

INTRODUCTION

Virology laboratories have historically used methods such as DFA and culture to diagnose six or seven respiratory virus infections, including Influenza A and B, Parainfluenza types 1 to 3, Respiratory Syncytial Virus (RSV), and Adenovirus. With traditional methods such as DFA and culture that use microscopy, result turn around times are restricted especially in laboratories handling large volumes of respiratory specimens. In recent times, molecular testing including nucleic acid amplification tests (NAAT) have been developed for a number of respiratory viruses (1). Results of carefully controlled laboratory studies employing DFA, culture and PCR have indicated that traditional methods of diagnosis by DFA and culture have suboptimal sensitivity (2). The emergence of five new respiratory viruses since the year 2001, including human Metapneumovirus, SARS Coronavirus, Avian Influenza H5N1 and two new Coronaviruses HKU1 and NL63 has presented new challenges for the clinical laboratory. The absence of commercially available tests for these viruses leaves laboratories without the ability to diagnose these important emerging virus infections. Therefore, there is a need for new and improved diagnostic tests with improved sensitivity to diagnose both traditional and emerging respiratory virus infections.

We have developed a multiplex PCR test called the Respiratory Viral Panel (RVP) Test that can detect 20 different respiratory viruses in a single test (3). This test has been commercialized and is currently being sold as an IUO test by Luminex Molecular Diagnostics (formerly TmBioscience Corporation) as the ID-Tag™ RVP Kit. The RVP test is a qualitative multiplex PCR test and is indicated for the detection of clinically relevant upper and lower respiratory viral infections in symptomatic adult and pediatric populations. In pre-clinical evaluations of the test we demonstrated that this test identifies up to 30% more patients that are infected with respiratory viruses (4). In addition the test detected dual respiratory virus infections in 5-8% of symptomatic adult and pediatric patients.

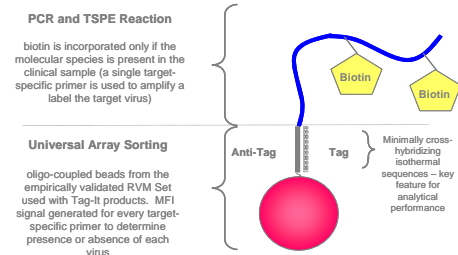
The objective of this study was to evaluate the ID-Tag™ RVP Test and compare its performance to the conventional DFA and culture tests for detecting respiratory viruses. This evaluation was part of a multi-center evaluation for a submission to the FDA for clearance as in vitro diagnostic device.

METHODS

- Specimens** – Nasopharyngeal (NP) specimens were collected under an ERB approval (St. Joseph's Healthcare) from 227 patients in Hamilton Ontario region in the winter of 2006. Specimens were divided into aliquots. One aliquot was processed in the routine virology laboratory for testing by DFA and shell vial culture (DHI) and a second aliquot was processed for ID-Tag™ RVP testing.
- DFA** – Direct fluorescent antibody staining of respiratory specimens was performed using standard methods. Briefly, cells were spun, washed, spotted in replicate and fixed onto microscope slides. Antigens were subsequently detected using FITC-labeled monoclonal antibodies (Diagnostic Hybrids Inc.) to the following viruses: Influenza A and B, Parainfluenza types 1 to 3, RSV, Metapneumovirus, and Adenovirus.
- Shell Vial Culture** – DFA negative specimens were tested by Shell Vial culture using commercially available R-Mix Shell Vials and monoclonal antibodies (Diagnostic Hybrids Inc.).
- Nucleic acid extraction**– Total nucleic acid (DNA+RNA) was extracted from an aliquot of cells (0.5 mL) using the Biomerieux MiniMag extraction kit and eluted in 50 uL elution buffer.
- ID-Tag™ RVP testing** – The ID-Tag™ RVP Test incorporates multiplex Reverse Transcription Polymerase Chain Reaction (RT-PCR) and multiplex Target Specific Primer Extension (TSPE) reactions with a proprietary Universal Tag sorting system (Luminex Molecular Diagnostics, formerly TmBioscience, Toronto) on the Luminex xMAP platform. The ID-Tag™ RVP Test was performed according to the manufacturer's protocol. Extracted nucleic acid (5 uL) was amplified by multiplex (16-mer) RT-PCR producing amplicons for each of the virus types/subtypes present in the specimen. The reaction products were treated with Shrimp Alkaline Phosphatase and Exonuclease I to inactivate remaining nucleotides and degrade left over primers. Multiplex TSPE was performed to detect viral DNA by elongation of hybridized primers and incorporation of biotin-dCTP. TSPE primers were chimeric by design and contained a "tag" oligo sequence that hybridizes to a complementary anti-tag oligo bound to 21 spectrofluorometrically-labeled microspheres (see figure). Following TSPE, the reaction was added to microwells containing bead-immobilized anti-tags complementary to the DNA tags on the primers (see figure). A fluorescent reporter molecule (streptavidin-phycoerythrin) is then bound to biotin-labeled TSPE products. Each tagged primer hybridizes only to its unique anti-tag complement; therefore, each colored bead represents a specific virus by virtue of the bead/anti-tag/tagged primer association. The beads are then analyzed on the Luminex 100 instrument containing two lasers; one laser identifies the color coded bead, and the other identifies the presence or absence of extended primer through the phycoerythrin reporter molecule. All viruses are identified in a single detection step and a summary report indicates which viruses are present in the sample. The entire assay takes about 5 hours to perform and can generate 20 virus results for each of 96 specimens on the plate.

- Discordant Testing**– All discordant DFA/culture and RVP specimens or RVP positives for targets not detected by DFA/culture, were tested by a second independent PCR with unique primers and the resulting amplicons were sequenced. The data from the second PCR was used to establish the true status of the specimen.

ID-Tag RVP Universal Array



RESULTS

A total of 227 NP specimens were tested by DFA plus culture and the ID-Tag™ RVP test. The ID-Tag™ RVP Test is configured with two positive controls to ensure proper performance of the test. *E. coli* MS 2 phage RNA is a positive control that is spiked into each specimen prior to extraction to ensure that extraction and reverse transcription has taken place. Failure to detect a positive signal for the MS 2 control indicates a failure at either the extraction step or the reverse transcription step and may indicate the presence of amplification inhibitors which could lead to a false negative result. A separate Bacteriophage Lambda DNA positive run control serves as a positive control for the PCR and TSPE steps.

Twenty-two of the 227 specimens either failed to give a signal for the internal control indicating extraction failure or were called equivocal for one or more targets and were therefore excluded from the analysis. The initial analysis was performed on results for the 8 viruses detected by both DFA/culture and RVP. Of the 205 specimens analysed, 179 gave concordant results: 113 were positive by both assays and 66 were negative by both assays. There were 26 discordant specimens including 22 RVP positive DFA/culture negative and 4 RVP negative DFA/culture positive. After resolution of the discordants using a second PCR, and using positivity by two or more tests as the Reference standard, DFA/culture had a sensitivity of 90.4% (113/125) and a specificity of 94.9% (75/79) while the RVP Test had a sensitivity of 100% (125/125) and specificity of 88.6% (70/79).

	DFA/Culture			Reference Std	
	+	-		+	-
RVP	113	22	113	4	
	4	66	12	75	
	117	88	125	79	
	Sensitivity 96.6% (113/117)		Sensitivity 90.4% (113/125)		
	Specificity 75.0% (66/88)		Specificity 94.9% (75/79)		

The ID-Tag™ RVP Test detected an additional 13 virus types or subtypes not detected by DFA/culture including Entero/Rhinovirus, OC43, 229E, NL63, HKU1, SARS-CoV, RSV type A, RSV type B, Parainfluenza type 4, and Influenza A subtypes H1, H3 and H5. The ID-Tag™ RVP Test detected an additional 26 respiratory virus infections that were not detected by DFA/culture. The additional infections included 22 Rhinovirus/Enteroviruses, 3 HKU1 Coronaviruses, and 1 NL63 Coronavirus. All of these additional positives were confirmed by a second PCR and sequencing. Analysing the data for all types of respiratory viruses detected by either DFA/culture or RVP, there were a total of 152 specimens that were positive for at least one respiratory virus. Setting the Reference Standard as positivity by two or more tests (DFA/culture, RVP and confirmatory PCR), the sensitivity of the ID-Tag™ RVP Test was 99.3% (151/152) compared to 74.3% (113/152) for DFA plus culture (the final call on a few specimens is still pending).

	Reference Std			Reference Std	
	+	-		+	-
RVP	125	9	151	10	
	0	70	1	42	
	125	79	152	52	
	Sensitivity 100% (125/125)		Sensitivity 99.3% (151/152)		
	Specificity 88.6% (70/79)		Specificity 80.8% (42/52)		

Some of the additional viruses detected by the RVP test were detected in positive specimens and therefore represented dual virus infections. In this small set of 227 patient specimens there were six (2.6%) dual infections. The six patients with dual respiratory virus infections had one of the following combinations: Influenza B plus HKU1 Coronavirus, Enterovirus plus Adenovirus, Enterovirus plus Influenza B, Rhinovirus plus RSV B, Metapneumovirus plus Influenza B, and RSV A plus Influenza B. Some of these combinations of respiratory viral infections have not previously been reported in the literature. In pre-clinical evaluations the ID-Tag™ RVP Test detected 5-8% dual infections and a few triple infections. The clinical significance of dual respiratory virus infections is currently being investigated.

CONCLUSIONS

- In a clinical evaluation of 227 nasopharyngeal specimens, the ID-Tag™ RVP Test was more sensitive than DFA plus culture for detecting eight conventional respiratory viruses (100% vs. 90.4%).
- The ID-Tag™ RVP Test detects additional respiratory viruses not routinely tested for by DFA/culture including human Metapneumovirus, Parainfluenza type 4, Rhinovirus/Enterovirus, and Coronaviruses OC43, NL63, HKU1 and identified Influenza A virus subtypes H1, H3, and H5.
- The ID-Tag™ RVP Test detected 34% additional viral infections (148 vs. 110) that were either missed by DFA/culture or not tested for by DFA/culture. Including the additional viruses not tested for by DFA/culture, the RVP test had an overall sensitivity of 99.3% for detecting any respiratory virus compared with 74.3% for DFA/culture.
- The ID-Tag™ RVP Test detected six dual respiratory virus infections not detected by DFA and culture. The clinical relevance of multiple respiratory tract infections in unknown and presently being evaluated.

DISCUSSION

The ID-Tag™ RVP Test detects SARS-CoV and the Asian lineage of Influenza A type H5N1 currently circulating in wild and domestic birds in South East Asia (data not shown) and could be used for national and global surveillance programs to track this potential pandemic strain of Influenza.

The ID-Tag™ RVP Test should allow hospital and public health laboratories to more accurately identify respiratory virus infections and to identify the causative agent in respiratory tract outbreaks in the community.

Since the test detects 95% of all known respiratory viruses (Bocavirus not currently detected), it can be used to establish the epidemiology of respiratory tract viral infections in various patient populations.

REFERENCES

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