex II RV16 assay was performed as described previously (Huh *et al.*, 2014). Anyplex II RV16 sets A and B were used, according to the manufacturer's instruction. Briefly, the assay was conducted in a final volume of 20 µL containing 8 µL cDNA, 5 µL 4× RV primer, and 5 µL 4× master mix with the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) under the following conditions: 50ºC for 4 minutes for activation of the Uracil-DNA Glycosylase (UDG) system to prevent contamination, denaturation at 95ºC for 15 minutes, followed by 50 cycles of 95ºC for 30 seconds, 60ºC for 1 minute, and 72ºC for 30 seconds. Catcher melting-temperature analysis (CMTA) was conducted by cooling the reaction mixture to 55ºC, maintaining the mixture at 55ºC for 30 seconds and heating the mixture from 55ºC to 85ºC (5 seconds/0.5ºC) with continuous fluorescent monitoring.

RESULTS

In this study, a total of 168 respiratory specimens collected prospectively from patients in a range of 4 months to 62 years old. The children < 7 year old constituted the majority (86.9%) of the specimens received in this study (Table 1). The specimens were predominantly nasopharyngeal secretions (76.7%, 129/168), bronchoalveolar lavages

(17.0%, 30/168), sputum (3.0%, 5/168) and tracheal secretion (2.4%, 4/168) (Table 1).

A concomitant comparison between Anyplex II RV16, viral culture and direct IF staining of clinical specimens was made for simultaneous detection of seven respiratory viruses, including IFV type A and B, PIV types 1, 2 and 3, AdV, HMPV and RSV. The positive rate of the Anyplex II RV16 assay (74.4%) for detecting respiratory viruses was higher than viral isolation (18.5%) and direct IF (14.9%). Only 4 respiratory viruses, AdV (7.1%, 12/168), IFV type A (0.6%, 1/168), PIV type 3 (1.2%, 2/168) and RSV (1.2%, 2/168), were detected by all three assays (Table 2).

There were 19 samples that showed discrepant results between viral culture and direct IF staining of clinical specimens. Viral culture detected 11 positive specimens (4 AdV, 2 IFV type A, 2 PIV type 3, 2 RSV and 1 IFV type B) but these specimens were found negative by direct IF staining of clinical specimens. Conversely, direct IF staining identified respiratory viruses in 7 clinical specimens (4 RSV, 1 AdV, 1 IFV type A and 1 HMPV) that were negative by viral culture.

Table 1. The basic characteristics of patients from week 47, 2012 through week 18, 2013

Patient characteristics	Value
Number of patients	122
Number of specimens	168
Sex ratio (male : female)	1.7 (77 : 45)
Age [total number (percentage)]	
< 7 years old	106 (86.9%)
7-18 years old	12 (9.8%)
> 18 years old	1 (0.8%)
Type of specimens [total number (percentage)]	
Nasopharyngeal secretions	129 (76.7%)
Bronchoalveolar lavage	30 (17.9%)
Sputum	5 (3.0%)
Tracheal secretions	4 (2.4%)
Results [total number (percentage)]	
Number of viral culture positive specimens	31 (18.5%)
Number of direct IF positive specimens	25 (14.9%)
Number of Anyplex II RV16 assay positive specimens	125 (74.4%)

Table 2. Detection of four respiratory viruses by viral culture, direct IF staining of clinical specimens and Anyplex II RV16 assay

Target virus	viral culture	Direct IF staining of clinical specimens	Anyplex II RV16 assay	Total (%)
AdV	+	+	+	12 (7.1%)
IFV type A	+	+	+	1 (0.6%)
PIV type 3	+	+	+	2 (1.2%)
RSV	+	+	+	2 (1.2%)
Total				17 (10.1%)
No virus detected	–	–	–	43 (25.6%)

These culture-positive and direct IF-positive results were consistent with the Anyplex II RV16 assay. The Anyplex II RV16 assay detected 94 (56.0%) and 100 (60%) positive specimens among culture-negative and direct IF-negative specimens.

Table 3 demonstrates the overall distribution of respiratory viruses detected by the three methods. HRV (34.5%, 58/168), AdV (25.6%, 43/168), HBoV (9.5%, 16/168), HEV (7.7%, 13/168), and RSV (7.7%, 13/168) were the five predominant viruses detected by the Anyplex II RV16 assay (Table 5). Subtype A and B strains of RSV can be differentiated by the Anyplex II RV16 assay, but not by the conventional methods (Table 3). Additionally, Anyplex II RV16 assay detected additional respiratory viruses (PIV type 4, HRV, HBoV, HCoV OC43, 229E, and NL63) that cannot be detected by viral culture and direct IF staining of clinical specimens. No PIV type 1 and HCoV 229E was detected in our study. Multiple viral infections were detected in 47 (28%) patients, which were missed by viral culture and direct IF. Most of the mixed infection was caused by HRV and AdV (Table 3). Our result showed that PIV type 2, PIV type 4 and HCoV OC43 were found to be associated with other viruses in our patient samples (Table 5). All of the multiple respiratory viral infections were detected in children < 7 years old.

DISCUSSION

Early and rapid detection of viral respiratory pathogens is crucial with the advent of clinical management to reduce nosocomial transmission and unnecessary overuse of antibiotics (Woo *et al.*, 1997). Respiratory viruses can be detected by viral culture, direct immunofluorescent antigen staining, immunochromatographic (ICG) assay, RT-PCR, or real time PCR (Chan *et al.*, 2001; Liolios *et al.*, 2001; Falsey *et al.*, 2003; Cazacu *et al.*, 2004a; Cazacu *et al.*, 2004b; Fader, 2005; van de Pol *et al.*, 2006). In Malaysia, major respiratory viruses such as AdV, HEV, RSV, IFV, and PIV are most commonly identified by routine viral culture and IF methods. However, these detection methods are usually time-consuming with low sensitivity, and the results are often affected by specimen quality, specimen transport, and technical skill. Other tests such as ICG assay can provide very rapid viral identification, but it have the limitation of low sensitivity (Cazacu *et al.*, 2004a; Fader, 2005). Moreover, the previous study showed that the use of multiplex molecular assays can markedly reduce cost compared to those for conventional methods, including viral culture and direct IF staining (Mahony *et al.*, 2009).

The nucleic acid-based detection methods such as multiplex PCR-based assay are a useful alternative for viral pathogens detection. A previous study reported that the RT-PCR-enzyme hybridization assay was 100% sensitive and 93% specificity (Liolios *et al.*, 2001). However, these PCR methods often have an intrinsic limitation of a high rate of false positives (Bellau-Pujol *et al.*, 2005). The Anyplex II RV16 assay is a newly introduced multiplex real-time PCR assay using the dual priming oligonucleotides (DPO) and the melting curve analysis method of TOCE technology (Cho *et al.*, 2013; Kim *et al.*, 2013). The principal components of the Anyplex II RV16 assay are DPO primer pairs, "Pitcher" and "Catcher," which are important to accomplish a single signal generation in real time. The DPO system enables a broad range of annealing temperatures and affords high specificity to reduce false positive result (Kim *et al.*, 2013).

In our diagnostic laboratory, a viral culture requires up to 7-14 days of observation time for cytopathic effect. Also, IF assay used in our laboratory suffered a drawback of low sensitivity with a large number of false negative results. In this study, we compared the performance of the Anyplex II RV16 assay with conventional viral culture and direct IF staining of clinical specimens. The concordance rate between these three methods was 35.7% (60/168). In agreement with a previous study (Choi *et al.*, 2006), our results showed that the positive rate of the multiplex real-time PCR method for detecting major respiratory viruses was higher than viral culture and direct IF staining of clinical specimens. The Anyplex II RV16 assay detected all positive clinical specimens that

Table 3. Distribution of respiratory viruses identified by Anyplex II RV16 assay, viral culture and direct IF staining of clinical specimens

Target virus	Virus subtype	Total positive specimen number by Anyplex II RV16 (%)	Single virus	Two viruses	Three viruses	Four viruses	Five viruses	Total positive specimen number by viral culture (%)	Total positive specimen number by direct IF staining of clinical specimens (%)
AdV		43 (25.6%)	17 (10.1%)	12 (7.1%)	11 (6.5%)	1 (0.6%)	2 (1.2%)	15 (8.9%)	13 (7.7%)
HEV		13 (7.7%)	4 (2.4%)	6 (3.6%)	1 (0.6%)	1 (0.6%)	1 (0.6%)	4 (2.4%)	NA
RSV	A	6 (3.6%)	2 (1.2%)	2 (1.2%)	–	–	2 (1.2%)	4 (2.4%)[a]	6 (3.6%)[a]
	B	7 (4.2%)	3 (1.8%)	3 (1.8%)	1 (0.6%)	–	–	–	–
IFV	A	9 (5.3%)	5 (3.0%)	2 (1.2%)	1 (0.6%)	–	1 (0.6%)	3 (1.8%)	2 (1.2%)
	B	5 (3.0%)	4 (2.4%)	–	1 (0.6%)	–	–	1 (0.6%)	–
PIV	1	–	–	–	–	–	–	–	–
	2	2 (1.2%)	–	1 (0.6%)	–	–	1 (0.6%)	–	–
	3	13 (7.7%)	5 (3.0%)	6 (3.6%)	2 (1.2%)	–	–	4 (2.4%)	3 (1.8%)
	4	1 (0.6%)	–	1 (0.6%)	–	–	–	NA	NA
HBoV		16 (9.5%)	3 (1.8%)	5 (3.0%)	6 (3.6%)	–	2 (1.2%)	NA	NA
HCoV	OC43	6 (3.6%)	–	3 (1.8%)	2 (1.2%)	1 (0.6%)	–	NA	NA
	229E	–	–	–	–	–	–	NA	NA
	NL63	1 (0.6%)	1 (0.6%)	–	–	–	–	NA	NA
HMPV		11 (6.5%)	7 (4.2%)	4 (2.4%)	–	–	–	–	1 (0.6%)
HRV		58 (34.5%)	27 (16.1%)	21 (12.5%)	8 (4.8%)	1 (0.6%)	1 (0.6%)	NA	NA
Total			78 (46.4%)	33 (19.6%)	11 (6.5%)	1 (0.6%)	2 (1.2%)	31 (18.5%)	25 (14.9%)

[a] Viral culture does not differentiate RSV A from RSV B. The number of 4 means the counting of RSV irrespective of any RSV subtype.

were positive by viral culture and direct IF staining. The viability and low titer of the respiratory viruses in the specimens might be the reasons for the failure of the IF and culture methods to detect viruses in Anyplex II RV16-positive samples.

HRV and HCoV are the primary causal agents of upper respiratory tract infections. However, these two pathogens cannot be detected by the routine methods of viral culture and IF staining of clinical specimens. Thus, the ability of the Anyplex II RV16 assay to detect HRV and HCoV is an advantage for the differential diagnosis of lower respiratory tract infections. Kim and collaborators also reported that the Anyplex II RV16 assay had an improved sensitivity for HCoV detection (Kim *et al.*, 2009). HMPV was first discovered in 2001 (van den Hoogen *et al.*, 2001) and reported as the causal virus responsible for 5-7% of lower respiratory tract infection in pediatric patients (Al-Sonboli *et al.*, 2006; Cheng *et al.*, 2006; van de Pol *et al.*, 2006). This respiratory virus is difficult to be cultured in cells routinely used in diagnostic laboratories, such as Vero, A549, and MDCK cells (van den Hoogen *et al.*, 2001). In this study, HMPV was not isolated by viral culture. A total of 11 (6.5%, 11/168), including of 4 co-infection cases (2 HMPV/HRV and 2 HMPV/HCoV OC43) were detected by the Anyplex II RV16 assay. Among the 11 Anyplex II RV16-positive samples, only 1 positive sample was detected by direct IF of clinical samples.

Multiple respiratory viruses were detected in 47 (28.0%, 47/168) respiratory specimens in this study. As previously noted, most of the co-infections was related to HRV (32, 19.0%) and AdV (26, 15.5%) (Kim *et al.*, 2013). Noh *et al.* (2013) reported that HRV was the most frequently identified concurrently respiratory virus in influenza patients during the 2011-2012 season. In our study, HRV was the most commonly associated with AdV in pediatric patients. Bosch *et al.* (2013) indicated that HRV and AdV are less likely to be the single causal agent of respiratory infection in children. Our results revealed that greater number of HRV and AdV were involved in mixed infections. In contrast, when detected, IFV type A and B were mainly found to be the single infectious agent of ARTI. Two previous studies demonstrated that influenza co-infections are rare, and no influenza co-infections were detected by multiplex PCR in 2,273 clinical influenza samples (Palacios *et al.*, 2009; Chidlow *et al.*, 2010). However, Lam *et al.* (2013) showed that co-infection of influenza B and *Streptococcus pneumoniae* can cause severe pneumonia, septic shock, and acute kidney injury. Additionally, it is uncommon that more than 4 or 5 respiratory viruses were detected in 4 samples. These results were unlikely to represent false positive results because of the integrity of the non-target control in the assay. The clinical significance and the impact of the multiple respiratory viral infections on the disease severity have not been clearly determined in this study. However, a previous study demonstrated that the severity of clinical illness was not markedly different between patients with multiple viruses and patients with single virus infections (Noh *et al.*, 2013).

In this study, our results highlighted the superiority of the Anyplex II RV16 assay for the simultaneous identification of the common respiratory viruses. The Anyplex II RV16 assay is rapid and highly sensitive for routine respiratory disease diagnostic services. Although cost analysis study was not performed in this study, however, based on the previous report, the use of advanced molecular multiplex assay would make significant savings for hospitals when the number of days in viral isolation, the length of hospitalization, antibiotic usage as well as all other medical procedures are taking into consideration. Also, the Anyplex II RV16 assay can detect mixed infection. Thus, clinical use of Anyplex II RV16 assay will be a potential option in early diagnosis and treatment of ARTIs, especially when the clinical symptoms are taken into consideration. Other complementary efforts including traditional viral culture, IF, ICG assay or sequencing methods could be selectively used to detect respiratory viruses, in particular, cases according to the laboratory's needs and facilities.

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