

COMPARISON OF ELECTRONIC MICROARRAY TO ENZYME HYBRIDIZATION ASSAY FOR MULTIPLEX RT-PCR DETECTION OF COMMON RESPIRATORY VIRUSES IN CHILDREN.

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ABSTRACT

The NGEN RVA [Nanogen, Inc. San Diego, CA; Prodesse, Inc. Waukesha, WI] is a pair of analyte specific reagents that allow for the multiplex RT-PCR and electronic microarray detection of influenza (Flu) A and B, respiratory syncytial virus (RSV) A and B, and human parainfluenza virus (HPIV) types 1, 2, and 3. We evaluated these reagents as an assay in our laboratory in comparison with the Hexaplex (Prodesse), a multiplex RT-PCR-enzyme hybridization assay (EHA), and conventional viral cell culture (CC). Comparisons included the detection of respiratory viruses from whole virus stocks (ATCC) and from frozen pediatric respiratory specimens collected at Children's Hospital of Wisconsin between 1991 and October 1998. The whole virus stocks demonstrated analytical limits of detection (LOD) for the RVA of 10² (HPIV-2), 10³ (HPIV-1, Flu B, RSV A), 10⁹ (RSV B), 10¹ (Flu A), and 10² (HPIV-3) TCID₅₀/ml, respectively. The Hexaplex had better analytical LOD overall. A total of 420 respiratory specimens were evaluated. The agreement on positive samples, negatives samples and 95% confidence intervals (CI) between the RVA and Hexaplex assays were: Flu A 88% (72-97), 100% (99-100); Flu B 97% (82-100), 100% (99-100); RSV 91% (79-98), 100% (99-100); HPIV-1 100% (90-100), 100% (99-100); HPIV-2 69% (39-91), 100% (98-100); HPIV-3 82% (65-93), 100% (99-100), respectively. After the retesting of 6 indeterminates and 20 discrepant, overall agreement improved to 96% on the positives, and 100% on negatives, with only 8 specimens still discrepant. The RVA had a sensitivity of 84% (74-91) and a specificity of 100% (98-100) compared to prior CC results on 115 samples. The sensitivity increased to 96% if current Hexaplex negative samples are considered true negatives. Cost comparison demonstrated that the Hexaplex was favored in low volume use but the RVA used much less technician time. The RVA reagents allow for a rapid, sensitive, and specific assay for detecting 7 of the most common respiratory viruses in children.

INTRODUCTION

Many agents cause upper and lower respiratory infections (1-10) including a growing list of viruses. The seven most common viruses being: Flu A, B; RSV A, B; and HPIV 1, 2, 3 (8, 9). Human metapneumovirus (A, B), adenoviruses (51 serotypes, subtypes A-F), human coronaviruses (229E, OC43, NL63, and others), and rhinoviruses (>100 serotypes) also cause LRI in children and adults, but their exact proportion of illness is still being determined. Molecular detection of common respiratory viruses has been widely accepted as the "gold standard" in terms of sensitivity and specificity compared to CC and rapid antigen methods (4, 8). One of the barriers to acceptance of molecular tests has been their high costs both in terms of reagents and technician time. Multiplex RT-PCR-EHA detection of the seven most common respiratory viruses (Hexaplex®) has been used widely for accurate, fast, and cost effective diagnosis in hospitalized children, adults, and immunocompromised patients (8). Analyte specific reagents (ASRs) are reagents that laboratories can use to develop and validate their own assays. We used the NGEN RVA ASRs and an electronic microarray (NanoChip® 400) as an assay method to detect and differentiate the amplified products from the same primer sequences in the field tested multiplex RT-PCR primer mix used in the Hexaplex assay.

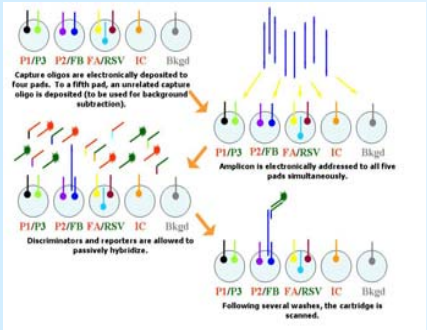
This microarray detection platform may offer advantages to mid and high throughput laboratories by decreasing technician time and overall assay time. We compared the RVA to EHA as detection methods for multiplex RT-PCR in the detection of Flu A, B, RSV A, B, and HPIV-1, 2, 3.

MATERIALS AND METHODS

212 respiratory specimens were thawed and split into two 400µl aliquots. RNA transcript controls were obtained from Prodesse. RNA extraction was carried out using the High Pure Viral Nucleic Acid Kit (Roche). Three µl of each duplicate sample was used in a standard RT reaction. The cDNA product of this reaction was split and used in two separate multiplex PCR reactions (10µl each). One reaction used 40µl of the Hexaplex Supermix for the assay while the other used 40µl of a similar RVA primer mix with the 3' primers lacking biotinylation. Following amplification, 50µl of material (amplified with Hexaplex Supermix) underwent post PCR purification using the QIAquick DNA purification kit (Qiagen). Following purification 5µl of amplified material was used in an EHA using 96 well neutravidin-coated microtiter plates. The optical density (OD) of each well was measured at 450 nm on a spectrophotometer. Following amplification, 9µl of the material (amplified with RVA primers) was diluted into 63µl of CAPdown Sample Buffer A and placed on the NanoChip 400 instrument; no post-PCR processing was required. The automated detection of the respiratory viruses was carried out on the NanoChip 400 in three distinct steps: Capture Oligonucleotide Addressing, Amplicon Addressing and Hybridization, and Reporting (Figure 1). Positive samples were determined by comparing the fluorescent signal corresponding to each virus to the signal obtained on a pad containing a nonspecific capture oligonucleotide. Frozen aliquots of specimens which were either positive by EHA and negative by RVA or positive by RVA but negative by EHA were thawed and retested starting from both the purified RNA sample and from the post amplification PCR product sample. The percentage agreement on positive samples ("sensitivity") and negative samples ("specificity") between the two assays and 95% confidence intervals (CI) were calculated using standard methods (35). Calculations were performed prior to discrepant analysis and after discrepant analysis.

RESULTS

Figure 1. Schematic layout for the detection of seven respiratory viruses on the NanoChip electronic microarray.



Circles represent pads on microarray. Straight blue lines represent amplicons. Dual colored lines represent discriminators and red/green lines with "cotton balls" represent reporter signals.

Table 1. Limits of Detection (LOD) of multiplex RT-PCR-EHA (Hex) and multiplex RT-PCR-electronic microarray (RVA) in serial 10 fold serial dilutions of ATCC whole virus (TCID₅₀). Positives are in bold.

TCID ₅₀ /ml	HPIV-1		HPIV-2		HPIV-3		Flu A		Flu B		RSV A		RSV B	
	Hex ^a	RVA ^a	Hex	RVA	Hex	RVA	Hex	RVA	Hex	RVA	Hex	RVA	Hex	RVA
	OD	SN	OD	SN	OD	SN	OD	SN	OD	SN	OD	SN	OD	SN
1 X 10 ⁴	4.000	43.9	4.000	34.5	4.000	32.7	4.000	52.6	4.000	32.5	N/A	N/A	4.000	22.2
1 X 10 ³	4.000	42.6	4.000	38.9	4.000	14.4	4.000	36.7	4.000	29.2	4.000	34.4	4.000	22.9
1 X 10 ²	4.000	40.1	4.000	33.1	3.073	4.1	3.241	8.7	4.000	26.4	4.000	35.9	3.231	27.1
1 X 10 ¹	4.000	34.8	3.773	33.5	0.526	2.0	2.686	2.6	4.000	27.1	4.000	48.9	1.672	24.8
1 X 10 ⁰	4.000	12.8	3.662	28.1	1.189	0.9	0.888	1.2	1.652	12.6	4.000	43.4	1.187	4.0
1 X 10 ⁻¹	2.782	4.4	1.496	10.8	1.849	0.8	0.047	1.3	0.072	5.8	4.000	2.6	0.793	0.8
1 X 10 ⁻²	1.428	1.4	1.928	6.2	0.046	1.0	0.052	1.1	0.069	0.9	2.099	1.2	0.506	1.1

^a For Hexaplex, an optical density reading < 0.300 is considered negative. ^b For RVA, a signal to background ratio < 2.3 is considered negative.

Table 2. Cost comparison of two "In-House" multiplex RT-PCR assays to detect 7 common respiratory viruses

Sample #		Manual extraction			Automated extraction		
		RVA	Hexaplex ^a	Hexaplex ^b	RVA	Hexaplex ^a	Hexaplex ^b
6 (8) ^c	Reagent costs	428	358	321	448	378	341
	Tech Time ^d (hrs)	1.8	2.4	2.4	1.0	1.45	1.45
	Linear Time (hrs)	5.8	6.5	6.5	4.75	4.95	4.95
	Total Cost/Sample ^e	59	52	48	59	52	47
14 (16)	Reagent costs	813	715	640	853	755	682
	Tech Time (hrs)	2.45	4	4	1.05	2.2	2.2
	Linear Time (hrs)	7.05	7.75	7.75	6.65	6.2	6.2
	Total Cost/Sample	55	51	46	55	51	46
30 (32)	Reagent costs	1583	1430	1210	1667	1510	1363
	Tech Time (hrs)	4.65	8	8	1.45	4.4	4.4
	Linear Time (hrs)	10.65	13.5	13.5	9.45	9.4	9.4
	Total Cost/Sample	53	51	44	53	51	46

^a Hexaplex with manufacturer's costs for all reagents and supplies. ^b Hexaplex with manufacturer's costs for the supermix and probes, all other costs are for reagents made in the laboratory. ^c The number in parenthesis includes controls. ^d Tech time at 525/hr. ^e Costs are in U.S. dollar.

Table 3a. Agreement between multiplex RT-PCR EHA (Hexaplex) and multiplex RT-PCR electronic microarray (RVA) in the detection of respiratory viruses in children.

	Sample #'s	Agreement % On positives	Agreement % On negatives	Ind. ^a	Dis. ^b
HPIV-1	14	100 (90-100) ^c	100 (98-100)		
HPIV-2	35	69 (39-100)	100 (99-100)	2	6
HPIV-3	34	82 (65-93)	100 (99-100)		6
Flu A	33	88 (72-97)	100 (99-100)	1	4
Flu B	29	97 (82-100)	100 (99-100)	1	1
RSV	44	91 (79-98)	100 (99-100)	2	3
TOTAL	189	90 (85-94)	100 (99-100)	6	20

3b. Agreement after the retesting of 20 discrepant samples.

	Sample #'s	Agreement % On positives	Agreement % On negatives	Ind. ^a	Dis. ^b
HPIV-1	34	100 (90-100) ^c	100 (99-100)		
HPIV-2	16	75 (48-93)	100 (99-100)		4
HPIV-3	34	100 (90-100)	100 (99-100)		
Flu A	34	97 (85-100)	100 (99-100)		
Flu B	30	93 (78-99)	100 (99-100)		2
RSV	45	98 (88-100)	100 (99-100)	1	1
TOTAL	193	96 (92-98)	100 (99-100)	1	8

^aInd.=indeterminates, ^bDis.=discrepant, ^c95%Confidence Interval.

Table 5. Comparison of RVA to cell culture^a in detecting respiratory viruses from pediatric patients.

	Isolate #'s	Sensitivity (95%CI) ^b	Specificity (95%CI)
HPIV-1	20	100 (83-100)	100 (96-100)
HPIV-2	15	40 ^c (16-68)	100 (96-100)
HPIV-3	4	100 (40-100)	100 (97-100)
Flu	16	100 (79-100)	100 (96-100)
RSV	24	83 ^c (63-95)	100 (96-100)
TOTAL	79	84 ^{d,e} (74-91)	100 (99-100)

^aIncludes two RSV results determined by DFA and two by EIA. ^bCI=confidence interval. ^c5 samples were negative and 1 falsely positive by Hexaplex. True sensitivity may be 67% (95%CI:30-93). ^dIncludes 4 samples negative by Hexaplex. True sensitivity may be 100% (95%CI:86-100).

CONCLUSIONS

- We compared two "in-house" assays that differed in their primer mixes (Hexaplex has 3' primer biotinylation, RVA does not), and their PCR product detection strategies.
- Both assays demonstrated excellent LODs against whole virus in analytical testing.
- Clinical sample testing demonstrated agreement ranging between 69-100% depending on virus and after discrepant analysis was 96% on positive samples and 100% on negative samples.
- The Hexaplex was less expensive for small sample numbers and had slightly better analytical and clinical sensitivity.
- The RVA assay offered increasing cost savings as the number of clinical samples increased or in situations with limited technician time.
- This automated detection assay demonstrated high level of agreement with the Hexaplex and cell culture.
- The RVA assay needs to be further optimized and validated in clinical situations and with fresh specimens to better establish its performance characteristics.
- Further improvements in its analytical LOD for HPIV-3 may be clinically useful in samples with low copy number like in elderly and immunocompromised patients.
- Until FDA-cleared point-of-care testing devices are available, we must utilize current technology (in-house developed tests and ASRs) for the greatest benefit of patients and society.

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