

PAPER**PATHOLOGY/BIOLOGY**

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Use of an Automated Nested Multiplex Respiratory Pathogen PCR Panel Postmortem in the Pediatric Forensic Setting*

ABSTRACT: Respiratory pathogens have been detected in forensic investigations using multiple techniques; however, no study has examined the use of automated, nested, multiplex polymerase chain reaction (ANM-PCR), commonly used in living patients, in the forensic setting. This retrospective study assessed the utility of ANM-PCR in detecting respiratory pathogens in the pediatric forensic setting. Respiratory samples from 35 cases were tested for up to 20 respiratory pathogens. 51.4% of these cases yielded a positive ANM-PCR result, 20% of which were considered the cause of or contributory to death. The most commonly detected pathogens were rhinovirus/enterovirus and respiratory syncytial virus, and these were the only pathogens determined to play a significant role in cause of death. The sampled sites and postmortem intervals tested did not affect the likelihood of a positive or negative test. ANM-PCR panels are effective, affordable, and rapid ancillary tools in evaluating cause of death in the forensic pediatric population.

KEYWORDS: forensic science, pediatrics, respiratory syncytial virus, rhinovirus, enterovirus, influenza, adenovirus, multiplex polymerase chain reaction, nested polymerase chain reaction, respiratory infection

Respiratory infections are a significant cause of infant and childhood mortality worldwide (1). Numerous respiratory pathogens are potentially fatal, from those expected to cause severe symptoms, such as respiratory syncytial virus (RSV), to more common, seemingly innocuous pathogens like rhinoviruses and enteroviruses. RSV infection commonly results in childhood hospitalization in the U.S.A., and the risk of mortality is greater in children less than a year old and/or those with a complex chronic health condition (2). Human rhinoviruses and enteroviruses were shown to be fatal in 2.1% of children admitted to intensive care in a recent study, and mortality in those cases was associated with an immunocompromised state, bacterial coinfection, and/or severity of illness (3).

Molecular techniques have recently become useful as rapid diagnostic tools due to innovations in automation, decreased cost, and resultant increased accessibility. Such techniques are already changing the epidemiologic landscape among hospitalized patients (3). In the past, multiple techniques including histology, immunohistochemistry (IHC), electron microscopy, Gram stain, culture, serology, and molecular techniques have been implemented to detect respiratory pathogens in the forensic setting (4–17). Molecular techniques have included enzyme-linked immunosorbent assay, *in situ* hybridization, next-generation sequencing and multiple variations on polymerase chain reaction (PCR) including PCR,

reverse transcriptase PCR, real-time methods, and nested, multiplex PCR. In addition, these PCR techniques have been performed on fresh, frozen, formalin-fixed, and paraffin-embedded tissues (5–9,11–16). Postmortem studies confirmed that PCR techniques are more sensitive at detecting respiratory pathogens compared to IHC and viral culture (7,9,14,18,19). Traditionally, PCR-based methods were complex and required extensive training in molecular biology, as well as ample time, laboratory space, and personnel for the running of assays. However, automated, nested, multiplex PCR (ANM-PCR) can now rapidly and thoroughly evaluate respiratory infections in living patients. This technology offers automated detection of multiple pathogens at once using nested PCRs which have increased sensitivity over traditional PCR (20). ANM-PCR is technically streamlined so that minimal training and little hands-on time with samples is needed for successful analysis, thus making sophisticated molecular techniques more readily available to laboratories that do not have space and/or funding to run a full molecular laboratory. To our knowledge, no prior study has been conducted assessing an ANM-PCR respiratory pathogen panel on fresh postmortem samples. The potential benefits of such analysis include a greater breadth of pathogen detection with minimal labor and decreased turn-around time of results. As costs have decreased, implementation of sophisticated molecular testing in the postmortem setting has become possible. The purpose of the current study was to determine the utility of ANM-PCR respiratory pathogen panels in evaluating cause of death in a pediatric population.

Materials and Methods

To ascertain the utility of an ANM-PCR respiratory pathogen panel in aiding cause of death determination, such tests were

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performed by six forensic pathologists on 35 of 145 coronial pediatric decedents from December 1, 2011, to June 30, 2015, in the Medical and Forensic Autopsy Section of the Department of Pathology and Laboratory Medicine at the Medical University of South Carolina. The decedents ranged from 2 weeks to 12 years of age. Cases were selected for PCR on the basis of the attending pathologists' suspicion that a respiratory illness may have contributed to death. Samples were acquired via postmortem mucosal swabs from nasal, nasopharyngeal, tracheal, and/or bronchial regions. Samples were then analyzed using an ANM-PCR panel of respiratory pathogens, specifically the BioFire FilmArray[®](20) (BioFire Diagnostics, Inc., Salt Lake City, UT). Briefly, each sample was acquired from a patient in the form of a mucosal swab, which was placed into BD Universal Viral Transport for Viruses, Chlamydiae, Mycoplasmas and Ureaplasmas (Becton, Dickinson and Company, Franklin Lakes, NJ). Specimens were transported in person to the microbiology laboratory within 2 h from the time of procurement. Upon receipt in the microbiology laboratory, hydration solution was injected into a compartmentalized pouch where the ANM-PCRs occur, the patient sample was mixed with denaturing buffer and added to the pouch, the pouch was then inserted into the analyzer, and the remaining reactions and analyses were performed by the bench top instrument. Two process controls were included in each pouch, an RNA process control and a PCR2 control. The RNA Process Control assay targets an RNA transcript from the yeast *Schizosaccharomyces pombe*. The yeast is present in the pouch in a freeze-dried form and becomes rehydrated when sample is loaded. The control material is carried through all stages of the test process, including lysis, nucleic acid purification, reverse transcription, first-stage PCR, dilution, second-stage PCR, and DNA melting. A positive control result indicates that all steps carried out in the FilmArray RP pouch were successful. The PCR2 Control assay detects a DNA target that is dried into wells of the array along with the corresponding primers. A positive result indicates that the second-stage PCR was successful. Both control assays must be positive for the test run to pass. When either control fails, the controls field of the test report will display "Failed" and all results will be listed as invalid. External positive and negative controls were run with each new lot of reagents.

Over the course of the study, the respiratory pathogen panel detected from 15 (version 1) to 20 (version 2) potentially pathogenic agents. The initial panel screened for adenovirus, coronavirus (229E and HKU1), human metapneumovirus, human rhinovirus/enterovirus, influenza (A, A/H1, A/H1 2009, A/H3, and B), parainfluenza (1–4), and RSV. The most recent panel also included coronavirus (OC43 and NL63), *Bordetella pertussis*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*. Rhino- and enteroviruses are grouped together because multiplex PCR tests cannot reliably distinguish between them due to their high degree of genetic similarity (3). Complete autopsies, including histology and toxicology, were conducted on each case. Due to the retrospective nature of this study, investigational techniques were not standardized. At minimum, each autopsy investigation included examination of each body cavity and organ, including the cranial cavity and brain. Histologic sections were submitted on all of the study cases and typically included heart, lung, liver, and kidney tissue. Bacterial cultures were performed in some cases at the discretion of the pathologist. Vitreous electrolytes were regularly performed on nontraumatic cases without antemortem documentation of electrolyte levels. The contribution of each positive PCR result to the cause of death was critically examined and interpreted based on the autopsy findings and

known circumstances surrounding death by the certifying pathologist.

For data analysis, forensic reports were reviewed for the tested cases. The swab location was categorized as nasal, nasopharyngeal, tracheal, bronchial, or respiratory site, not otherwise specified, based on information provided in the forensic reports and laboratory information system. Postmortem intervals were estimated based on information within the forensic reports and rounded to the nearest hour. The positive ANM-PCR results were divided into three categories based on the determined contribution to the cause of death: (i) significant, (ii) not significant, or (iii) undetermined. The contribution of ANM-PCR results to cause of death was determined solely on statements within the final reports directly related to the cause of death or ANM-PCR results. "Significant" results were clearly reflected in the report as causing or contributing to death, "not significant" results were those in which the test result was clearly not contributory to the death based on documented statements of the certifying pathologist, and results of "undetermined significance" were those in which the role of the pathogen remained indeterminate relative to cause of death based on the final report. Chi-square analyses were performed using GraphPad Prism version 6.0h, ©1994–2015 (GraphPad Software, Inc., La Jolla, CA).

Results

Respiratory pathogen PCR panels were completed in 24.1% (35/145) of 0- to 12-year-old decedents examined (Fig. 1). Of those, 18 (51.4%) yielded positive PCR results. Potentially pathogenic agents detected included rhinovirus/enterovirus (13 cases), RSV (five cases), adenovirus (two cases), coronavirus NL63 (two cases), and influenza B (one case) (Fig. 2).

In 38.9% (7/18) of cases with a positive respiratory pathogen ANM-PCR result (or 20% of total tested cases), a detected pathogen was considered significant to the cause of death (Fig. 3). Three (16.7%) of the cases with a positive ANM-PCR result were determined not significant in the cause of death. The role of the detected pathogen in the eight remaining ANM-PCR-positive cases remained undetermined. Of the 23 cases in which the decedent was under 1 year of age, 10 (43.5%) had a positive

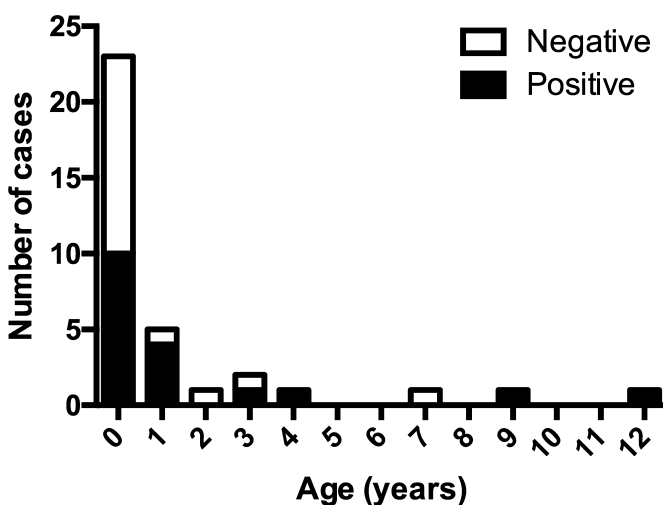


FIG. 1—Age distribution of the 35 decedents on which automated, nested, multiplex PCR (ANM-PCR) respiratory pathogen panel was performed. Positive and negative ANM-PCR results are depicted.

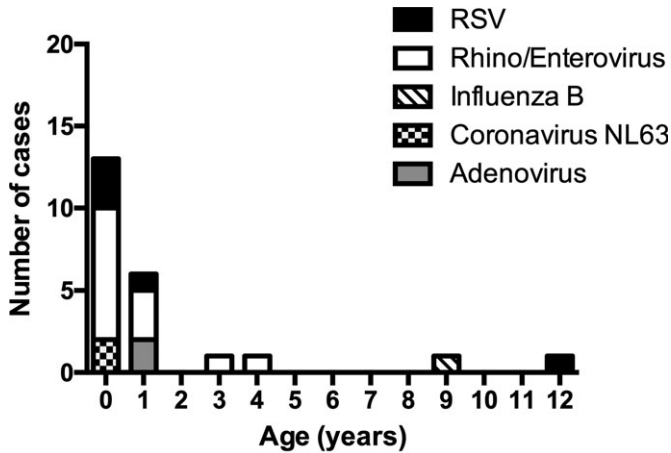


FIG. 2—Respiratory pathogens and age distribution. The 18 cases with positive results from automated, nested, multiplex PCR (ANM-PCR) for potential respiratory pathogens are depicted. RSV, respiratory syncytial virus.

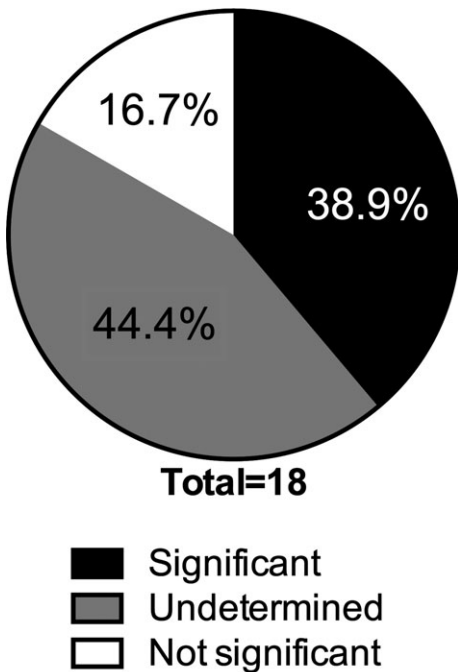


FIG. 3—Proportions of positive automated, nested, multiplex PCR (ANM-PCR) results that were not significant, undetermined, or significant to cause of death.

ANM-PCR result. Of these, 40% (4/10) were significant, 50% (5/10) were of undetermined significance, and 10% (1/10) were not significant to cause of death. The significance of each detected potential pathogen to cause of death is depicted in Fig. 4.

Rhinovirus/enterovirus was the most commonly detected potential pathogen (12/18, 66.7%). Results in 33.3% (4/12) of cases that tested positive for rhinovirus/enterovirus were significant to the cause of death, 58.3% (7/12) were of undetermined significance, and 16.7% (2/12) had no significance. RSV was the second most commonly detected pathogen (5/18, 27.8%). Sixty percent (3/5) of RSV positive results were significant to the cause of death, and 40% (2/5) were of undetermined

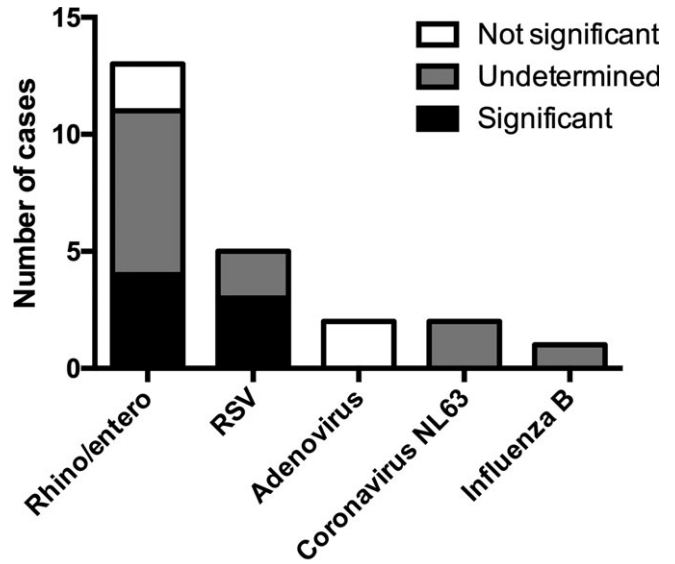


FIG. 4—Relative frequency of pathogen detection by automated, nested, multiplex PCR (ANM-PCR) and the significance to cause of death. The detected potential pathogens are on the y-axis. RSV, respiratory syncytial virus.

significance. The three remaining detected potential pathogens (adenovirus, coronavirus NL63, and influenza B) were determined not to be significant to the cause of death.

Coinfections/exposures were documented by ANM-PCR in four cases, all of which were in decedents ≤ 1 year of age (Table 1). One case was positive for rhinovirus/enterovirus and coronavirus NL63. A second case was positive for rhinovirus/enterovirus, RSV, and coronavirus NL63. A third case demonstrated coinfection with rhinovirus/enterovirus and RSV. The contributions of the potential pathogens in these three cases to cause of death remained undetermined. A fourth case was positive for coinfection with rhinovirus/enterovirus and adenovirus. This sample was sent to an outside laboratory for further characterization, which revealed the presence of coxsackievirus A6. Coxsackievirus A6 encephalitis was determined to be the cause of death.

Samples from seven cases with positive PCR results for rhinovirus/enterovirus, including the case just mentioned, were ultimately sent to the Centers for Disease Control for further viral subtyping by reverse transcription, seminested PCR amplification of the genetic sequence encoding the viral capsid protein VP1 (21). These cases were submitted for further viral typing in large part due to the enterovirus D68 outbreak in 2014 in which more than 1000 positive cases were reported, many of which involved children with significant respiratory disease (22). The pathogens identified on further workup of these seven cases were enterovirus D68, coxsackievirus A4, coxsackievirus A6, human rhinovirus 49, human rhinovirus B6, human rhinovirus 77, human rhinovirus A63, and parechovirus. Three of these pathogens (coxsackievirus A6, human rhinovirus 49, and human rhinovirus 77) were significant to the cause of death.

For sixteen of the 35 cases tested, the cause of death remained unexplained (Table 1). Eight of these 16 (50%) cases yielded a positive ANM-PCR result, all of which detected rhinovirus/enterovirus. One case had known antemortem respiratory viral PCR testing, and the results were congruent with postmortem ANM-PCR panel results.

TABLE 1—Coronial cases (35) on which automated, nested, multiplex PCR for potential respiratory pathogens was performed.

Age	Sex	Swab Location	PCR Result*†	COD	Significance of PCR to COD	PMI (Nearest Hour)
0	F	Nasopharyngeal	Negative†	Undetermined	n/a	29
0	M	Nasopharyngeal	Negative*	Positional asphyxia	n/a	19
0	M	Nasopharyngeal	Negative†	Undetermined	n/a	6
0	M	Respiratory site, NOS	Negative†	Asphyxia due to overlay	n/a	10
0	M	Respiratory site, NOS	Negative*	Sudden unexplained infant death associated with prematurity (preterm birth)	n/a	26
0	M	Nasopharyngeal	Negative*	Probable asphyxia due to overlay	n/a	25
0	M	Respiratory site, NOS	Negative*	Sudden unexpected infant death	n/a	25
0	F	Respiratory site, NOS	Rhino/entero, CorNL63†	Undetermined	Undetermined	24
0	F	Nasal	RSV†	RSV bronchiolitis with associated acute pneumonia	Significant	25
0	M	Tracheal	Rhino/entero (entero D68)†	Undetermined	Undetermined	<12
0	M	Respiratory site, NOS	Rhino/entero (HRV-49)†	Acute and organizing pneumonia complicating HRV-49 infection	Significant	3
0	M	Nasopharyngeal	Rhino/entero (HRV-77)†	Diffuse alveolar damage due to rhinovirus infection	Significant	21
0	F	Nasopharyngeal	Rhino/entero (HRV-A63)†	Undetermined, sudden unexpected death in infancy	Undetermined	40
0	F	Respiratory site, NOS	Negative†	Complications of perinatal hypoxic-ischemic encephalopathy	n/a	24
0	F	Nasopharyngeal	RSV†	Respiratory bronchiolitis due to RSV infection	Significant	13
0	M	Nasopharyngeal	Rhino/entero, RSV, CorNL63†	Undetermined	Undetermined	23
0	M	Nasopharyngeal	Negative*	Positional asphyxia with smothering (wedging)	n/a	Unknown
0	F	Nasopharyngeal	Negative†	Undetermined	n/a	6
0	F	Respiratory site, NOS	Rhino/entero‡ (HRV-B6)†	Sudden unexpected death in infancy	Undetermined	23
0	M	Nasopharyngeal	Negative*	Complications of extreme prematurity	n/a	17
0	M	Respiratory site, NOS	Negative*	Sudden unexpected infant death	n/a	20
12	M	Bronchial	RSV*	Lymphocytic myocarditis complicating RSV infection	Significant	21
3	M	Nasopharyngeal	Rhino/entero*	Septic complications of necrotizing gastroenteritis	Not significant	24
3	M	Bronchial lavage	Negative†	Idiopathic pulmonary hypertension, following <i>Pneumocystis jirovecii</i> pneumonia, complicating bone marrow transplant for familial hemophagocytic lymphohistiocytosis	n/a	24
2	M	Nasal	Negative†	Undetermined	n/a	21
7	M	Nasopharyngeal	Negative†	Complications of porencephaly (congenital brain anomaly)	n/a	20
4	M	Nasopharyngeal	Rhino/entero†	Probable electrolyte/pH abnormalities due to gastrointestinal viral syndrome	Undetermined	26
1	F	Nasopharyngeal	Adeno†	Congenital cardiac anomalies: ventricular septal defect, patent foramen ovale, aortic coarctation	Not significant	26
9	M	Nasopharyngeal	Influenza B†	Acute bronchopneumonia in the setting of polymicrogyria	Undetermined	8
1	M	Nasopharyngeal	Negative†	Complications of congenital heart defects	n/a	24
1	M	Nasopharyngeal	Adeno, Rhino/entero (coxsackievirus A6)†	Encephalitis due to coxsackievirus A6 infection	Significant	24
0	M	Nasopharyngeal	Rhino/entero†	Undetermined	Not significant	14
1	F	Nasopharyngeal	Rhino/entero†	Undetermined	Significant	24
0	M	Nasopharyngeal	Negative†	Undetermined (sudden unexpected death in infancy)	n/a	28
1	M	Nasopharyngeal	RSV, Rhino/entero (coxsackievirus A4, parechovirus)†	Sudden unexpected death in childhood	Undetermined	29

COD, cause of death; CorNL63, coronavirus NL63; F, female; HRV, human rhinovirus; M, male; PMI, postmortem interval; Rhino/entero, rhinovirus/enterovirus; RSV, respiratory syncytial virus.

*Results obtained using BioFire FilmArray® version 1.

†Results obtained using BioFire FilmArray® version 2.

‡Congruent antemortem and postmortem automated nested multiplex PCR panel results.

Sampling of the airways occurred along multiple sites along the respiratory tract, including nasal, nasopharyngeal, tracheal, bronchial, and unspecified sites (Fig. 5). Only a single site was sampled in each case, and the site was determined at the discretion of the pathologist as no definitive gold standard existed. Each of these sample sites yielded at least one positive ANM-PCR result. Chi-square analysis revealed no significant difference in the number of positive and negative ANM-PCR results from the different sites ($\chi^2(1, n = 35) = 0.5362, p = 0.4640$).

Positive PCR results were acquired in postmortem intervals ranging from 3 to 40 h, and 40 h was the longest postmortem

interval in the dataset. Chi-square analysis was performed using four postmortem interval categories: <12, 12–24, 24–36, and >36 h, and there was no significant difference in the number of positive and negative ANM-PCR results from the postmortem interval ranges tested ($\chi^2(1, n = 34) = 0.02738, p = 0.8686$; Fig. 6).

Manners of death in cases not tested using ANM-PCR were as follows: one suicide, 20 natural deaths, 42 accidents, 10 homicides, and 37 undetermined. Causes of death in these cases were as follows: 35 asphyxiations, 20 traumatic deaths, 22 with natural disease, and 33 that remained undetermined.

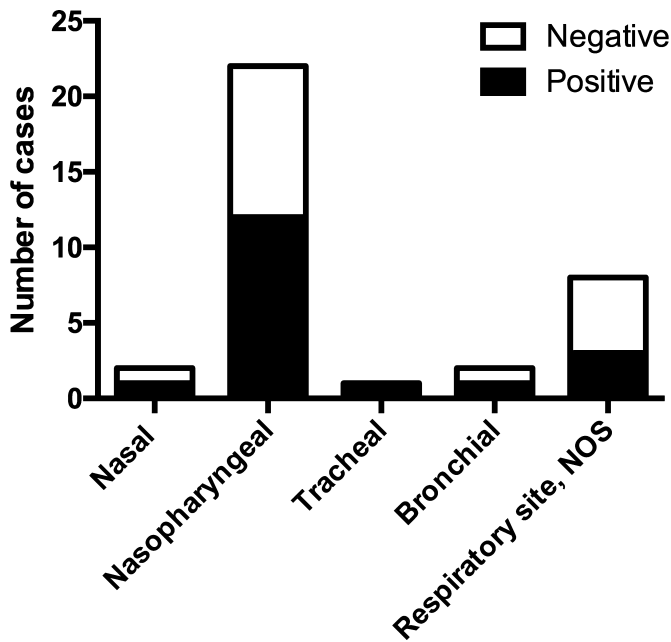


FIG. 5—Relationship of sampling location to pathogen detection. Sampling locations for all 35 cases are represented. Results of the automated, nested, multiplex PCR (ANM-PCR) panel for potential respiratory pathogens are depicted as positive or negative. NOS, not otherwise specified.

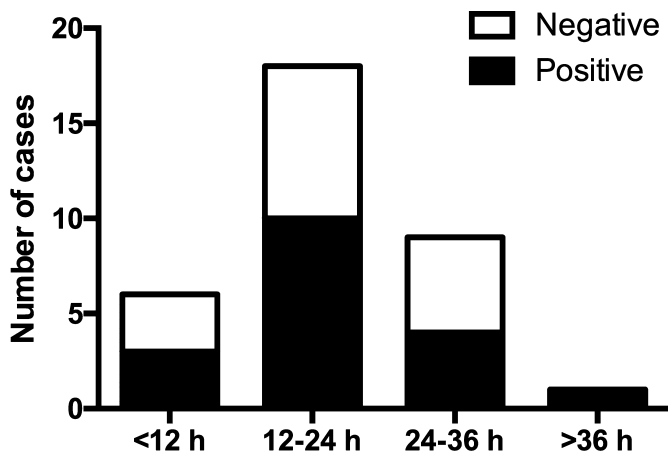


FIG. 6—Relationship between postmortem interval and pathogen detection. Postmortem interval categories for the 34 cases with known time interval of death are represented. The postmortem intervals are rounded to the nearest hour. Results of the automated, nested, multiplex PCR (ANM-PCR) panel for potential respiratory pathogens are depicted as positive or negative. h, hours.

Discussion

These results indicate that an ANM-PCR respiratory pathogen panel currently used for diagnostic purposes in living patients can be applied at the time of autopsy to aid in determining the cause of death. In the current study, at least 24.1% (35/145) of pediatric forensic cases warranted the use of a respiratory pathogen ANM-PCR panel based on pathologist suspicion. 51.4% of these cases yielded a positive ANM-PCR result, and of the positive cases 38.9% (20% of tested cases) yielded a result that was significant to the cause of death. In decedents under 1 year of age, 40% of cases that tested positive for a respiratory pathogen were significant to the cause of death.

Of the 20 potential pathogens included in the panel, only five were detected in pediatric decedents during this study period. The ratio of detected to tested pathogens is low, but the use of molecular techniques, such as PCR and IHC, to diagnose respiratory pathogens has historically been limited by the need to identify specific potential infections prior to testing to choose appropriate primers or antibodies, thus leaving many potential infectious agents untested and potentially overlooked. A benefit of the ANM-PCR panel is the ability to cast a wide net with a sensitive technique without significantly increasing turn-around time or cost if an unexpected pathogen is involved. The available panel expanded during the course of the present study from 15 to 20 potential pathogens, and panels will likely continue to include more candidate pathogens in the future for the same or decreasing cost.

Rhinovirus/enterovirus infections are common; thus, it was not surprising that 72.2% (13/18) of positive cases documented exposure to these potential pathogens. In approximately half of these cases, the role of these viruses in the cause of death was unclear. Explanations exist regarding these trends: (i) these viruses are extremely common with typically mild presentations, and (ii) shedding of these viruses can occur for weeks following acute illness (23). Despite the low level of significance of these potential pathogens to cause of death, it remains important to include such pathogens in respiratory pathogen panels. Previous studies have indicated that these viruses can contribute to death, although the incidence is low (3).

Respiratory syncytial virus was the second most commonly detected potential pathogen and exhibited the highest percentage of cases that were significant to cause of death (60%). Forensic pathologists are positioned to enhance public knowledge of the epidemiology of respiratory pathogens, such as RSV, among decedents from the community that have not been hospitalized (7). Continued use of ANM-PCR respiratory pathogen panels in the forensic setting will broaden understanding of significant respiratory pathogens, such as RSV, in the community.

During the course of this study, sample sites from the nasal cavity to the lower airways were utilized, all of which yielded at least one positive ANM-PCR result. Furthermore, there was no significant difference in potential pathogen detection among the sites tested. In living patients, the nasopharyngeal swab is ideal as it is relatively less invasive than the more inferior samples sites, such as tracheal and bronchial mucosae. At the time of autopsy, the entire respiratory tract is available for sample acquisition. Therefore, the existence of a site or sites more amenable to potential pathogen detection using ANM-PCR is possible. Furthermore, different pathogens may demonstrate different optimal sample sites. At this time, data are not available regarding the optimum sample site(s) for detection of potential respiratory pathogens by ANM-PCR in the forensic setting. Further testing, possibly including testing multiple sites from a single decedent, could clarify the presence or absence of the optimal sample site for postmortem respiratory pathogen detection using automated PCR techniques.

Detection of potential respiratory pathogens by ANM-PCR from swabs of the respiratory tract was possible at the highest postmortem interval tested in the current study, 40 h. In addition, there was no significant difference in detection of potential respiratory pathogens among the postmortem interval ranges tested. Postmortem interval has not routinely been reported in other studies, but future investigation into the reliability and outer limits of detection of potential respiratory pathogens by

molecular methods is necessary for further validation of such techniques.

The cost and time investments for implementing these tests are relatively low. The instrument costs c. \$35,000, and each pouch, in which the ANM-PCR reactions take place, is about \$140. The time required for inoculating the sample into the pouch and placing the pouch on the machine is 3 min, and the results are reportable in about an hour. Furthermore, one can successfully learn to prepare the samples and operate the machine with little training.

Of the 110 cases that were not tested using ANM-PCR, half (55/110) were due to either asphyxia or trauma with significant autopsy or scene findings sufficient to explain the cause of death. In addition, many of the untested cases in which a natural disease process contributed to the cause of death exhibited florid disease that would preclude ANM-PCR testing. However, cause of death in 33 of the untested cases remained undetermined. It is impossible to determine whether ANM-PCR testing would have proved beneficial in these cases. Future, carefully standardized, prospective studies of ANM-PCR in the forensic setting should be performed to develop solid guidelines for its future implementation.

The current study had several limitations that might be improved upon in future investigations. Epidemiologically, the study was limited by the small sample size, so generalizations regarding these potential pathogens in the community cannot yet be extrapolated. The respiratory pathogen panel tested did not include all existing potential pathogens, so pathogens may have been present that were not included in the panel. Furthermore, potential pathogens that were added to the panel later in the study may have been undetectable in earlier cases. The respiratory pathogen status of cases that were not tested remains unknown. The sample sites tested were left to the pathologists' discretion. The contribution of the potential pathogens detected to the cause of death was up to the interpretation of the pathologist based on all of the elements of each individual case. Thus, it is possible that two pathologists could disagree regarding the significance of potential pathogens to cause of death. The sample sites were not standardized. The postmortem intervals did not exceed 40 h, so the utility of the ANM-PCR respiratory pathogen panel beyond 40 h is unclear. Also, low sample size and lack of experimental controls disallowed any measurement of sensitivity of the test at any time point.

In summary, regular use of ANM-PCR panels postmortem could have a significant impact on our knowledge of public health and epidemiology. Results of the current study demonstrate that this affordable, easy-to-use technology is useful in the evaluation of the cause of death in the pediatric forensic population. Further study to identify ideal sample sites and postmortem time intervals for the optimum use of such technology is warranted in both pediatric and adult populations.

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