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Serology Enhances Molecular Diagnosis of Respiratory Virus Infections Other than Influenza in Children and Adults Hospitalized with Community-Acquired Pneumonia

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ABSTRACT Both molecular and serological assays have been used previously to determine the etiology of community-acquired pneumonia (CAP). However, the extent to which these methods are correlated and the added diagnostic value of serology for respiratory viruses other than influenza virus have not been fully evaluated. Using data from patients enrolled in the Centers for Disease Control and Prevention (CDC) Etiology of Pneumonia in the Community (EPIC) study, we compared real-time reverse transcription-PCR (RT-PCR) and serology for the diagnosis of respiratory syncytial virus (RSV), human metapneumovirus (HMPV), parainfluenza virus 1 to 3 (PIV1, PIV2, and PIV3), and adenovirus (AdV) infections. Of 5,126 patients enrolled, RT-PCR and serology test results were available for 2,023, including 1,087 children below the age of 18 years and 936 adults. For RSV, 287 (14.2%) patients were positive by RT-PCR and 234 (11.6%) were positive by serology; for HMPV, 172 (8.5%) tested positive by RT-PCR and 147 (7.3%) by serology; for the PIVs, 94 (4.6%) tested positive by RT-PCR and 92 (4.6%) by serology; and for AdV, 111 (5.5%) tested positive by RT-PCR and 62 (3.1%) by serology. RT-PCR provided the highest number of positive detections overall, but serology increased diagnostic yield for RSV (by 11.8%), HMPV (by 25.0%), AdV (by 32.4%), and PIV (by 48.9%). The method concordance estimated by Cohen's kappa coefficient (κ) ranged from good (for RSV; $\kappa = 0.73$) to fair (for AdV; $\kappa = 0.27$). Heterotypic seroresponses observed between PIVs and persistent low-level AdV shedding may account for the higher method discordance observed with each of these viruses. Serology can be a helpful adjunct to RT-PCR for research-based assessment of the etiologic contribution of respiratory viruses other than influenza virus to CAP.

KEYWORDS respiratory virus infections, community-acquired pneumonia, PCR assays and serology, serology, PCR assays

Community-acquired pneumonia (CAP) is a leading cause of morbidity and mortality worldwide (1, 2). In the United States, despite high pneumococcal vaccine coverage among children, CAP remains a common cause of hospitalization for all ages and an

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infectious cause of death among adults (3–6). Although often associated with clinically mild infections, respiratory viruses can also cause severe lower respiratory tract illnesses, including pneumonia, particularly in young children and elderly adults. Respiratory syncytial virus (RSV), human metapneumovirus (HMPV), parainfluenza viruses (PIVs), and adenovirus (AdV), among other respiratory viruses, have frequently been associated with CAP (5–11).

In recent years, advanced molecular diagnostic methods, such as real-time reverse transcription-PCR (RT-PCR) assays, have been used to better define the role of respiratory viruses in CAP etiology and have dramatically increased the frequency of virus detection (12, 13). However, efforts to establish a true etiology for CAP by RT-PCR alone have their limitations. For example, RT-PCR is susceptible to false-positive results due to target contamination (14). Most importantly, some respiratory viruses are detected by RT-PCR in the upper respiratory tracts of both symptomatic and asymptomatic children, making it difficult to determine whether the pathogen is associated with lower respiratory tract disease (5, 6, 15–17). Alternatively, in some CAP studies, a significant number of adults are RT-PCR negative despite clinical and epidemiological suspicion of viral infection (5, 16, 18).

By measuring the convalescent-phase serum antibody response to acute viral infection, serology might help resolve these discrepancies and support RT-PCR in providing a more accurate assessment of infectious etiology. Molecular studies of respiratory virus infections that have included serology have generally increased the number of acute infections identified (12, 19, 20). However, the added diagnostic value of serology for the etiologic assessment of CAP in U.S. populations has not been fully evaluated. In this study, we compared serology and RT-PCR methods for the detection of RSV, HMPV, PIV, and AdV infections in patients enrolled in the Centers for Disease Control and Prevention (CDC) Etiology of Pneumonia in the Community (EPIC) study (5, 6) and assessed the added value of serology for the determination of the etiology of CAP among hospitalized U.S. patients.

RESULTS

Comparison of RT-PCR and serology by patient age. Among 5,126 enrolled patients, 2,082 (40.6%) for whom both respiratory specimens and paired sera were available were considered for analysis. Compared with patients who did not have specimens available for comparison, children aged 0 to 6 months, the elderly (≥ 65 years), and those who died were less likely to be tested by both RT-PCR and serology ($P, < 0.0001$ for each comparison). The proportions of patients with chronic comorbidities, intensive care unit (ICU) admission, and invasive mechanical ventilation were similar among patients tested and not tested by both methods. Following serologic testing, 59 (3.0%) additional patients were excluded from the analysis: 49 with inconclusive serology results due to high enzyme immunoassay (EIA) background signals with a control antigen and 10 for whom antibody titer increases or decreases in response to multiple viruses indicated possibly mismatched serum pairs. Of the 2,023 remaining patients, among whom 1,087 (53.7%) were children < 18 years old (median age, 2 years) and 936 (46.3%) were adults (median age, 57 years), 1,876 (92.7%) had radiographically confirmed pneumonia according to the study radiologist.

Serology and RT-PCR assays agreed most often for RSV (62.3% agreement; κ , 0.73) and least often for AdV (17.7% agreement; κ , 0.27) (Table 1). For RSV overall, 287 (14.2%) patients were positive by RT-PCR and 234 (11.6%) were positive by serology; for HMPV, 172 (8.5%) were positive by RT-PCR and 147 (7.3%) by serology; for the PIVs, 94 (4.6%) were positive by RT-PCR and 92 (4.6%) by serology; and for AdV, 111 (5.5%) were positive by RT-PCR and 62 (3.1%) by serology. RT-PCR provided the highest number of positive detections overall, but serology increased the diagnostic yield for RSV (by 11.8%), HMPV (by 25.0%), AdV (by 32.4%), and PIV (by 48.9%). Serology added the fewest new cases among infants under the age of 6 months and the most among older children and adults for all viruses tested. Serology contributed proportionately the most new cases of PIV infection in children between the ages of 7 months and 5 years and

TABLE 1 Comparison of RT-PCR and serology for 2023 CAP patients by age

Assay	Age group	Total no. of patients	Total no. (%) positive	No. (%) with the following result(s):				κ (95% CI) ^a	% increase in diagnostic yield (95% CI) with positive serology results ^b
				Positive by RT-PCR	Positive by serology and serology	Positive by RT-PCR, negative by serology	Positive by serology, negative by RT-PCR		
RSV	0-6 mo	128	54 (42.2)	53 (41.4)	16 (12.5)	15 (11.7)	38 (29.7)	1 (0.8)	1.9 (0, 6)
	7 mo-2 yr	421	167 (39.7)	149 (35.4)	148 (35.2)	130 (30.9)	19 (4.5)	18 (4.3)	12.1 (7, 18)
	3-5 yr	208	49 (23.6)	47 (22.6)	33 (15.9)	31 (14.9)	16 (7.7)	2 (1.0)	4.3 (0, 12)
	6-17 yr	330	18 (5.5)	14 (4.2)	12 (3.6)	8 (2.4)	6 (1.8)	4 (1.2)	28.6 (6, 78)
	18-64 yr	623	18 (2.9)	14 (2.2)	13 (2.1)	9 (1.4)	5 (0.8)	4 (0.6)	28.6 (5, 82)
≥65 yr	313	15 (4.8)	10 (3.2)	12 (3.8)	7 (2.2)	3 (1.0)	5 (1.6)	50.0 (9, 167)	
All ages	2,023	321 (15.9)	287 (14.2)	234 (11.6)	200 (9.9)	87 (4.3)	34 (1.7)	0.73 (0.69, 0.78)	11.8 (8, 16)
HMPV	0-6 mo	128	20 (15.6)	19 (14.8)	10 (7.8)	9 (7.0)	10 (7.8)	1 (0.8)	5.3 (0, 20)
	7 mo-2 yr	421	83 (19.7)	67 (15.9)	67 (15.9)	51 (12.1)	16 (3.8)	16 (3.8)	23.9 (13, 38)
	3-5 yr	208	34 (16.3)	23 (11.1)	24 (11.5)	13 (6.3)	10 (4.8)	11 (5.3)	47.8 (21, 90)
	6-17 yr	330	26 (7.9)	21 (6.4)	16 (4.8)	11 (3.3)	10 (3.0)	5 (1.5)	23.8 (5, 54)
	18-64 yr	623	38 (6.1)	30 (4.8)	20 (3.2)	12 (1.9)	18 (2.9)	8 (1.3)	26.7 (9, 56)
≥65 yr	313	14 (4.5)	12 (3.8)	10 (3.2)	8 (2.5)	4 (1.3)	2 (0.6)	16.7 (0, 63)	
All ages	2,023	215 (10.6)	172 (8.5)	147 (7.3)	104 (5.1)	68 (3.4)	43 (2.1)	0.62 (0.55, 0.69)	25.0 (17, 33)
PflV1, -2, -3	0-6 mo	128	8 (6.3)	7 (5.5)	4 (3.1)	3 (2.3)	4 (3.1)	1 (0.8)	14.3 (0, 67)
	7 mo-2 yr	421	48 (11.4)	28 (6.7)	36 (8.6)	16 (3.8)	12 (2.9)	20 (4.8)	71.4 (38, 128)
	3-5 yr	208	22 (10.6)	14 (6.7)	16 (7.7)	8 (3.8)	6 (2.9)	8 (3.8)	57.1 (21, 133)
	6-17 yr	330	19 (5.8)	13 (3.9)	14 (4.2)	8 (2.4)	5 (1.5)	6 (1.8)	46.2 (11, 135)
	18-64 yr	623	28 (4.5)	19 (3.0)	14 (2.2)	5 (0.8)	14 (2.2)	9 (1.4)	47.4 (14, 107)
≥65 yr	313	15 (4.8)	13 (4.2)	8 (2.6)	6 (1.9)	7 (2.2)	2 (0.6)	15.4 (0, 50)	
All ages	2,023	140 (6.9)	94 (4.6)	92 (4.6)	46 (2.3)	48 (2.4)	46 (2.3)	0.47 (0.38, 0.56)	48.9 (34, 69)
AdV	0-6 mo	128	16 (12.5)	15 (11.7)	5 (3.9)	4 (3.1)	11 (8.6)	1 (0.8)	6.7 (0, 29)
	7 mo-2 yr	421	84 (20.0)	70 (16.6)	27 (6.4)	13 (3.1)	57 (13.5)	14 (3.3)	20.0 (10, 32)
	3-5 yr	208	15 (7.2)	9 (4.3)	8 (3.8)	2 (1.0)	7 (3.4)	6 (2.9)	66.7 (17, 200)
	6-17 yr	330	13 (3.9)	9 (2.7)	6 (1.8)	2 (0.6)	7 (2.1)	4 (1.2)	44.4 (7, 150)
	18-64 yr	623	12 (1.9)	6 (1.0)	6 (1.0)	4 (0.6)	2 (0.3)	5 (1.0)	100.0 (14, 400)
≥65 yr	313	7 (2.2)	2 (0.6)	6 (1.9)	1 (0.3)	1 (0.3)	5 (1.6)	250.0 (40, 800)	
All ages	2,023	147 (7.3)	111 (5.5)	62 (3.1)	26 (1.3)	85 (4.2)	36 (1.8)	0.27 (0.14, 0.40)	32.4 (21, 45)

^aCohen's kappa coefficient (κ) indicates the level of agreement as follows: <0 to 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; 0.81 to 1, very good. CI, confidence interval.

^bCalculated by dividing the number of serology-positive, RT-PCR-negative specimens by the sum of the number of specimens positive by both methods and the number of specimens testing negative by serology and positive by RT-PCR.

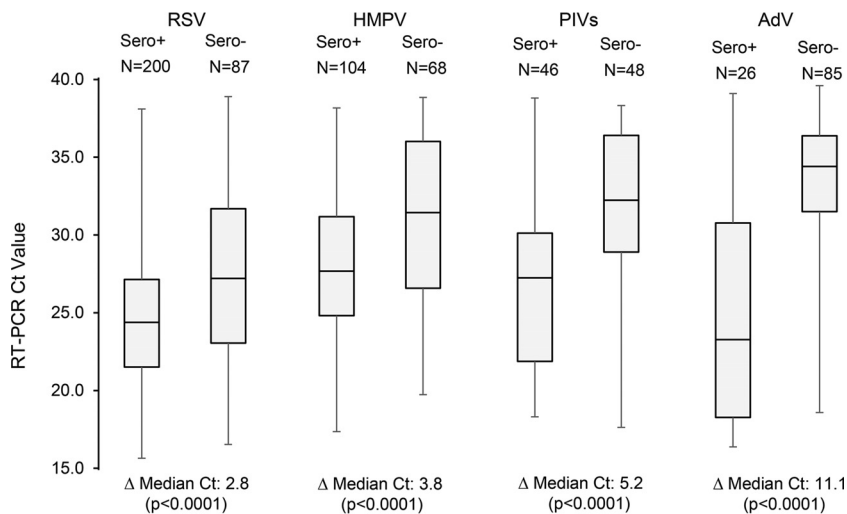


FIG 1 Box-and-whisker plots of C_T values of RT-PCR-positive respiratory specimens, comparing patients with different seroresponse statuses. Whiskers mark the 10th and 90th percentiles, and boxes are bounded by the 25th and 75th percentiles, with bisecting lines representing median C_T values. The sample size (N) is indicated for each category. Differences (Δ) in median C_T values for each category are given, as well as P values calculated by the Mann-Whitney U test.

the most new cases of AdV infection in older adults, although few adult AdV infections were identified overall.

Comparison of RT-PCR and serology by C_T values. As shown in Fig. 1, median virus loads were significantly higher (lower cycle threshold [C_T] values) in patients who developed a diagnostic seroresponse than in nonresponders for all viruses, ranging from a median difference of 2.8 ($\Delta 2.8$) in C_T values for RSV to $\Delta 11.1$ in C_T values for AdV ($P < 0.0001$). Also, the proportion of patients with a seroresponse declined with decreasing virus loads; proportions of patients with high virus loads ($C_T \leq 25$) and diagnostic seroresponses ranged from 66.7% for AdV to 75.7% for RSV, while proportions of patients with low virus loads ($C_T > 35$) and diagnostic seroresponses ranged from only 4.9% for AdV to 18.8% for RSV (Table 2).

Heterotypic antibody responses. Positive associations were found between seroresponses to PIV1 and seroresponses to PIV2 or PIV3 (Table 3), as expected given the antigens known to be shared between these viruses. Among 28 and 54 patients who showed increases in the levels of diagnostic antibodies to PIV1 and PIV3, respectively, 21 (75.0%) were concurrent increases; among 28 and 33 patients who showed increases in the levels of antibodies to PIV1 and PIV2, 3 (10.7%) were concurrent increases. In contrast, there was no codetection by RT-PCR among any of the PIVs.

A positive association ($P < 0.01$) in seroresponses was also evident with RSV and HMPV. Among 234 and 147 patients who showed antibody rises in response to RSV and HMPV, respectively, 28 (12.0%) had concurrent rises, of which 26 were in children below the age of 6 years. However, only 4 of these 28 patients were positive for both RSV and HMPV by RT-PCR; 13 of the remaining 24 patients were RT-PCR positive only for RSV, 8 were positive only for HMPV, and 3 were negative for both. In most cases, the dominant seroresponse corresponded with the RT-PCR-positive virus (data not shown). No positive associations in seroresponse were found with other virus combinations, although codetection of AdV with all other respiratory viruses was more common by both methods.

Comparison of RT-PCR and serology with NREVSS seasonality data. As an external validation of the serology and RT-PCR test methods used in this study, we compared the collection dates of positive specimens with NREVSS (National Respiratory and Enteric Virus Surveillance System) seasonal virus circulation data from the four cities where the EPIC study was conducted. Serology- and RT-PCR-positive specimens

TABLE 2 Comparison of RT-PCR and serology for CAP patients by cycle threshold value

C _T ^a	RSV		HMPV		PIV1, -2, -3		AdV					
	No. positive by RT-PCR	No. (%) positive by RT-PCR and serology	No. (%) positive by RT-PCR and negative by serology	No. (%) positive by RT-PCR and negative by serology	No. (%) positive by RT-PCR and negative by serology	No. (%) positive by RT-PCR and negative by serology	No. (%) positive by RT-PCR and negative by serology	No. (%) positive by RT-PCR and negative by serology				
≤20	37	27 (73.0)	10 (27.0)	2	1 (50.0)	1 (50.0)	5	4 (80.0)	1 (20.0)	13	11 (84.6)	2 (15.4)
>20 ≤ 25	111	85 (76.6)	26 (23.4)	37	27 (73.0)	10 (27.0)	18	13 (72.2)	5 (27.8)	8	3 (37.5)	5 (62.5)
>25 ≤ 30	78	60 (76.9)	18 (23.1)	62	43 (69.4)	19 (30.6)	26	18 (69.2)	8 (30.8)	17	4 (23.5)	13 (76.5)
>30 ≤ 35	45	25 (55.6)	20 (44.4)	48	30 (62.5)	18 (37.5)	23	8 (34.8)	15 (65.2)	33	6 (18.2)	27 (81.8)
>35 < 40	16	3 (18.8)	13 (81.2)	23	3 (13.0)	20 (87.0)	23	4 (17.4)	19 (82.6)	41	2 (4.9)	38 (92.7)
Total	287	200 (69.7)	87 (30.3)	172	104 (60.5)	68 (39.5)	95	47 (49.5)	48 (50.5)	111	26 (23.2)	85 (76.6)

TABLE 3 Virus codetection by serology

Test(s) and virus	No. of patients found positive	No. (%) of patients with codetection ^a				
		HMPV	PIV1	PIV2	PIV3	AdV
Serology						
RSV	234	28 (12.0)*	4 (1.7)	4 (1.7)	6 (2.6)	12 (5.1)
HMPV	147		0	1 (0.7)	1 (0.7)	5 (3.4)
PIV1	28			3 (10.7)*	21 (75.0)*	2 (7.1)
PIV2	33				0	2 (6.1)
PIV3	54					2 (3.7)
AdV	62					
Serology and RT-PCR						
RSV	200	4 (2.0)	0	0	1 (0.5)	2 (1.0)
HMPV	104		0	0	0	3 (2.9)
PIV1	7			0	0	0
PIV2	16				0	0
PIV3	24					0
AdV	26					

^aAsterisks indicate a *P* value of <0.01 by Fisher's exact two-tailed test.

showed close temporal correspondence with each other and with the NREVSS seasonal circulation patterns for RSV, HMPV, PIV3, and PIV2 (Fig. 2). The specimens found positive for PIV1 by RT-PCR corresponded with the 2011–2012 biennial fall-winter peak of NREVSS activity, but PIV1-positive serologies were also common during preceding peaks in PIV3 NREVSS activity. This likely reflects a one-way heterotypic antibody response between these viruses. The specimens found positive for AdV by RT-PCR and serology were more irregularly dispersed throughout the study period and showed less-clear correspondence with the late-winter and spring peak detection patterns described by the NREVSS.

DISCUSSION

The EPIC study was designed to determine the etiology and burden of CAP in the United States so as to guide future prevention and control strategies (5, 6). To achieve this goal, both molecular assays and serology studies were employed. The inclusion of serology allowed us to assess its ability to improve the accuracy of these estimates. Overall, RT-PCR and serology showed good concordance for RSV and HMPV, and serology increased the diagnostic yields for all virus infections, ranging from 11.8% for RSV to 48.9% for PIV. Despite some discordance, the validity of both test methods was supported by several lines of evidence. The proportion of diagnostic seroresponses was higher for all patients with high virus loads (lower C_T values), which would be predicted for primary virus infections and is consistent with case-control studies that have shown higher virus loads among RT-PCR-positive symptomatic patients than among matched asymptomatic controls (9, 16, 21). The relative prevalences of virus detection and patterns of age distribution found in this study were similar for RT-PCR and serology and were consistent with those reported in other CAP studies (9, 10, 19). Finally, for most viruses, positive test results obtained by both methods were temporally concordant with each other and with the seasonal circulation patterns of the respective respiratory viruses described by regional NREVSS data over the study period.

Test method discordance occurred with all respiratory viruses studied and was particularly pronounced with PIV and AdV ($\kappa < 0.5$). Several factors could account for method discordance. RT-PCR-negative results for serology-positive patients, which ranged from 10.6% for RSV to 32.9% for PIV, could be due to (i) respiratory specimens of poor quality or from suboptimal locations (upper versus lower respiratory tract), (ii) repeat viral infections, leading to the development of an anamnestic immune response and rapid viral clearance (22, 23), (iii) viral genome sequence polymorphisms resulting in diminished RT-PCR sensitivity, (iv) intercurrent viral infections between acute- and convalescent-phase specimen sampling, or (v) a false-positive seroresponse due to increases in the titers of nonspecific or heterotypic antibodies (see discussion below).

Serology-negative results for RT-PCR-positive patients, which ranged from 27.1% for RSV to 57.8% for AdV, could be due to (i) late collection of the acute-phase serum after virus infection, masking a rise in the level of the diagnostic antibody (pneumonia is a late stage in the development of respiratory symptoms), (ii) failure of immune recognition or immune immaturity, as described for RSV (24), (iii) antigenic polymorphisms among currently circulating virus strains, resulting in a diminished seroresponse, or (iv) concurrent virus shedding from an earlier infection unrelated to the acute illness, a common feature of persistent AdV infections (see discussion below).

More infections were detected by RT-PCR than by serology for all viruses tested. It is generally recognized that detection of RSV, HMPV, and PIVs by RT-PCR in the upper respiratory tract is sufficient to explain clinical illness (9, 16, 17). Even in the absence of serologic confirmation, a positive RT-PCR result with these viruses would be sufficient for disease association. For example, RT-PCR detected RSV in 38 children younger than 6 months who did not develop a seroresponse, which is consistent with studies that have shown that infants do not develop effective immune responses to RSV, especially following initial infection (25). Because subclinical infections with RSV are rare, RSV was likely causally related to the illness in these cases. In contrast, AdV is often detected in a high proportion of persons without respiratory symptoms, complicating disease attribution (9, 16, 17). Using data from the EPIC study, Self et al. (15) compared the prevalence of respiratory virus detection in case patients with that in matched asymptomatic controls. Although significantly more viruses were detected in case children than in controls for all viruses studied, AdV was detected in a relatively high proportion of controls (3.1%) compared with case children with CAP (6.4%). Persistent subclinical AdV shedding from the upper respiratory tract may account for this finding (26) and may also explain the low proportion of increases in the levels of diagnostic antibodies in children found positive for AdV by RT-PCR and the high proportion of RT-PCR-positive samples containing low virus loads found in our study (Fig. 1). This suggests that RT-PCR alone may overestimate the true number of AdV infections etiologically linked with CAP. Nevertheless, it is important to recognize AdV infections because of their potential to cause severe, sometimes fatal pneumonia (27).

Heterotypic antibody responses among PIVs due to shared antigenic determinants can complicate the interpretation of PIV serology (28). Had serology results been tallied separately for PIV1 and PIV3 in this study, the number of PIV1 infections would have been substantially overestimated; thus, we combined the serology results for these viruses. Serology appeared at first to be particularly effective in identifying RT-PCR-negative PIV infections among children aged 1 to 5 years. However, heterotypic responses following unrecognized exposure to other related viruses may account for some of these positive findings. For example, natural mumps virus infections have been shown to induce heterotypic antibody responses to PIV, and it is conceivable that measles, mumps, and rubella (MMR) vaccination could have contributed to antibody rises seen with some of the study children (29, 30). The first dose of MMR vaccine is given between the ages of 12 and 15 months, and a second dose is given at the age of 4 to 6 years, which is consistent with the age of children showing the highest relative proportion of RT-PCR-negative PIV seroresponders. Increasing evidence suggests that PIV4 is an important contributor to acute respiratory illness in young children (31). Although we did not test for PIV4 in our study, PIV4 infections might produce heterotypic antibodies that cross-react in the other PIV seroassays. Improvements in PIV serodiagnostics are needed in order to achieve more-specific identification.

Interestingly, we also found evidence of possible serologic cross-reactions between RSV and HMPV in some study patients. Simultaneous antibody rises in response to both viruses were detected in 28 patients, only 4 of whom were confirmed positive for both viruses by RT-PCR. In contrast to the pattern for PIV, the seasonal peaks of RSV and HMPV activity in the United States overlap, increasing opportunities for true coinfections. However, as we and others have shown, these viruses share antigenic sites on their nucleocapsid (32) and fusion (33, 34) proteins, and though less common, heterotypic antibody responses following infections with these vi-

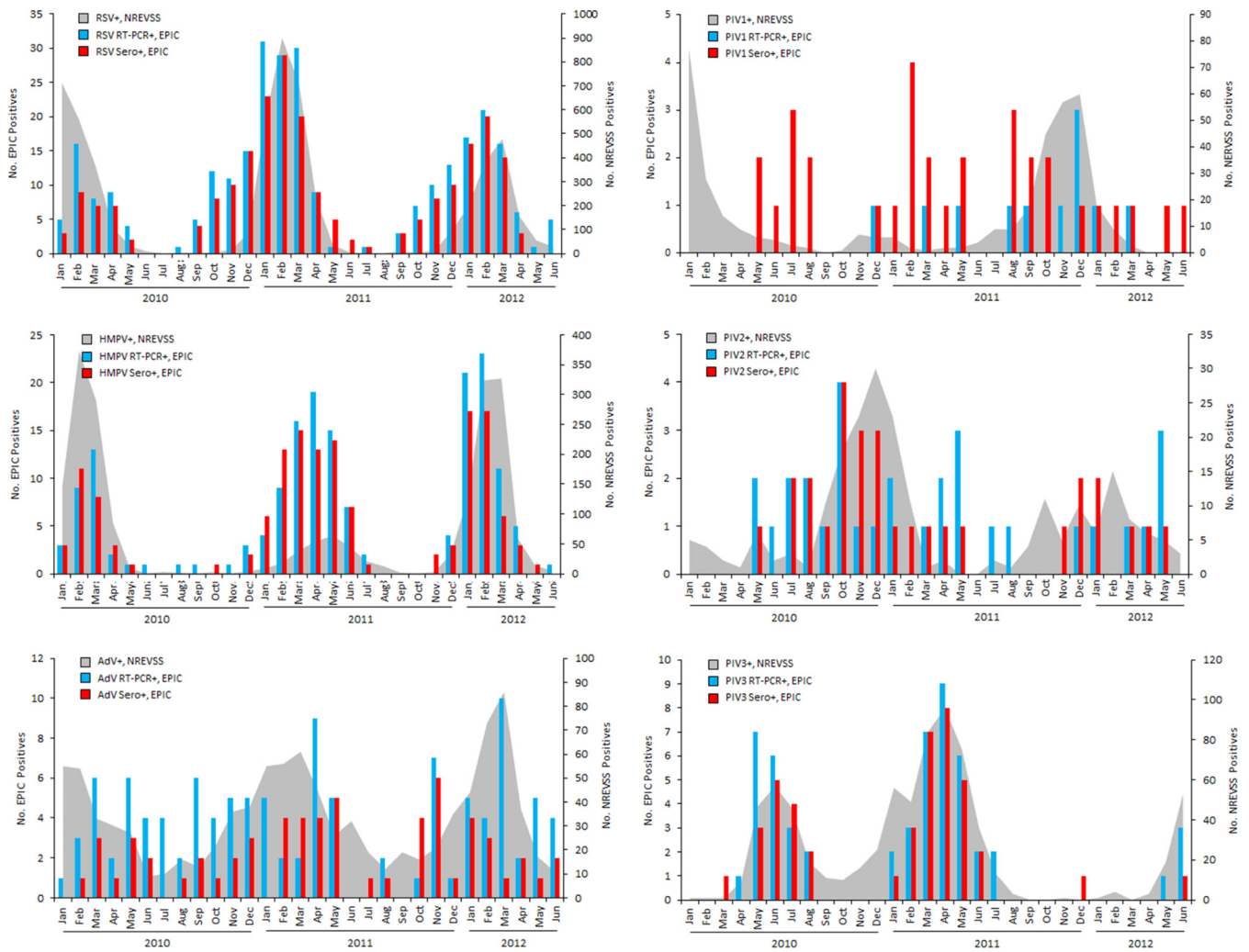


FIG 2 Temporal distribution of serology- and RT-PCR-positive test results (colored bars) superimposed on CDC National Respiratory and Enteric Virus Surveillance System (NREVSS) regional surveillance data for each of the viruses studied. The NREVSS data shown here were collected on a weekly basis between January 2010 and June 2012 from reporting centers located in the metropolitan areas of the four cities where the EPIC study was conducted.

ruses have been demonstrated in children presenting with acute febrile respiratory illnesses (32). As with PIV, serologic coresponses to RSV and HMPV should be interpreted with caution.

This study had several limitations. No “gold standard” reference assay to compare method performance was available. Although patients with positive results obtained by both methods or by neither method could be classified with some confidence, discordant results could not be easily resolved. Serum specimens from asymptomatic controls were not available for assessment, and more than half of the EPIC study patients did not have convalescent-phase serum samples for testing. No serologic assays were available for the antigenically diverse rhinoviruses that were identified in 27% of children and 9% of adult patients (5, 6). We did not assess the role of coinfections with other respiratory pathogens, on which a separate study has been published (15). Finally, accurate laboratory detection of acute virus infections does not necessarily equate with disease causation. CAP etiology must be determined on a case-by-case basis, with full assessment of the clinical, demographic, and epidemiological context of the laboratory test results. Ultimately, we found that the addition of serology in the EPIC study provided clear diagnostic benefit, but its inclusion in future studies must be weighed against the added cost and challenges of test interpretation.

MATERIALS AND METHODS

Patients and specimens. From 1 January 2010 to 30 June 2012, children and adults admitted to eight hospitals (three located in Chicago, IL, one in Memphis, TN, three in Nashville, TN, and one in Salt Lake City, UT) were enrolled in the EPIC study if they met a clinical and radiographic case definition of CAP (5, 6). Most patients had combined naso- and oropharyngeal (NP/OP) swabs collected within 3 days of hospital admission, and 42% also had acute-phase blood samples drawn at admission and convalescent-phase blood samples obtained 2 to 10 weeks later (mean time interval, 32.5 days). NP/OP swabs were collected in universal transport media. Serum was separated from clotted blood, and all samples were aliquoted and stored at -70°C prior to testing. Only patients with complete NP/OP samples and both acute- and convalescent-phase serum samples were included in this study. In addition, for the previously published primary analysis of the EPIC study (5, 6), only patients with CAP as determined by a study radiologist were included; however, for this analysis, we included all enrolled patients because of the limited number of specimens available for analysis.

Real-time RT-PCR assays. CDC-developed real-time RT-PCR assays for multiple respiratory pathogens, including RSV, HMPV, PIV1 to PIV3, and AdV, were performed on NP/OP swabs at each study site as described previously (6). Quality assurance and monitoring procedures, including on-site inspections, external proficiency testing, and standardized protocols, were performed at the sites (35). Specimens with virus-specific RT-PCR cycle threshold (C_T) values of <40 and with a positive RT-PCR test result for the human RNase protein housekeeping gene were considered positive for the respective viral pathogen. Although an imperfect measure of virus load, C_T values were used as a semiquantitative surrogate for virus loads in the upper respiratory tracts of RT-PCR-positive patients to assess the relationship between viral loads and detectable seroresponse.

Serology assays. Serum samples were tested at the CDC. Serology for RSV, HMPV, PIV1 to -3, and AdV was performed by in-house indirect IgG enzyme immunoassays (EIAs) using viral culture lysate antigen as described previously (36). A ≥ 4 -fold rise in the IgG antibody titer was considered a positive seroresponse and diagnostic for acute virus infection. Serum specimens that gave high background signals with the uninfected cell control antigen in the EIA, or in which the titer of antibody rose or fell in response to multiple viruses, indicating possibly mismatched serum pairs, were excluded from the analysis. Due to known serologic cross-reactions among PIVs (see below), test results for these viruses were combined.

Because different methods (hemagglutination inhibition and microneutralization assays) were used for influenza serology, and influenza vaccination of some patients complicated interpretation of their serology results, a comparison of influenza serology and PCR is planned for a separate report.

NREVSS data. Positive test results obtained in this study were graphed by diagnostic method and collection date against regionally reported data obtained from the CDC National Respiratory and Enteric Virus Surveillance System (NREVSS) in order to assess the temporal concordance of the diagnostic methods with respiratory virus circulation patterns observed independently over the study period. NREVSS is a laboratory-based passive surveillance network that monitors temporal and geographic patterns associated with the detection of respiratory and other viruses in the United States. Data are collected from collaborating universities, community hospitals, state and county public health departments, and commercial laboratories that report viruses by a combination of PCR, antigen detection, or culture isolation methods on a weekly basis. For this analysis, NREVSS data were obtained each year between 2010 and 2012 from a range of 15 to 27 different reporting centers located in the metropolitan areas of the four cities where the EPIC study was conducted.

Statistical analysis. The overall agreement between serology and PCR for the respective respiratory viruses was measured using Cohen's kappa coefficient (κ). Agreement was defined as poor (κ , <0.20), fair (κ , 0.21 to 0.40), moderate (κ , 0.41 to 0.60), good (κ , 0.61 to 0.80), or very good (κ , 0.81 to 1.0) as described previously (37). The increased diagnostic yield contributed by serology was calculated by the following formula: [(specimens positive by serology and negative by RT-PCR)/(specimens positive by both methods + specimens negative by serology and positive by RT-PCR)]. To assess the relationship between patient age and diagnostic yield, patients were further divided into 6 age groups (0 to 6 months, 7 months to 2 years, 3 to 5 years, 6 to 17 years, 18 to 64 years, and ≥ 64 years).

To determine if a higher viral load was associated with the development of a diagnostic seroresponse (a ≥ 4 -fold rise in the antibody titer), C_T values among RT-PCR-positive patients who did and did not develop a seroresponse were compared using the Mann-Whitney U test. Because heterotypic (cross-reactive) seroresponses can occur following infection with some respiratory viruses, leading to potential false-positive results, the numbers of patients with seroresponses to more than one virus were evaluated in order to determine if the association was greater than that expected by chance (28, 32). To determine if potential heterotypic antibody responses between viruses occurred in this study, we compared the number of cooccurring seroresponses for all virus combinations with those expected by chance using Fisher's exact test. P values of <0.05 were considered statistically significant.

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The Etiology of Pneumonia in the Community (EPIC) study conducted by the CDC,

three U.S. children's hospitals, and five U.S. adult medical centers estimated the burden of community-acquired pneumonia hospitalizations among U.S. children and adults from 1 January 2010 to 30 June 2012.

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