

Evidence from Multiplex Molecular Assays for Complex Multipathogen Interactions in Acute Respiratory Infections[∇]

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While most diagnostic processes cease with the detection of the first relevant infectious agent, newer multiplexed molecular methods which provide simultaneous analysis of multiple agents may give a more accurate representation of the true pathogen spectrum in these samples. To examine this in the context of respiratory infections, acute-phase respiratory specimens submitted to our clinical diagnostic microbiology/virology laboratory for our routine VIRAP diagnosis protocol during the spring 2006 peak respiratory infection season were processed in parallel by analysis with Genaco (QiaPlex) ResPlex I and ResPlex II molecular diagnostic panels. A total of 1,742 specimens were examined for 21 relevant targets each. The resulting data reveal that multiple infections are frequent and provide evidence for complex interactions between different infectious agents. Statistically relevant association patterns (both positive and negative) were observed between particular pathogens. While some interactions we observed are substantiated by prior reports in the literature, several specific patterns do not appear to have been reported previously. In addition, we report preliminary clinical evidence which supports a hypothesis that these coinfections are medically relevant and that effective treatment for severe respiratory tract infections will increasingly require diagnosis of all involved pathogens, as opposed to single-pathogen reporting.

The majority of infection diagnoses proceed via an approach which assumes a single-agent etiology. This assumption is self-validating because diagnostic processes end with detection of the first relevant pathogen. While coinfections are more commonly accepted as occurring in respiratory infections than in many other clinical settings, this diagnostic bias towards single-pathogen detection and subsequent treatment is still prevalent. This is true in the case of our clinical diagnostic virology/microbiology laboratory, which serves as a regional referral center for acute-phase respiratory specimen diagnosis through the VIRAP program (23). Based on a commercial direct fluorescence assay (DFA) (SimulFluor screen; Chemicon Inc., Temecula, CA) for seven pathogens (adenovirus, parainfluenza virus 1 [PIV-1], PIV-2, PIV-3, influenza A virus, influenza B virus, and respiratory syncytial virus (RSV) A/B combined), with referral of DFA-negative specimens to viral cell culture, the VIRAP program processes several thousand specimens annually. Diagnosis proceeds via a flow approach tailored to seasonal and case parameters such that the most likely causal agents are assayed first, with the diagnostic process ending at the first positive result.

While approximately 35% of all VIRAP specimens have a pathogen identified by DFA, the extended turn-around time and limited detection spectra of those ~65% of specimens referred to viral cell culture have led us to examine alternative second-line diagnostic methods. Our prior successful pilot

study of the multiplex molecular assays sold by Genaco Biomedical Products (now Qiagen Inc.) (8) led us to a larger prospective study of the method, with parallel specimen processing by VIRAP and ResPlex I/II panels.

(Some preliminary aspects of these data were presented previously, at the Third International Symposium of the Chinese American Association for Clinical Microbiology, Shenzhen, China, October 2006; the First Asian Pacific Symposium on Advanced Molecular Technologies, Hong Kong SAR, China, October 2006; and the CACMID/AMMI Canada Annual Conference, Halifax, Nova Scotia, Canada, 14 to 18 March 2007.)

MATERIALS AND METHODS

VIRAP specimens consist exclusively of nasopharyngeal wash (NPW) samples obtained by trained staff with a uniform protocol (24). All samples were taken from cases of acute respiratory infection for diagnostic purposes, with approximately half being inpatient (pediatric and maternal) and the other half being mixed outpatient specimens; in total, approximately 80% of specimens were pediatric and 20% were adult. From January through April 2006, for each specimen where sufficient volume was present, an aliquot was stored at -80°C ; a total of 1,742 specimens were incorporated into the analysis. All appropriate institutional ethics reviews were approved prior to commencement of this study.

Stored samples were thawed once and immediately processed for nucleic acid extraction. Two-hundred-microliter aliquots of NPW were mixed with an equal volume of phosphate-buffered saline and extracted by a Qiagen BioRobot M48 machine (Qiagen Inc., Hilden, Germany), using the manufacturer's MagAttract M48 virus mini kit. In accordance with the instructions for processing of phosphate-buffered saline-containing samples, Qiagen protease was resuspended in Qiagen AVE buffer instead of protease resuspension buffer. Purified nucleic acids were eluted in a 50- μl volume and frozen at -80°C prior to further study.

Extracts were thawed a single time and analyzed by ResPlex I and ResPlex II panels (Genaco Biomedical Products, Huntsville, AL) in accordance with the supplied protocols. Extracts were analyzed simultaneously by both panels, with 10- μl aliquots of extract serving as templates in 50- μl PCR (ResPlex I) and

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reverse transcription-PCR (RT-PCR) (ResPlex II) mixtures, using the supplied primer mixes. Qiagen HotStarTaq master mix was used for the PCRs, and Qiagen OneStep RT-PCR master mix was used for the RT-PCRs, without the inclusion of RNasin. Both PCR and RT-PCR mixtures were thermocycled together under the provided RT-PCR conditions (identical to the PCR conditions except for an initial 42°C 30-min RT step, during which the PCRs were effectively idled prior to the hot start). Following thermocycling, amplicons were detected by mixing 5- μ l portions of amplification products with either ResPlex I beads (PCR products) or ResPlex II beads (RT-PCR products) in hybridization buffer at 52°C for 10 min. Streptavidin-phycoerythrin conjugate was added, and mixtures were incubated at 52°C for a further 5 min prior to the addition of stop buffer and analysis on a Bio-Rad BioPlex instrument (Bio-Rad Laboratories, Hercules, CA) running Bio-Rad BPM 4.1 software. The BioPlex instrument was calibrated to the high RP1 value, with assays run at 50 beads per class minimum, at a plate temperature set to 52°C. Raw mean fluorescence intensity data from each run were exported to Excel for storage and data analysis. Normally, samples were batch processed from (RT)-PCR through data collection in sets of 46 specimens, with one negative control (distilled H₂O amplification template) and one positive control (supplied with the ResPlex reagents) for each run of the ResPlex I and ResPlex II panels.

The assay cutoff value for each of the 21 targets was set at a mean fluorescence intensity of 1,250, as validated by our prior study (8). Both ResPlex I and ResPlex II panels contain a human X chromosome marker as an internal control for the sample collection, nucleic acid extraction, amplification, and detection/classification steps; the lower bound limit for this signal as a control for each specimen was set at the average plus 10 standard deviations of the negative control X chromosome signals for 36 duplicates each for ResPlex I and ResPlex II. A specimen was thus required to generate a minimum X chromosome signal of 415.8 (ResPlex I) or 942.6 (ResPlex II) for data from the respective panel to be accepted for further analysis.

RESULTS

A total of 36 specimens (2.1%) failed to reach the ResPlex I internal control (X chromosome marker) cutoff, and 145 specimens (8.3%) failed to reach the ResPlex II internal control cutoff; 30/36 specimens which failed to reach the ResPlex I cutoff also failed to reach the ResPlex II cutoff (83%). In a small number of cases, particularly in the case of a ResPlex II-only internal control failure, specific pathogens were still detected. Because of the input specimen type (NPW), it is possible that a specimen could have small numbers of human cells while still having appreciable titers of cell-free pathogen; specimens failing to reach the positive control cutoff for either panel I or II were thus considered undetermined for any individual panel targets reported as negative, but a positive target signal was taken as valid evidence for the presence of that specific pathogen.

For six of the seven pathogen types detected by VIRAP DFA, concordance between the assay methods was good, ranging from 100% agreement (PIV-2) to 84% agreement (RSV-B), with the VIRAP DFA method being taken as the reference; for most of the shared targets, concordance was above 90%. The seventh shared pathogen (adenovirus) gave very low concordance values (<10%) and is discussed further below. As expected for an amplification-based method, the ResPlex panels detected more positive samples for all target species than those detected by VIRAP; these likely originated from low-pathogen-titer specimens below the DFA detection limit. Based on the observed concordance, our earlier validation study including some of the bacterial as well as viral targets (8), preliminary data on ResPlex assay specificity from other groups (25), and specificity evaluation of related Templex-based assays (12), we believe that false-positive results arising from the ResPlex I/II assays are, at most, rare events and, if

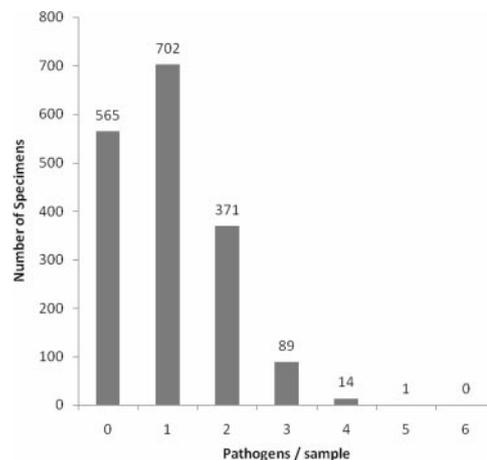


FIG. 1. Pathogen prevalence per sample. The histogram shows the number of pathogens detected per sample by ResPlex I/II assay of 1,742 total specimens.

present at all, are unlikely to skew the large data set analyzed here. A single likely exception to this was observed in the case of PIV-3-positive samples; while there was good concordance between VIRAP and ResPlex results (21/22 [96%] PIV-3-positive samples by VIRAP were also PIV-3 positive by ResPlex II), ResPlex II also reported PIV-1 positivity for 20/21 samples and PIV-4 positivity for 19/21 of the same specimens (not supported by our VIRAP data), indicating a cross-reactivity between the PIV-1, -3, and -4 targets in the ResPlex assay. To ensure that this did not introduce errors into our data set, all 92 samples with any PIV-1, -3, or -4 signal above the cutoff were filtered so as to score positivity only for the highest signal among these three targets. In the majority of cases, one of the three target values was very substantially elevated over the other two, suggesting that this was a valid filter.

A total of 1,177 specimens out of 1,742 scored positive for one or more pathogens by ResPlex assay (68%); the remaining 565 specimens scored negative. A histogram of the number of pathogens detected per sample is shown in Fig. 1. The observed prevalence for all pathogens detected by the ResPlex I/II assay is shown in Fig. 2, in comparison with the prevalence by VIRAP for the seven shared targets. A number of pathogens detected by ResPlex assay but not VIRAP were found to be frequent in our sample population (particularly *Streptococcus pneumoniae*, *Haemophilus influenzae*, human metapneumovirus [hMPV], the coxsackie virus/echovirus [CVEV] group, and rhinovirus). None of these is particularly surprising, and our ResPlex observed prevalence values appear to be generally in line with published incidences for all of the target species (1, 6, 7, 13, 17, 19, 20, 26, 29), although some differences among the values reported in these references suggest that regional variation is to be expected.

In order to examine whether there were patterns of association between pathogens found in individual specimens, all samples from the study were grouped according to pathogen positivity by ResPlex analysis. Samples positive for *H. influenzae* targets 1, 2, and 3 were taken as a single group, as were the two adenovirus subgroups and PIV-1, -2, -3, and -4; all other ResPlex targets were taken individually. *Legionella* was ex-

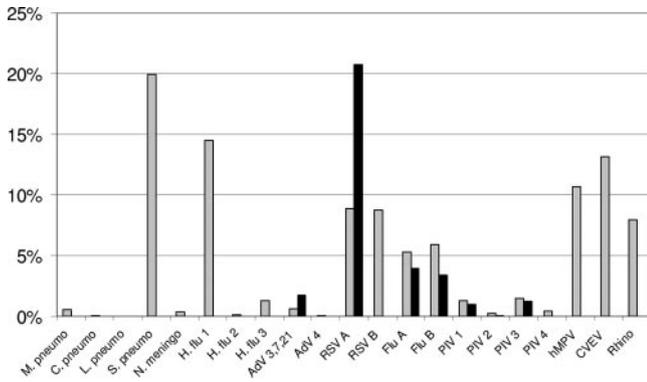


FIG. 2. Pathogen prevalence by ResPlex I/II assay and VIRAP DFA (as obtained with the SimulFluor assay according to the manufacturer's protocols). Light bars, ResPlex I/II prevalence values for all samples; dark bars, VIRAP DFA prevalence values for the same sample set. Note that VIRAP examines only adenovirus, RSV, influenza A virus, and influenza B virus, and PIV-1 to PIV-3 and that it groups RSV-A and RSV-B together; the indicated VIRAP RSV-A prevalence should thus be compared against the ResPlex RSV-A and RSV-B prevalence values combined. Abbreviations: M. pneumo, *Mycoplasma pneumoniae*; C. pneumo, *Chlamydia pneumoniae*; L. pneumo, *Legionella pneumophila*; S. pneumo, *Streptococcus pneumoniae*; N. meningitidis, *Neisseria meningitidis*; H. flu 1, *Haemophilus influenzae* (all types); H. flu 2, *Haemophilus influenzae* (strains a, b, c, and d); H. flu 3, *Haemophilus influenzae* (strains e and f); AdV, adenovirus; RSV, respiratory syncytial virus; Flu, influenza virus; PIV, parainfluenza virus; hMPV, human metapneumovirus; CVEV, coxsackie virus/echovirus family; Rhino, rhinovirus.

cluded from the comparison, as no positive samples were present among the specimens we examined. Sample sets with a background of positivity for each of the 14 groups were then analyzed for the prevalence rate of each of the 21 ResPlex targets (except *Legionella*). The results of this analysis are presented numerically in Table 1; for comparison, the overall prevalence frequency for each pathogen from Fig. 2 is included (last row), and for statistical significance calculations, the size of each background positivity group is indicated (last column). A blank diagonal "self-identity" line through this table indicates where sample groups cross the assays for the pathogen set in which they were already selected as positive.

Cells of particular interest within this table are those which contain numbers markedly different from the overall prevalence value at the bottom of that row. Higher-than-overall-prevalence values indicate a potential positive correlation between the background pathogen (as indicated by row) and the test pathogen (column); that is, the test pathogen occurs more frequently in the indicated background than in the entire specimen set as a whole. Conversely, a lower-than-overall-prevalence value for a cell indicates a possible negative correlation between the background and test pathogens.

In order to select only those values for which meaningful interpretations can be made, all pathogen pairs in Table 1 were evaluated through 2 × 2 Pearson's chi-square analysis with Bonferroni-Holm P value correction for multiple comparisons (22); in cases where Pearson's chi-square analysis was constrained by low expect values, Fisher's exact test was employed. Eight pathogen pairs resulting in significant P values (<0.05) by this method are indicated in Table 1 and given in Table 2, along with the test method employed, the uncorrected P value

^a Upper and lower bounds show 90% confidence intervals around the overall prevalence values. Blank cells mark the self-identity diagonal through the table. Values in bold show significant or suggestive elevations in prevalence; *italic* values show significant or suggestive decreases in prevalence. * values flagged as significant by our first analysis method (chi-square or Fisher's exact test) and further detailed in Table 2. Values shown in bold or *italic*, but without an asterisk, were flagged as suggestive values by our second analysis approach (using confidence intervals). See the legend to Fig. 2 for an explanation of abbreviations.

Pathogen positivity background	Prevalence (%) of pathogen														No. of samples making up background positivity group							
	M. pneumoniae	C. pneumoniae	S. pneumoniae	N. meningitidis	H. flu 1	H. flu 2	H. flu 3	ADV 3,7,21	ADV 4	RSVA	RSVB	Flu A	Flu B	PIV-1		PIV-2	PIV-3	PIV-4	hMPV	CVEV	Rhinovirus	
M. pneumoniae	0	0	20	0	10	0	0	0	0	10	0	0	0	0	0	0	0	0	0	10	0	10
C. pneumoniae	1	0	0	0	100	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0
S. pneumoniae	0	0	0	1	0	0	4*	0	0	13	11	6	6	1	0	2	0	0	14	14	8	347
N. meningitidis	0	0	50	0	21*	0	0	0	0	17	0	67*	0	0	0	0	0	0	0	0	0	6
H. influenzae	0	0	29*	0	0	0	0	0	0	8	10	5	10	1	0	2	0	0	14	14	9	278
ADV	0	0	0	0	0	0	0	0	0	8	0*	0	0	0	0	0	0	0	0	0	8	12
RSV-A	1	0	28	0	14	1	1	1	0	2*	7	7	7	1	0	0	0	0	8	8	5	155
RSV-B	0	0	26	0	18	0	1	1	0	7	0	0	7	1	0	0	0	0	5	4	4	153
Flu A	0	0	18	0	13	0	2	2	0	7	0	4	4	0	0	0	0	0	7	2*	7	92
Flu B	0	0	21	4*	24	0	3	0	0	0*	1	4	3	1	0	1	0	0	2	2*	1	103
PIV	0	0	17	0	15	0	2	0	0	2	3	7	7	0	0	0	0	0	2	3*	3	186
PIV-1	0	0	20	0	15	0	2	0	0	5	3	1*	1*	0	0	0	0	0	2*	1*	7*	229
PIV-2	0	0	20	0	15	0	1	1	0	5	4	1	1	0	0	1	0	0	1*	1*	8	139
PIV-3	0	0	20	0	15	0	1	1	0	9	9	5	6	1	0	1	0	0	11	11	8	145
PIV-4	0	0	20	0	15	0	1	1	0	9	9	5	6	1	0	1	0	0	11	13	8	90
hMPV	0	0	21.5	0	13.1	0.0	0.9	0.9	0.2	10.0	9.9	6.2	6.8	1.8	0.4	2.0	0.7	11.9	9.5	14.5	8	6.9
CVEV	0.9	0.2	18.3	0.1	0.0	0.0	0.3	0.3	0.0	7.8	7.7	4.4	5.0	0.9	0.0	1.0	0.2	11.8	11.8	14.5	8	6.9
Rhinovirus	0.3	0.0	18.3	0.1	13.1	0.0	0.9	0.3	0.0	7.8	7.7	4.4	5.0	0.9	0.0	1.0	0.2	11.8	11.8	14.5	8	6.9
Overall prevalence	1	0	20	0	15.9	0.2	1.8	0.9	0.2	10.0	9.9	6.2	6.8	1.8	0.4	2.0	0.7	11.9	9.5	14.5	8	6.9
Upper bound	0.9	0.2	21.5	0.1	15.9	0.2	1.8	0.9	0.2	10.0	9.9	6.2	6.8	1.8	0.4	2.0	0.7	11.9	9.5	14.5	8	6.9
Lower bound	0.3	0.0	18.3	0.1	13.1	0.0	0.9	0.3	0.0	7.8	7.7	4.4	5.0	0.9	0.0	1.0	0.2	11.8	11.8	14.5	8	6.9

TABLE 1. Cross-correlation of pathogen prevalence rates by ResPlex assay^a

TABLE 2. Pathogen pairs with significant codetection rates^a

Pathogen 1	Pathogen 2	Test	<i>P</i> value	Bonferroni-Holm <i>P</i> value	Benjamini-Hochberg <i>P</i> value
CVEV	Rhinovirus	Chi-square	1.61E-42	3.30E-40	
RSV-A	hMPV	Chi-square	1.22E-05	2.49E-03	
hMPV	CVEV	Chi-square	1.35E-05	2.75E-03	
<i>H. influenzae</i>	<i>S. pneumoniae</i>	Chi-square	3.93E-05	7.94E-03	
RSV-B	hMPV	Chi-square	4.77E-05	9.59E-03	
hMPV	Rhinovirus	Chi-square	1.34E-04	2.67E-02	
<i>N. meningitidis</i>	Flu B	Fisher's exact	1.58E-04	3.14E-02	
<i>S. pneumoniae</i>	H. flu 1	Chi-square	1.68E-04	3.32E-02	
	H. flu 3	Fisher's exact	5.24E-04		1.19E-02
Flu B	CVEV	Chi-square	9.04E-04		1.85E-02
RSV-A	Flu B	Chi-square	1.99E-03		3.71E-02
Flu A	CVEV	Chi-square	2.35E-03		4.02E-02
RSV-A	RSV-B	Chi-square	2.64E-03		4.16E-02

^a See the legend to Fig. 2 for an explanation of abbreviations.

observed, and the Bonferroni-Holm-corrected *P* value for the interaction. The uncorrected *P* value results were also analyzed with the Benjamini-Hochberg adjustment (5), which indicated a further five significant interactions, as indicated in Tables 1 and 2. Following this analysis, in order to examine our data for weaker but still potentially suggestive interactions, we took an approach of calculating the 90% confidence intervals for each Table 1 cell's prevalence value and for the overall prevalence frequency of each target pathogen; if these values were non-overlapping, the result was considered suggestive. While this method lacks some statistical rigor due to the fact that the confidence intervals were not calculated for totally independent populations, it is simple and analogous to how one might consider these data if they were presented as a bar graph. Cell values (high and low) flagged as suggestive by this approach are also highlighted in Table 1. As expected, all interactions determined as significant by the more rigorous first approach were also detected by this method, and a small number of additional pathogen interaction pairs were highlighted as being of potential interest. We comment further on the relevance of this observation in Discussion.

DISCUSSION

Of the seven shared pathogen targets between the ResPlex panels and our current DFA method, adenovirus was the only one for which we observed poor concordance between methods. For this target, while the two assay methods reported similar overall prevalence levels, each of the assays frequently reported a sample as positive while the other assay reported the sample as negative. We feel this may have arisen in large part from two compounded issues. Firstly, the DFA detects 41 serovars of adenovirus, while the ResPlex assay is directed against only 4 serovars; it is therefore not overly surprising that there may be DFA-positive samples which are ResPlex negative. Conversely, for these four serovars, the molecular method may be much more sensitive than the DFA method in detecting residual or persisting adenovirus which could be missed by the antigen-based assay (in particular, previous studies have shown this DFA assay to miss some adenovirus infections) (16). Beyond this one target, based on our previous work with the ResPlex panels (8) and the good agreement overall in

pathogen prevalence values between the ResPlex data and either our paired VIRAP DFA data (where available) or literature values, we feel that the ResPlex I/II data presented here represent an accurate data set of pathogen prevalence in our specimens. From these data, a number of observations can be made.

Firstly, codetection of pathogens was the norm, not the exception, among our acute-phase respiratory samples. Our data averaged 1.02 pathogens per sample, including 565 specimens reporting no pathogen; it would not be unreasonable to assume that these nonclassified specimens had, as a minimum, one unidentified respiratory pathogen responsible for the symptomatic presentation. If this conservative estimate of single-pathogen infection is true for these 565 samples, our actual average number of pathogens per specimen value rises to 1.34. The fact that such unidentified pathogens are present in our sample set and would by inclusion increase the average number of pathogens per sample is hardly speculative; the ResPlex I/II assay panels do not include several important pathogens, including bocavirus and members of the coronavirus family (such as strains OC43, 229E, and NL63), which are reported to occur at frequencies of approximately 12% and 5 to 10%, respectively (13, 14, 15, 26), and would thus certainly be expected to be present within our samples. Our results in this regard differ markedly from those of Weigl and coworkers (27), who observed coinfections in a remarkably small percentage of specimens in their study. This may be attributable, to a large extent, to two factors. The first is methodological differences, as Weigl et al. employed a classical high-multiplicity RT-PCR technique; aside from the difficulty in optimizing all of the individual reactions within such a multiplex reaction to work under the same thermocycling conditions, these assays can suffer from a quenching effect whereby one successful target amplification in a sample can suppress amplification of other targets present and thus have a tendency to underrepresent codetection frequencies. In contrast, the Tempex ("QiaPlex") method used here is effectively a simplex PCR which avoids this quenching effect (12) and can thus more accurately detect the simultaneous presence of multiple assay targets. The second factor may be that Weigl et al. were primarily examining viral targets, for which we also observed low codetection frequencies, as commented on below.

Our data provide statistical evidence demonstrating that coinfections are not random; there are clear correlations for the occurrence of certain pathogens, as shown in Tables 1 and 2. The major infectivity-enhancing interactions we observed included an enhancement between *S. pneumoniae* and *H. influenzae*, a very large (and to our knowledge previously unreported) enhancement between *Neisseria meningitidis* and influenza B virus (on the order of eightfold, either way), and an enhancement between the CVEV group and the rhinovirus group. While the mechanisms behind these enhancements may vary from case to case, a number of plausible candidates have been discussed widely (for reviews, see references 4 and 10).

Beyond the 13 strongly supported interactions shown in Table 2, we saw a number of weaker but suggestive interactions indicated by our second analysis method, as indicated in Table 1. While our data set is not large enough to give strong statistical support to any of these, we believe the example of *S. pneumoniae* in an RSV-B-infected background is potentially meaningful in this regard and warrants additional discussion. Our data show that in a background of RSV-B positivity, *S. pneumoniae* prevalence is 26%, which is greater than its overall prevalence of 20% in our samples. This interaction approached but did not cross the threshold for significance ($P < 0.05$) by our first analysis method and escaped, by a tiny margin (1.3%), being flagged as suggestive by our second confidence interval approach. The reverse situation—RSV-B positivity in an *S. pneumoniae* background—does not meet significance criteria for either approach. A review of the literature not only reveals long-standing epidemiological evidence for coinfection by these two agents (2, 10, 21) but also provides convincingly demonstrated mechanisms centered around the ability of *S. pneumoniae* adherence to the RSV-B glycoprotein G (gG) expressed either on the surfaces of infected cells or on free virions to enhance *S. pneumoniae* adherence and infection (11). Either route enables previously present RSV-B to increase the infectivity of *S. pneumoniae*. Our elevated prevalence value in this case thus detects a documented phenomenon, despite not being quite large enough to be picked by either of our statistical analysis methods. We believe that this example highlights that even a relatively large data set such as the one presented here may not be large enough to detect some real interactions and thus that beyond the well-supported interactions shown in Table 2, the weaker interactions indicated as suggestive by our second screening method may be worthy of further directed study.

In all cases of observed enhancement, a major concern is whether these may be spurious results arising from cross-detection of the involved targets; however, as discussed previously, we have not seen evidence of cross-reaction of these target sets. In the case of the observed CVEV/rhinovirus targets, the large number of serotypes represented in each group means that our prior tests for cross-reactivity have not been exhaustive, and it is conceivable that some serovars of each group could cross-react with the target for the other. We can, however, state that in many samples where a strong positive signal was seen for one or the other of these two targets, the signal was restricted to that target only; thus, our data indicate that any such cross-reactivity cannot be a general occurrence, if it happens at all.

Our data include cases of clearly supported or suggestive

pathogen cosuppression, where infection with one pathogen reduces the risk for infection by the other. Interestingly, most of these interactions (Table 1) are reciprocal and occur between single-stranded RNA viruses. One possible explanation for this could be activation of nonspecific antiviral functions (including protein kinase R, the 2-5A system, Mx proteins, and apoptotic pathways) (3, 18, 28) by the first infecting agent; inhibition of initiation of infection by a second RNA virus in the face of multiple already activated antiviral responses would be a plausible outcome.

All of these data beg the question of whether codetection of multiple respiratory pathogens in a sample has clinical relevance. In particular, while both *H. influenzae* and *S. pneumoniae* can be important pathogens, they are also well known to occur in apathogenic carrier states; more detailed clinical analysis of outcomes when they occur simultaneously with other respiratory pathogens is clearly desirable for our understanding of how to interpret their detection in these cases. An initial line of evidence comes from a review of particularly sick pediatric patients in our intensive care unit who had samples included in this study. Seven such patients with clinical diagnoses of RSV bronchiolitis were selected based purely on severity of presentation; when the ResPlex data for these cases were examined, we found that five of seven patients had multiple pathogens present (one patient had no pathogen detected; one patient was positive for RSV alone; one patient was positive for *S. pneumoniae* plus influenza A virus; two patients were positive for *H. influenzae*, *S. pneumoniae*, and RSV; and two patients were positive for *H. influenzae*, RSV, and CVEV). While preliminary, these data suggest that flow-scheme diagnostics, which would have ceased with diagnosis of a single viral pathogen in these examples, might have led to exclusion of antibiotic therapy when it was potentially warranted.

We hope that the data presented herein will be a starting point for further examination of this topic. While our total specimen set was of appreciable size, some of the individual positivity subgroups were too small to yield meaningful data; there are several cell values in Table 1 which appear to be highly suggestive yet do not reach our definition of statistical relevance with the current sample size. Until a much larger data set examining respiratory specimens for multiple pathogens is available, a more thorough interpretation of the results presented here is therefore difficult. Based on our results, we concur with the recent opinion of Freymuth et al. (9) and feel that DFA backed with a multiplex molecular method is the current best practice in respiratory diagnostics. As such, we hope that the needed data will increasingly be available in the future as we and other clinical diagnostic laboratories move toward this approach.

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