Direct Screening of Clinical Specimens for Multiple Respiratory Pathogens Using the Genaco Respiratory Panels 1 and 2

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Abstract: We report here on the results of a pilot study comparing our clinical diagnostic virology laboratory's current methods of respiratory pathogen detection against the Genaco Respiratory Infections Panels 1 and 2. These assays employ xMap (Luminex) liquid phase bead conjugated array technology to facilitate automated detection of PCR and RT-PCR products, which provides potential for levels of assay multiplexing above those currently practical with either conventional gel-resolved or real-time methods. In the study presented here we used the Genaco panels to simultaneously screen previously analyzed clinical specimens (nasopharyngeal washings) for twenty-one important pathogens. Our results indicate the Genaco panels met or exceeded our current methods' sensitivity and specificity although allowing for detection of a wider range of infectious agents than practical by current diagnostic laboratory practices. In addition, the Genaco panels provided data on the presence of multiple respiratory pathogens in single specimens, which would otherwise be missed in most instances. To our knowledge, this study represents the first trial of these panels on standard clinical specimens in a routine diagnostic setting.

Key Words: Luminex, xMap, Templex, respiratory infection

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Detection and classification of respiratory pathogens is of particular clinical importance due to the fact that pathogens with potentially high morbidity and mortality rates (and public health significance) may present with initial symptoms indistinguishable from other, less clinically serious pathogens. Currently available diagnostic methods include culture-based, antigen-based (FA or direct antigen detection), and molecular techniques. Of these, molecular assays have the potential for highest sensitivity, with assay turn-around times (TATs) on the order of a few hours and foreseeable capability to be run in high-throughput batch processes. A major weakness of molecular assays, however, has been that although an assay can be well optimized for a particular target (as a "Simplex" assay), combination of reagents to detect multiple pathogens in a single reaction (a "Multiplex" assay) generally results in loss of sensitivity for each of the individual target species. In addition, there are limits as to how many target species can be effectively detected out of a multiplex molecular test. For fluorescent-reporting real-time assays, physical limits are imposed by the requirement for nonoverlapping spectral ranges of each reporting dye; in most instruments this limits a multiplex assay to at most five targets. Gel-resolved endpoint analyzed molecular assays can avoid this problem, but require clearly size-resolved alternate target amplicons which inherently leads to differential target amplification efficiencies; effectively such assays rarely include more than 8 or 10 targets (see for example review by Markoulatos et al[1]). These suffer from the additional drawback of requiring more sample handling postassay, thereby increasing assay TATs and cost although hindering smooth adaptation to high-throughput applications.

Recently developed xMap technology in the form of various iterations of the Luminex instrument (including the BioRad BioPlex and Qiagen LiquiChip) provide a possible method to overcome both drawbacks of multiplex molecular assays.[2–7] Briefly, the device uses a set of 100 types of uniquely color-addressed fluorescent polystyrene beads which serve as a surface for coupling analytes (in this case, a set of oligonucleotide probes specific to possible target amplicons; each particular probe is coupled to a specific color bead). After a massively multiplex PCR reaction which generates fluorescently labeled products for any target templates present, hybridization to the bead mixture with liquid-phase kinetics occurs. The Luminex instrumentation then samples the reaction and classifies beads individually in a manner reminiscent of flow cytometry: each bead is analyzed for color (and thus analyte being assayed) and simultaneously analyzed for presence or absence of that analyte. Data presented herein suggests that the high specific fluorescence of product amplicons overcomes the loss in sensitivity common to multiplex analysis, although the singlicate analysis of each bead allows for effective discrimination between as many as 100 discrete analyte species per sample.

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Genaco (Genaco Biomedical Products, Inc, Huntsville AB) has recently developed two commercially available respiratory pathogen screening panels based on this approach. Panel 1, directed at DNA pathogens, screens for the presence of *M. pneumoniae*, *L. pneumophila*, *C. pneumoniae*, *N. meningitidis*, *S. pneumoniae*, *H. influenzae* (all strains), Adenovirus (types 3, 7, 21; and type 4, as two groups); and *A. baumannii*. Panel 2 is directed at RNA pathogens and screens for the presence of SARS, Influenza A, Influenza B, Respiratory syncytial virus (RSV) A, RSV B, Parainfluenza virus (PIV) 1, PIV 3, human Metapneumovirus (hMPV), Rhinoviruses, Coxsackie viruses, and Echoviruses.

We have conducted a brief trial of these reagent panels against a set of routine clinical nasopharyngeal wash (NPW) specimens which had been submitted for diagnosis, tested, and found to contain pathogens identified by our hospital laboratory VIRAP program[8] using DFA methods (SimulFluor; Chemicon International, Inc, Temecula CA) with the exceptions as outlined further below. We selected two clinical samples positive for each of the following pathogens: Influenza A, Influenza B, RSV A (classified as RSV by DFA, the further subclassified as non-B by an in-house real-time RT-PCR method), RSV-B (classified as RSV by DFA, the further subclassified as B by an in-house real-time RT-PCR method), PIV 1, PIV 2, PIV 3, hMPV (NPW specimens supplied by Laboratoire de Sante Publique du Quebec, and identified both by that laboratory and our laboratory as hMPV positive by RT-PCR methods), and Adenovirus. As the assay employed here does not screen for PIV 2, inclusion of these two specimens served a negative control function to examine possible cross-reactivity between related pathogens.

MATERIALS AND METHODS

Respiratory Infections 1 and Respiratory Infections 2 reagent panels were obtained from Genaco and used according to the supplied protocols. All primers and capture probes contained within these kits have been developed by Genaco and are proprietary.

All samples had been previously frozen at $-80^{\circ}C$; no samples had been previously thawed more than once. Samples were thawed, and 140 µl aliquots of each NPW were processed for nucleic acid extraction by Qiamp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany), eluted in 60 µl volumes. Ten microlitre aliquots of each eluate (plus two blanks, 10 µl sterile dH_2O each) were used as templates for 50 µl PCR reactions and 50 µl RT-PCR reactions using the HotStarTaq Master Mix Kit and OneStep RT-PCR Kit, respectively (both from Qiagen) according to the manufacturer's instructions. Thermocycling parameters used were those suggested by Genaco: for Respiratory Panel 1, this was (in sequence) 95°C, 15 minutes; 15 cycles of (94°C, 30 s; 52°C, 1 min; 72°C, 1 min); 6 cycles of (94°C, 15 s; 70°C, 1 min 30 s); 30 cycles of (94°C, 15 s; 52°C, 15 s; 72°C, 15 s); and finally 72°C, 3 minutes. Respiratory Panel 2 cycling conditions start with a reverse transcription step of 50°C, 30 minutes, and then proceed as per cycling conditions for Respiratory Panel 1. After thermocycling, 5 µl of reaction product was mixed with Res 1 or Res 2 bead set (for PCR or RT-PCR products, respectively) and hybridized at 52°C for 10 minutes. Ten microlitres of Streptavidin-PE in the supplied Detection Buffer was then added, followed by a further five minutes at 52°C and addition of 120 µl of Stop buffer (supplied). Reactions were kept at 52°C in the dark before analysis on a BioPlex xMap reader (BioRad Laboratories (Canada) Ltd, Missasauga, ON, Canada) at the default instrument settings. The BioPlex is a rebranded Luminex 100 instrument with changes in the fluorescence detector gain settings (as described further below and in Results) and with additional calibration and data analysis software.

Genaco recommends a positivity cut-off value of 250 mean fluorescence intensity (MFI) for this assay when run on a Luminex 100 instrument. As our version of the device (a Bio-Plex) uses higher excitation laser and detector gain settings than the Luminex 100, comparison of our signal values with examples from the Genaco literature[9] was performed (see Results). The Bio-Plex instrument reported an approximately five-fold higher average signal intensity for both blank and positive specimens. Based on this we have chosen a five-fold increase in cut-off value to 1250 MFI for this study.

RESULTS

Results from this study are shown in Table 1 (results from Genaco Panel 1) and Table 2 (results from Genaco Panel 2). Our initial analysis of this data included selection of a signal cut-off criteria for these assays as run on the Bio-Plex instrument. To determine this, we observed the first 24 "below cutoff" values reported by Genaco[9] for the Respiratory Infections 2 panel as run on a Luminex 100. Values ranged from 7 to 88 MFI, with an average of 18.9 MFI and a standard deviation of 16 MFI. We compared these values with 24 presumed negative signals from our Panel 2 results (see Table 2; all 20 results from the SARS 1 reporter, and the first 4 results from the SARS 2 reporter). The values for these 24 specimens range from 41.5 to 110 MFI, with an average of 79.7 MFI and a standard deviation of 20 MFI. Our apparent increase in background signal intensity was therefore (79.7/18.9) or 4.2 fold. We then examined Genaco's reported above-cutoff (positive) signals for Influenza A, Influenza B, RSV A, RSV B, PIV 1, and hMPV as determined on a Luminex 100[9]. For each of these pathogens, we then compared the value we observed on the Bio-Plex for that pathogen-specific assay in our duplicate samples known to be positive for that pathogen (see Table 2). These pathogen-specific known positive samples gave signal intensities raging from 2.4 fold higher than Genaco's reported Luminex 100 results (RSV B) to 8.3 fold higher (for hMPV). Across these 6 pathogens and 12 data points from our results, the average positive signal intensity increase of the Bio-Plex data compared

TABLE 1. Results on Specimens Tested with Genaco Respiratory Panel 1

Sample #	VIRAP ID	A. bau	M. pneumo	C. pneumo	L. pneumo	S. pneumo	Meningo	H. flu	Ad 3, 7, 21	Ad 4
1	Inf A	122.5	50	62	46	**22,693**	93.5	339	73	60.5
2	Inf A	202.5	128	60.5	103	121.5	108.5	413	69	82
3	Inf B	180	88	102	112.5	143.5	109.5	**6509**	57.5	98.5
4	Inf B	167	119	115.5	119.5	111.5	115	408	108	120
5	PIV 1	195	139	120	126	**20,227**	97	410	108	76
6	PIV 1	129.5	117.5	80	67	128	113	446	151	85.5
7	PIV 2	157	114	103.5	102	118	105.5	570	97	116.5
8	PIV 2	201	157	91.5	122	150.5	104	589	140	99.5
9	PIV 3	155	69	80.5	116	**6939**	99	549	134	87.5
10	PIV 3	210	126	129	125	112	92	**12,685**	480	156
11	Adenovirus	159	90	85	126	131	106	541	**14,736.5**	105
12	Adenovirus	160	119	91	139	107	80	450.5	**17,039**	77.5
13	RSV A	159.5	100	86	128	108	96	479.5	133	107.5
14	RSV A	120	101	131.5	43	385.5	129.5	922	118	55
15	RSV B	193	111	119	109.5	162	115	440	103.5	151.5
16	RSV B	131	50.5	151	113	119	101	**1619.5**	91	75
17	hMPV	163	80	104	109	161.5	115	362.5	86.5	65.5
18	hMPV	128.5	113	89	89	601	62.5	**17,328.5**	94	70
19	blank	126	78.5	73	47.5	74.5	85	407	71	59
20	blank	110.5	49	80	73	81	53	299.5	63.5	86

Values represent unmodified fluorescence intensity values as reported by the Bio-Plex instrument. Individual samples are in single rows; "Sample" indicates the pathogen previously detected in each specimen, as described in detail within the main text. Numbers in bold face indicate a positive score as described in the main text. Each column represents particular pathogen-specific signals with pathogen names abbreviated as follows: A. bau (*A. baumannii*); M. pneumo (*M. pneumoniae*); C. pneumo (*C. pneumoniae*); L. pneumo (*L. pneumophila*); S. pneumo (*S. pneumoniae*); Meningo (*N. meningitidis*); H. flu (*H. influenzae*); Ad 3, 7, 21 (Adenovirus serogroups 3, 7, and 21); Ad 4 (Adenovirus serogroup 4).

with the Luminex 100 data was 5.6 fold. Based on both these negative and positive sample results, we chose a signal cut-off level of 5 times that recommended by Genaco for the Luminex 100, or 1250 MFI. Similar results were observed by equivalent examination of the Panel 1 data (not shown) and this cut-off value was applied to both panels. Examination of the very high signal to noise ratios observed (see Tables 1 and 2) there seems to be little likelihood this cutoff will result in false negative results.

TABLE 2. Results on Specimens Tested with Genaco Respiratory Panel 2

Sample #	VIRAP ID	SARS 1	SARS 2	SARS 4	Flu A	Flu B	PIV 1	PIV 3	RSV-A	RSV-B	hMPV	C&E	Rhino
1	Inf A	65	63.5	76	**17,917**	495	103	305	111	10,959	44.5	104	81
2	Inf A	41.5	68.5	113	**20,366.5**	695	67.5	417.5	54	105	87.5	89.5	**2067**
3	Inf B	63	84	61	200.5	**22,933**	60	103	86.5	105.5	117	52	61
4	Inf B	79	59	43	175	**21,717.5**	41.5	80	83	86	59	59	61
5	PIV 1	87	52	69.5	322	830	**22,276**	**1722**	76.5	85.5	63.5	**1266.5**	159.5
6	PIV 1	70.5	69.5	94	279.5	347	**21,778.5**	367.5	64	93	69	102	71
7	PIV 2	84	74.5	32.5	293.5	143	76.5	153	41	107.5	67	50	97
8	PIV 2	68.5	45.5	67	274	143	80	148	65.5	121	59	73	70
9	PIV 3	68	61	91.5	**1,3894**	251.5	37.5	234.5	72	80	46	555	121.5
10	PIV 3	63	91	87	278.5	169	**5896**	**19,770**	77.5	99	88.5	834	271
11	Adenovirus	77	37.5	30.5	252.5	161	52.5	183	73.5	55	46	74	83
12	Adenovirus	95	11	63	319.5	182	42.5	249.5	33	103	67	941	215
13	RSV A	81	64.5	85	272	102.5	65	247	**15,567**	92.5	95	43.5	93
14	RSV A	138	64	66	254.5	69	54	198.5	**20,227**	73.5	55.5	65	65
15	RSV B	103.5	125	89.5	247	193	78	237	149	**7564.5**	93	642	112
16	RSV B	91	40	104	252.5	180	82	229	265.5	**16,324**	54.5	410	441.5
17	hMPV	110	102	76	196	187.5	43	276	53	129	**20,088**	295	85
18	hMPV	81	116	48.5	251	174	39	252	60.5	104	**15,325**	77.5	67.5
19	blank	71	40	95	221	174	50	320.5	65.5	66	44.5	43.5	93
20	Blank	105	69.5	63	270	323.5	27	631	27.5	147	67	55	50

Values represent unmodified fluorescence intensity values as reported by the Bio-Plex instrument. Individual samples are in single rows; "Sample" indicates the pathogen previously detected in each specimen, as described in detail within the main text. Numbers in bold face indicate a positive score as described in the main text. Each column represents particular pathogen-specific signals with pathogen names abbreviated as follows: SARS 1, 2, 4 (SARS Coronavirus Types 1, 2, and 4, respectively); Flu A, Flu B (Influenza A and Influenza B); PIV 1, 3 (Parainfluenza viruses 1 and 3); RSV-A, -B (Respiratory syncytial virus type A and type B); hMPV (human Metapneumovirus); C&E (Coxsackie and Echoviruses); Rhino (Rhinovirus).

DISCUSSION

Respiratory Panels 1 and 2 as developed and marketed by Genaco represent a novel approach to solving the intrinsic difficulties in highly multiplex (RT-) PCR analysis through application of liquid phase bead-based array (Luminex) technology. Independent initial studies on purified positive control specimens indicate that the method has sensitivity levels ranging from ~80 to 100 genome copies/assay (eg, for human Metapneumovirus, Influenza B, Enterovirus; comparable sensitivity to real-time simplex PCR) through ~2000 genome copies/assay (Influenza A; roughly 1/10 to 1/100 the sensitivity of simplex real-time PCR.[10] To our knowledge this represents the first report of the application of this technology to real clinical specimens. Although we have not in this context ascertained the analytical sensitivity of the individual pathogen-specific assays, our results clearly demonstrate good clinical sensitivity for the pathogens examined in as much as we observed no false negative results.

With the exception of only one specimen (sample 9, originally scored as PIV 3 by DFA but reported as Influenza A and *S. pneumoniae* by the Genaco assay) there was complete concordance between the Genaco assay results and those originally obtained by our diagnostic virology laboratory. We have reexamined this one discrepant specimen with the Xpect Influenza direct antigen test (Remel Inc, Lenexa, KS), where a weak positive Influenza A signal was confirmed suggesting that the Genaco assay results were correct and our original DFA diagnosis was in error on this specimen. In the case of Specimen 5, there is a weak PIV 3 signal (1722 MFI) in the presence of a very strong PIV 1 signal (22 276 MFI); given the close similarity between these pathogens we suspect this may be due to cross hybridization. As the other PIV 1 specimen (Specimen 6) had a similarly high PIV 1 signal (21 778.5 MFI) without appreciable PIV 3 signal (367.5 MFI) this would not seem to occur in all cases and may depend on minor sequence variations in PIV 1 strains. Based on this we would caution that for this particular pathogen pair, appearance of a weak positive PIV 3 signal in the presence of a strong PIV 1 signal should not be taken as definitive evidence for coinfection. Examination of a larger PIV 1 sample size should allow for development of a more rigorous guideline in these cases, ie PIV 3 signals of "N-fold" less than a concurrent PIV 1 signal should be discounted. Overall, we thus believe the Genaco assay to have shown 100% correlation with our prior DFA and/or molecular diagnoses for the pathogens examined in this study. We found no other evidence for false positives with the 1250 MFI cutoff employed here; see in particular the PIV 2 and blank specimens, where no signals approach the positivity cutoff.

Multiplex diagnosis has the potential for interpretative challenges, depending on the number of pathogens detected. The concept of respiratory infections with multiple pathogens has been accepted for many years[11] but the recent development of multiplex PCR assays for the detection of multiple respiratory pathogens has documented this further and resulted in recent publications.[12–14] Semple et al also reports that infants with dual RSV and hMPV infection have a 10 fold increase in relative risk of admission to a pediatric intensive care unit for mechanical ventilation.[12] In our small study 8/18 patient specimens had more than one organism detected (see Tables 1 and 2); four (samples 1, 2, 5, and 10) indicated dual viral infections, of which one (sample 1) also had Streptococcus pneumoniae detected and another (sample 10) had Haemophilus influenzae. Streptococcus pneumoniae was detected concurrently with a virus in Sample 9, and Haemophilus influenzae was detected concurrently with a virus in samples 3, 16, and 18. Both of these organisms can represent asymptomatic carrier state or normal flora[15]; but in certain cases they could represent a bacterial infection after initial viral destruction of the respiratory epithelium.

For economy both of cost and technologist time, our current laboratory diagnostic schemes routinely stop analysis and report with the first relevant pathogen detected in a specimen and would therefore not have been expected to indicate these coinfections. From a laboratory medicine perspective, this ability of multiplex assays such as the Genaco panels to report the presence of multiple respiratory pathogens in a specimen without additional cost or sample processing steps may be of use in allowing us to clearly define the importance of dual viral/viral or viral/bacterial infections and their relevance to the clinical presentation and appropriate treatment strategies. If the presence of multiple respiratory pathogens is a predictor of clinical severity as suggested by Semple et al,[12] assays such as the Genaco panels examined here could play an important role in the management of severe respiratory infections.

Based on our results, we believe the method to be both accurate and robust enough to be used in a routine clinical setting. We hope to further validate this through a much larger prospective comparison study of the Genaco panels versus our current DFA panels over the 2005 to 2006 peak respiratory viral season.

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