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J Infect Chemother 23 (2017) 859–861

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Note

Evaluation of Anyplex™ II RV16 and RB5 real-time RT-PCR compared to Seeplex® RV15 OneStep ACE and PneumoBacter ACE for the simultaneous detection of upper respiratory pathogens

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ARTICLE INFO

Article history:
Received 15 May 2017
Received in revised form
19 July 2017
Accepted 26 July 2017
Available online 19 August 2017

Keywords:
Multiplex PCR
Upper respiratory infections
Molecular diagnostics

ABSTRACT

This prospective study was performed to evaluate and compare the performance of the multiplex PCR Seeplex® assays and Anyplex™ II assays. From May 2014 until April 2016, a total of 247 respiratory samples were collected in Okinawa, Japan. Multiple respiratory pathogens were detected in 37% of patients with positive results. The most prevalent pathogens were influenza A virus and respiratory syncytial virus B. Despite minor differences in capabilities, both the Seeplex® assays and Anyplex™ II assays can be easily implemented in diagnostic or research laboratories to optimize the detection and management of respiratory pathogen induced diseases.

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Diagnostic decisions for acute upper respiratory tract infections are increasingly relying on molecular testing methods. Multiple studies have shown the utility of multiplex PCR methods able to detect and differentiate up to 20 pathogens in one assay [1–7]. Additionally, early diagnosis of respiratory pathogens is known to be valuable to clinical management and improving patient outcomes by reducing complications, time spent in the hospital, antibiotic use, and unnecessary laboratory testing [8,9]. However, few reports evaluate and compare the utility of newly developed assays in Japan.

The aim of this study was to evaluate and compare the performance of the multiplex PCR Seeplex® assays and Anyplex™ II assays as diagnostic aides for a currently ongoing, larger, and more comprehensive study of upper respiratory infections in Okinawa, Japan. Samples were collected prospectively, in a non-selective manner, from patients within Okinawa, Japan. Between May 2014 and April 2016, a total of 247 respiratory samples (237 nasopharyngeal swabs, 7 sputum, and 3 bronchoalveolar lavage fluid samples) were collected from 216 unique patients with acute respiratory symptoms (e.g., cough, sputum) visiting or admitted to the University of the Ryukyus Hospital or other affiliated hospitals. All patient identifying information was removed to protect patient confidentiality. The Institutional Ethics Committee of the University of the Ryukyus approved this study (H28-3-14-644).

Upon receipt, patient samples were stored at 4 °C until processing. Nucleic acids were extracted from all samples within 72 h using Ribospin™ vRD (Geneall Biotechnology Co., LTD, Seoul, South Korea), according to manufacturer's instructions with a final elution volume of 100 μl. Samples were tested using the Seeplex® PneumoBacter ACE detection (V3.0) and Seeplex® RV15 OneStep ACE detection (V1.1), which are DPO™ (Dual Priming Oligonucleotide) -based multiplex assays that maximize PCR sensitivity and specificity through the inhibition of non-specific priming, as well as, Anyplex™ II RB5 detection, and Anyplex™ II RV16 detection (V1.1) kits (Seegene, Inc., Seoul, South Korea), which utilize a multiplex real-time PCR based on tagging oligonucleotide cleavage extension (TOCE™). TOCE™ technology is not affected by sequence variations observed among DNAs and guarantees consistent melting curve analysis by introducing two components, the "pitcher" and "catcher," to accomplish a unique signal generation in real-time, according to the included user manual (Seegene, Inc).

The pathogens detected by the Seeplex® kits include: *Streptococcus pneumoniae, Haemophilus influenzae, Bordetella pertussis, Legionella pneumophila, Mycoplasma pneumoniae, Chlamydophila*

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http://dx.doi.org/10.1016/j.jiac.2017.07.013
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pneumoniae, influenza A virus, influenza B virus, human respiratory syncytial virus A, human respiratory syncytial virus B, human adenovirus, human metapneumovirus, human coronavirus 229E/NL63, human coronavirus OC43, human parainfluenza virus 1, human parainfluenza virus 2, human parainfluenza virus 3, human parainfluenza virus 4, human rhinovirus A/B/C, human enterovirus, and human bocavirus 1/2/3/4. The Anyplex™ kits do not include primers for *S. pneumoniae* and *H. influenzae* but do test for *Bordetella parapertussis* and have distinct primers for human coronavirus 229E and human coronavirus NL63.

The PCR products from the Seeplex® assays were detected using microchip electrophoresis on the MCE-202 MultiNA System (Shimadzu Corp., Kyoto, Japan). To analyze the samples for viral infection using the Anyplex™ assays, cDNA was first synthesized from extracted nucleic acids using the cDNA Synthesis Premix included (Seegene, Inc.). Anyplex™ assays were analyzed in real-time using the CFX96™ Real-time PCR System (Bio-Rad Laboratories Inc., Berkley, CA., USA). All target amplification by PCR was performed in the presence of a positive and negative control, to ensure quality results.

Pathogens were detected in 62% (134/216) of patients; single pathogens were found in 63%, whereas multiple pathogens were found in a total of 37% patients with positive results. Forty-seven patients had multiple pathogens detected with Seeplex®, whereas fifteen patients had multiple pathogens detected with Anyplex™. Influenza A and respiratory syncytial virus B (RSV B) were the most commonly detected viral pathogens (33/247 and 42/247, respectively), while *S. pneumoniae* and *H. influenzae* were the most commonly detected bacteria pathogens (29/193 and 24/193, respectively). No samples tested positive for *B. parapertussis*, coronavirus 229E, human bocavirus 1/2/3/4 or parainfluenza virus 4 (PIV4).

Statistical analysis using inter-rater agreement between the two assays shows high agreement rates, with a range of 95.5%–100% agreement. Kappa values ranged from 0 to 1.00, as shown in Table 1. Because *S. pneumoniae*, *H. influenzae*, and coronavirus 229E/NL63 were only included in the Seeplex® assays and *B. parapertussis*, coronavirus 229E, coronavirus NL63 were only included in the Anyplex™ assays, Kappa scores could not be calculated. Pathogens without any positive results or without a Kappa score were excluded from the table. Nevertheless, a high degree of correlation was found between the two assays. Agreement was highest for *M. pneumoniae, B. pertussis*, metapneumovirus and respiratory syncytial virus A. Low Kappa values, indicating poor agreement (i.e., those calculated for adenovirus and human enterovirus) likely reflect the differences in the genetic targets used among the two assays. For example, in addition to the adenovirus types detected by Seeplex®, the Anyplex™ II kit also detects types 2, 4 and 5. Moreover, the Anyplex™ II kit detects multiple strains of both coxsackievirus and echovirus as human enterovirus, according to the included user manual (Seegene, Inc.)

Both of these assay methods should be considered for use in a diagnostic laboratory for the detection of respiratory pathogens. Although, the workload for each of these assays is lighter when compared to culture detection, some downfalls do exist. For instance, the Seeplex® assays require post-amplification detection after nucleic acid extraction and amplification. Furthermore, because of the post-amplification detection, Seeplex® results are subject to more interpretation, creating the potential for false positive results. In contrast, the Anyplex™ assays do not require post-amplification detection; therefore, runtime is reduced. Real-time detection also eliminates the manipulation of amplified products, which minimizes problems associated with amplicon contamination and carryover [10]. Still, Anyplex™ II kits are measured against an internal threshold set by the manufacturer, which could potentially create false negative results. However, despite these minor shortcomings, the sensitivities and specificities are comparable with other PCR detection kits [1,6,7,11,12], and remain high when evaluated against bacteria and viral culture [12,13]. Kim et al., specifically states that Anyplex™ and Seeplex® kits are superior to the Luminex xTAG respiratory viral panel because they are time saving and less labor-intensive [6].

There are some limitations to this investigation. First, we did not compare a large enough volume of each respiratory pathogen, excepting influenza A, RSV B and rhinovirus, to calculate Kappa scores with dependable confidence intervals. This limited sample size affects the power of this study, as inadequate numbers of positive results can limit the accuracy of the Kappa score. Second, patient background and symptomatology beyond acute respiratory symptoms, were not collected as a part of this study. As a result, the predictive values of these tests could not be calculated. Additionally, culture was not used to compare results or to generate sensitivity and specificity. However, many reports have previously shown that PCR detection of pathogens has greater sensitivity and specificity than culture [12,13]. Last of all, samples were not collected sequentially and therefore cannot represent local epidemiology.

In conclusion, both of these assay methods correlate well and provide a useful method for the detection of common respiratory pathogens. Additionally, the multiplex design of these assays allows for a broad range of etiologies to be investigated simultaneously.

Table 1
Correlational analysis for Seeplex® and Anyplex™ II assays.

	Results (Seeplex®/Anyplex™ assay)				Total Agreement	Kappa	95% CI
Bacterial Pathogens (n = 193)	+/+	+/−	−/+	−/−			
Legionella pneumophila	3	1	2	187	98.40%	0.66	(0.20, 0.87)
Mycoplasma pneumoniae	2	0	0	191	100.00%	1	(0.22, 1.00)
Chlamydophila pneumoniae	1	1	0	191	99.50%	0.67	(0.03, 0.67)
Bordetella pertussis	6	0	0	187	100.00%	1	(0.60, 1.00)
Viral Pathogens (n = 247)							
Adenovirus	0	0	7	240	97.00%	0	(0.00, 0.00)
Coronavirus OC43	3	1	0	243	99.60%	0.87	(0.28, 0.87)
Enterovirus	0	1	2	244	98.70%	0.13	(0.00, 0.63)
Influenza A	27	0	6	214	97.50%	0.88	(0.76, 0.88)
Influenza B	5	1	4	237	98.00%	0.68	(0.30, 0.79)
Metapneumovirus	8	0	0	239	100.00%	1	(0.68, 1.00)
Parainfluenza virus 1	0	3	1	243	98.40%	0	(0.00, 0.47)
Parainfluenza virus 2	3	1	1	242	99.10%	0.72	(0.24, 0.98)
Parainfluenza virus 3	1	6	1	239	97.10%	0.24	(0.00, 0.43)
Respiratory syncytial virus A	2	0	0	245	100.00%	1	(0.22, 1.00)
Respiratory syncytial virus B	31	9	2	205	95.50%	0.82	(0.69, 0.88)
Rhinovirus	18	3	3	223	97.60%	0.85	(0.65, 0.94)

CI, confidence interval.

Conflict of interest

None to declare.

Acknowledgements

This work was partially supported by research funds from EIDIA Co., Ltd. (Tokyo, Japan). The authors would like to thank EIDIA Co., Ltd., especially Toshihiko Fujikawa, for supplying the Anyplex™ II kits and consumables used in this study. The sponsor (EIDIA CO., Ltd.) had no involvement in the study design, data interpretation, or preparation of the manuscript.

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