

Enhanced Identification of Viral and Atypical Bacterial Pathogens in Lower Respiratory Tract Samples With Nucleic Acid Amplification Tests

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The advantages of nucleic acid amplification tests (NAT) over conventional methods for the detection of pathogens in lower respiratory tract samples have not been established. NAT for respiratory pathogens were performed on 439 endotracheal tube (ETT) and bronchoalveolar lavage (BAL) samples. A potential pathogen was detected in 87 samples. Of 22 samples that tested positive by conventional methods, 15 tested positive for the same pathogen by NAT, 1 tested positive for a different pathogen, 2 had co-infections identified only by NAT, and 4 tested negative by NAT. An additional 73 pathogens were detected by NAT in 65 samples including 30 pathogens that were missed by conventional methods (19 adenovirus, 6 respiratory syncytial virus, 3 parainfluenza virus 1–4, 2 influenza A), 41 pathogens not routinely identified by conventional methods in most laboratories (23 rhinovirus, 8 human coronavirus OC43, 5 human metapneumovirus (hMPV), 2 human coronavirus 229E, 2 human coronavirus NL63, 1 *Chlamydomphila pneumoniae*) and 2 pathogens from samples where no respiratory virus testing was requested (1 influenza A, 1 parainfluenza virus). Four of 52 patients who had multiple BAL samples submitted on the same day had negative and positive results by NAT on different samples. NAT improves detection of potential pathogens from ETT and BAL samples. **J. Med. Virol. 78:702–710, 2006.** © 2006 Wiley-Liss, Inc.

KEY WORDS: bronchoscopy; pneumonia; polymerase chain reaction; respiratory tract infection; viral pneumonia

INTRODUCTION

Determining the etiologic agent of bronchoalveolar lavage infections (LRTI) promotes targeted therapy and prevents unnecessary investigations, antimicro-

bials, and hospitalization. Compared to detection in sputum, detection of pathogens in the lower tract is thought to have higher specificity for typical bacterial pathogens and higher sensitivity for atypical pathogens such as *Pneumocystis jirovecii*, *Mycobacterium* species, and cytomegalovirus [Bartlett et al., 2000], with the advantages being less clear for detection of respiratory viruses, *Mycoplasma pneumoniae* (MP) or *Chlamydomphila pneumoniae* (CP). Conventional methods of pathogen detection including antigen detection methods and traditional viral culture (TVC) do not usually allow for identification of newly identified viral pathogens such as human metapneumovirus (hMPV) [van den Hoogen et al., 2001] and the human coronaviruses (hCoV) OC43, 229E, and NL63 [Fouchier et al., 2004]. Nucleic acid amplification testing (NAT) has been used to rapidly detect viruses, MP and CP in nasopharyngeal samples (NPS) from patients with LRTI [Grondahl et al., 1999; Michelow et al., 2004; Miyashita et al., 2004], and may be more sensitive than conventional methods, especially for MP and CP [Miyashita et al., 2004].

The primary objective of this study was to determine if routine addition of NAT for multiple respiratory viruses, MP, and CP to diagnostic investigation of bronchoalveolar lavage (BAL) and endotracheal tube (ETT) samples would detect a significant number of pathogens that were missed by conventional methods. A secondary objective was to determine if testing by conventional or NAT methods would yield discordant results if multiple samples were submitted on the same day.

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METHODS AND MATERIALS

All samples obtained by ETT or bronchoscopic methods (protected brush specimen or BAL) at the University of Alberta Hospital and submitted to the Provincial Laboratory of Public Health (Microbiology), Edmonton for any microbiologic investigations from April 12, 2003 to July 30, 2004 that had sufficient volume for testing were used in the study.

Conventional Testing (Antigen Detection and Culture)

All conventional testing was performed as per submitter's request. Direct fluorescent antibody (DFA) tests for respiratory syncytial virus (RSV), parainfluenza virus (PIV), influenza A (Flu A) and B (Flu B) (ImagenTM, DakoCytomation Ltd.) were performed. Rapid respiratory culture (RRC) with mixed cell lines (mink lung (Mv1Lu) and NCI H292) and immunofluorescent staining (Light DiagnosticsTM, Chemicon[®] International) in a 24-well plate format [Lee et al., 1992; Huang and Turchek, 2000] was available from October 1, 2003 to July 30, 2004 only, and was performed when DFA tests were negative, not requested, or positive for PIV (as a confirmation of positive PIV DFA). TVC using standard cell lines (Rhesus Monkey Kidney cells and A549) was set up throughout the study unless a virus was already detected by DFA or RRC. Culture for MP was performed using respiratory *Mycoplasma* agar plates and a biphasic media with respiratory *Mycoplasma* agar and respiratory glucose broth.

Nucleic Acid Extraction

Samples were aliquoted and stored at -70°C until NAT. Preparation and extraction of respiratory specimens utilized the NucliSens[®] automated isolation reagents and extractor according to manufacturer's instructions (bioMérieux, St-Laurent, Qué., Canada) and 200 μl of each specimen was extracted into an elution volume of 100 μl [Boom et al., 1990].

Set Up and Analysis of Real-Time Nucleic Acid Tests (NATs)

The threshold cycle number for the real-time PCR assays and the cut-off for positive specimens in real-time NASBA was defined based on experiments using known positive and negative cultured controls and clinical specimens to maximize sensitivity without compromising specificity. Some data on method validation has already been published [Samuelson et al., 1998; Fox et al., 2002; Hibbitts and Fox, 2002, 2004; Hibbitts et al., 2003; Welti et al., 2003; Moore et al., 2004, 2006]. Additional experiments were undertaken during the validation and set up phases of the study to ensure the consistent performance of the published assays and to define the parameters for the new methods. A positive result in each assay was determined by the assessment of the threshold cycle number for each target in a similar

way to that described previously [Welti et al., 2003]. Forty-five cycles were run for each assay and a significant increase in signal above background, together with a typical amplification curve, were used to delineate a specimen as positive for the target in question.

Specificity of each assay was confirmed by using samples with high titers of other respiratory viruses and bacteria as well as by alignment of sequence data and use of specific probes in the assay (data not shown). The end point sensitivity of each assay was either defined by culture-based procedures [FluA, FluB, PIV1-4, RSV, adenovirus, rhinovirus (RV), MP, CP] or by cloning of target sequences and end point determination using a DNA plasmid (coronaviruses NL63, OC43, and 229E) or synthetic, transcribed RNA (hMPV). The acceptable sensitivity was $\leq 1\text{TCID}_{50}$ input for titrated viruses, ≤ 100 copies input for plasmid clones (DNA) or transcribed RNA and ≤ 1 cfu input for the bacteria for the optimized new assays and as quality control for the published methods.

Contamination was minimized by use of separate areas for clean master mix preparation, extraction, and preparation of specimens and controls, and set up of the NATs. Controls taken throughout each step in the procedure ensured that any problems related to possible contamination would be identified and results disregarded.

Pathogen-Specific Amplification and Detection by Nucleic Acid Sequence-Based Amplification (NASBA)

Flu A, Flu B, PIV 1-4, RSV, and RV amplification and detection was by in-house developed real-time NASBA similar to that described previously [Fox et al., 2002; Hibbitts and Fox, 2002, 2004; Hibbitts et al., 2003; Moore et al., 2004; Moore et al. (in press); Samuelson et al., 1998]. Five different NASBA reactions were set up for these targets with Flu A and Flu B in a single reaction tube, PIV 1 and 3 in a second tube, PIV 2 and 4 in a third tube, RSV (A and B) in a fourth reaction, and RV in the fifth. Any RV positive specimens were further tested by an enterovirus-specific NASBA [Fox et al., 2002] to ensure specificity (the RV NASBA has some cross-reaction with related non-rhinovirus picornaviruses at high copy number). All NASBA molecular beacon probes used fluorescein (FAM) as the 5' label with the non-fluorescent quencher 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) on the 3' end. A positive reaction in the first screen was followed up by a repeat test in a monoplex format to differentiate between the positive targets for multiplex assays. Primers and probes for real-time NASBA were either as described previously [Hibbitts and Fox, 2002; Hibbitts et al., 2003; Moore et al., 2004] were adapted to the real-time format from previous studies [Samuelson et al., 1998] or have not yet been published in any format (assays for Flu B and RSV). Primers and probes not previously published or adapted for this study are given in Tables I and II.

TABLE I. Real-Time NASBA Primers and Molecular Beacons Designed for This Study

Virus	Primer/probe identification*	Sequence 5' → 3'	Function
RSV A and RSV B	RSV P1 A + B	<i>AAATCTAATACGACTCACTATATAGGGGAYAGAGGATGGTAYTGTGA</i>	RSV generic T7 NASBA primer
	RSV A P2	CAATGGCTCTAGAGATGTGA	RSVA-specific NASBA primer
	RSV B P2	CTATAGCTCCAAGAGAAATAA	RSVB-specific NASBA primer
Influenza B	RSV MB A + B	CCATGCCDGGCTGCTTAYRTCTGTTTTCATGG	RSV generic molecular beacon
	Flu B pol P1	<i>AAATCTAATACGACTCACTATATAGGGGAYAGAGGCTATTCAACATCTGCGTCCATC</i>	Influenza B T7 NASBA primer
	Flu B pol P2	ATYACTTCATAYTGTGCTCA	Influenza B NASBA primer
Human rhinoviruses	Flu B pol MB	<i>CCAAGCCCTTGTCTTCTTAATAGTGTATAGCTTGG</i>	Influenza B molecular beacon
	HRV P1	<i>AAATCTAATACGACTCACTATATAGGGGAYAGAGGCTATTCAACATCTGCGTCCATC</i>	Rhinovirus T7 NASBA primer
	HRV P2	CTCCGGCCCTGAATGYGGCT	Rhinovirus P2 NASBA primer
	HRV MB	CCAAGCGAYGGACCRACTACTCTTTGGGCTTGG	Rhinovirus molecular beacon

*The T7 RNA polymerase tail/spacer region for P1 primer and the stem regions for the molecular beacons are given in italics. RSV, respiratory syncytial virus. Y = C + T, R = A + G, M = C + A, W = A + T, S = G + C.

TABLE II. Real-Time PCR Primers and Hydrolysis Probes Designed for This Study

Virus	Primer/probe identification	Sequence 5' → 3'	Function
Human coronavirus	HCov 229 E spike F	CACGGAAATTCCTTTT	Coronavirus 229E primer
	HCov 229 E spike R	CCGGTATATCCTTTAGCGAAA	Coronavirus 229E primer
	HCov 229 E spike probe	TGTTAAATTTGGCAGTGTATG	Coronavirus 229E (FAM/MGB) hydrolysis probe
	HCov OC 43 spike F	TTTTATTTCAGGCAGACTCATTTACTTGG	Coronavirus OC43 primer
	HCov OC 43 spike R	AGTTATGCTGGAAAAACACATACCAT	Coronavirus OC43 primer
	HCov OC 43 spike probe	ATTGATGCTGCTAAGATA	Coronavirus OC43 (FAM/MGB) hydrolysis probe
	HCov NL63 1a gene F	TGTTCTGTTTAACTTGGTTGCTTAG	Coronavirus NL63 primer
	HCov NL63 1a gene R	CCTTGGCAATAACTTTAATATCATGCT	Coronavirus NL63 primer
	HCov NL63 1a gene probe	TGTTTAGTCTTTTAGCTACTTCT	Coronavirus NL63 (FAM/MGB) hydrolysis probe
	HCov NL63 spike F	CTTTATCAACCACCTCCGTTTAACTTG	Coronavirus NL63 primer
	HCov NL63 spike R	CAGTGGCATTAATAATAACAAAACCA	Coronavirus NL63 primer
	HCov NL63 spike probe	ACCTGGTCTTAAATCT	Coronavirus NL63 (FAM/MGB) hydrolysis probe
	Ad2-F	CCAGGACGCCCTCGGAGTA	Adenovirus primer
	Ad2-R	AAACTTGTATTCAGGCTGAAGTACGT	Adenovirus primer
Ad2-probe	AGTTTGGCCGCGCCACCG	Adenovirus (FAM/TAMRA) hydrolysis probe	
Ad4-F	GGACAGGACGGCTTCGGAGTA	Adenovirus primer	
Ad4-R	CCTTGT CCC CAGACTGAAGTAGGT	Adenovirus primer	
Ad4-probe	CAGTTCGCGCGCMACAG	Adenovirus (FAM/TAMRA) hydrolysis probe	
Human metapneumovirus	hmpv-F-For1	CTAAATGTTGTGGCGCAATTTTC	Metapneumovirus primer
	hmpv-F-For2	CTAAATGTTGTGGCGCAATTTTC	Metapneumovirus primer
	hmpv-F-For2b	TAAATGTTGTGGCGCAATTTTCAGA	Metapneumovirus primer
	hmpv-F-Rev1	GTCTGCAGATGTTGGCAATGT	Metapneumovirus primer
	hmpv-F-Rev2	CCTGCAGATGTTGGCGCAATGT	Metapneumovirus primer
	hmpv-F-uni	ACACCAGCAATATC	Metapneumovirus (FAM/MGB) generic hydrolysis probe

Targets for amplification were nucleoprotein (Flu A), polymerase (Flu B), hemagglutinin–neuraminidase (PIV1-3), phosphoprotein (PIV-4), fusion protein (RSV A and B), and the 5' non-coding region (RV).

Amplification and detection of NASBA products utilized the NucliSens[®] EasyQ analyser (bioMérieux) with 5 µl template input in each case as described previously [Moore et al., 2004]. Appropriate positive and negative controls were included in each assay. The clinical samples were all tested under the optimized reaction conditions and a cut off value for a positive result was set at 20% above the negative control wild type signal.

Pathogen-Specific Amplification and Detection by Real-Time Polymerase Chain Reaction (PCR)

Five different real-time PCR assays were set up to complete the range of respiratory pathogens to be investigated in this study. Assays for hMPV, ADV, and hCoV OC43, 229E, and NL63 were designed in house utilizing hydrolysis probe (TaqMan) chemistry with amplification and analysis on the ABI 7000 [Applied Biosystems (ABI, Foster City, CA)]. Primer and probe design for in house assays utilized Primer Express software (ABI). The hCoV and hMPV assay design incorporated use of a black hole quencher on the 3' end of each hydrolysis probe whereas the ADV assay design utilized TAMARA as the 3' quencher/label. Targets for in house real-time PCR assays were fusion protein (hMPV), hexon protein (ADV), spike protein (separate primers and probes for OC43, 229E, and NL63), and replicase 1a (additional target for NL63). All newly designed real-time PCR assays utilized multiple primers and probes, as required, to ensure pick up of all known sequence variants (Table I).

Standard two-step (random cDNA) RT-PCR protocols with universal conditions and thermocycling profile were used for the detection of hCoV and hMPV (ABI reagents). Assays for hMPV and ADV utilized FAM as the reporting label. The single-gene target assays for 229E- and OC43-like viruses were run as a multiplex assay with different labels (FAM and VIC) to differentiate the group 1 and 2 viruses. NL63-like viruses were detected with primers for both spike gene and replicase 1a gene and FAM labeled-probes. The in-house designed ADV assay was run under universal cycling conditions (ABI) alongside the other in house assays at the cDNA stage. In addition to the in house developed real-time PCR assays described above, a multiplex assay for CP and MP was undertaken using previously described primers, probes, and procedures [Welti et al., 2003]. Appropriate positive and negative controls were included in each assay.

Epidemiologic Data

The age of the patient, their hospitalization status, and history of a transplant or underlying malignancy was recorded.

RESULTS

Samples

There were 797 BAL and ETT samples submitted during the study of which 439 (55%) had sufficient volume to attempt nucleic acid extraction. One or more NAT were performed on 399 BAL and 40 ETT samples from 130 females and 160 males, of which 241 were adults (median 57 years, range 17.0–89.0 years) and 49 were children <17 years of age (median 2.0 years, range 0.02–16.0 years) (Fig. 1). At the time the sample was obtained, the patient was an outpatient (n = 151), or

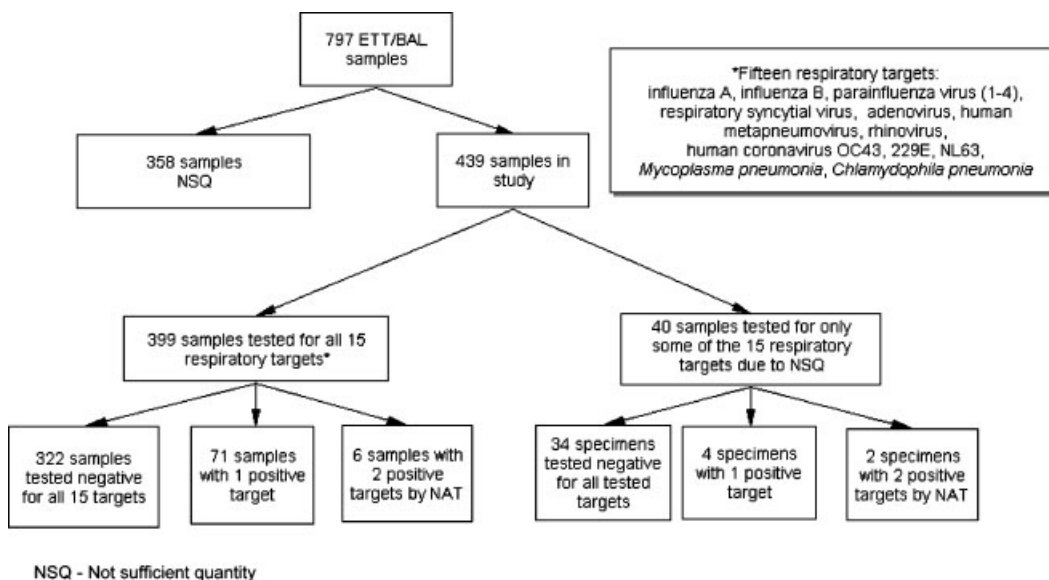


Fig. 1. NAT of bronchoscopic or endotracheal aspirate samples.

TABLE III. Summary of Results of Viral Detection in Lower Respiratory Tract Samples

	Flu A	Flu B	PIV	RSV	ADV	Mycoplasma
DFA	1/154 ^a	0/153	2/138 ^b	6/141 ^a	0/106	—
Viral culture (RRC or TVC)	0/439	0/439	7/439 ^c	0/439	5/439 ^d	—
NAT	4/434	0/434	11/436	11/434	22/436	—
Mycoplasma culture	—	—	—	—	—	2/246 ^e

ADV, adenovirus; DFA, direct fluorescent antibody; Flu A, influenza A; Flu B, influenza B; PIV, parainfluenza virus; RRC, rapid respiratory culture; RSV, respiratory syncytial virus; TVC, traditional viral culture.

^aRRC and TVC were not performed.

^bOne sample was also positive for RRC and was not tested by TVC and the other was not tested by RRC and negative by TVC.

^cTwo of the six positive samples were also tested by DFA for PIV and only one was positive. One positive sample was not tested by DFA and was negative by RRC.

^dOnly one ADV positive sample was tested by DFA for ADV and was negative and all were negative by RRC.

^eMycoplasma not *Mycoplasma pneumoniae* was detected in 23 samples and 42 Mycoplasma cultures were contaminated by other microbes.

admitted to the adult intensive care unit (ICU) (n = 145), adult neurosurgical ICU (n = 1), adult thoracic–cardiac surgical ICU or ward (n = 47), internal medicine ward (n = 48), pediatric ward (n = 42), or pediatric or neonatal ICU (n = 5).

Antigen Detection and Culture Results

Traditional respiratory virus testing was performed on 412 of the 439 samples (93.9%) and respiratory viruses were detected by DFA, RRC, or TVC in 11 inpatient and 9 outpatient samples (4.9% of the 412 samples) collected from 15 different patients (Table III). One of those 15 patients had the same virus detected from three samples submitted on the same day (PIV) and another patient had two different viruses (RSV by DFA and PIV by RRC, respectively) identified in two samples submitted one day apart. Two other patients had different viruses detected from samples collected 112 days (ADV and PIV) and 135 days (PIV and Flu A) apart. Antigen detection for at least one virus was performed in 158 of the 439 samples (36.0%) and was positive in 9 samples (5.7%). RRC was performed in 299 (68.1%) of the samples and detected viruses in 6 samples (2.0%), and TVC was performed in 399 samples (90.9%) and detected viruses in 6 samples (1.5%). Cultures were positive for MP in 2 of 246 samples (0.8%), with these 2 samples being collected by BAL on the same day from a pediatric oncology inpatient. No co-infections were detected by conventional methods.

Because of insufficient nucleic acid, 40 of the 439 samples were tested by NAT for <15 targets (Fig. 1), of which 6 samples tested positive. For the 399 samples that were tested for all 15 targets, 77 tested positive. In total, 83 of the 439 samples from 67 different patients tested positive for 91 pathogens by NAT: 75 samples tested positive for one target (3 Flu A, 0 Flu B, 3 PIV1, 0 PIV2, 5 PIV3, 1 PIV4, 9 RSV, 15 ADV, 19 RV, 5 hMPV, 8 hCoV OC43, 2 hCoV 229E, 2 hCoV NL63, 2 MP, and 1 CP), and 8 tested positive for two targets (4 ADV and RV and one each of Flu A and ADV, PIV4 and ADV, RSV and ADV, and PIV3 and RSV). Fifty of the samples that had positive NAT results were collected from inpatients and 33 of the samples from outpatients.

Comparison of Methods for Pathogen Detection

The results of conventional methods for detection of samples that tested positive by NAT for Flu A, PIV 1–4, RSV, ADV, and MP are shown in Table IV. For the 22 samples where pathogens were detected by conventional methods, 17 tested positive for the same pathogen by NAT with a second pathogen being identified by NAT in 2 of those samples. Another five samples tested negative by NAT but positive by conventional methods for PIV (n = 2, one detected by RRC and one by TVC), RSV (n = 1, detected by DFA), and ADV (n = 2, both detected by TVC). The child mentioned above with RSV by DFA and PIV by RRC one day apart had PIV3

TABLE IV. Comparison of Results for Conventional Detection Methods With NAT for Each Respiratory Virus

NAT versus DFA for specific virus	Flu A (n = 149)	Flu B (n = 148)	PIV (n = 136)	RSV (n = 137)	ADV (n = 103)
NAT positive DFA positive	1	0	2	5	0
NAT positive DFA negative	0	0	0	3	7
NAT negative DFA positive	0	0	0	1	0
NAT negative DFA negative	148	148	134	128	96
NAT versus viral culture (RRC or TVC) for specific virus	Flu A (n = 434)	Flu B (n = 434)	PIV (n = 436)	RSV (n = 434)	ADV (n = 436)
NAT positive viral culture positive	0	0	5	0	3
NAT positive viral culture negative	4	0	6	11	19
NAT negative viral culture positive	0	0	2	0	2
NAT negative viral culture negative	430	434	423	423	412

ADV, adenovirus; DFA, direct fluorescent antibody; Flu A, influenza A; MP, *Mycoplasma pneumoniae*; NAT, nucleic acid amplification test; PIV, parainfluenza virus; RRC, rapid respiratory culture; RSV, respiratory syncytial virus; TVC, traditional viral culture.

detected by NAT on both samples. NAT detected 74 pathogens that were not detected by conventional methods of which 29 samples from 27 patients had 31 pathogens that were missed by conventional methods (2 Flu A, 4 PIV, 6 RSV, and 19 ADV), 41 samples from 34 patients had pathogens not routinely identified by conventional methods in most laboratories (23 RV, 8 hCoV OC43, 5 hMPV, 2 hCoV 229E, 2 hCoV NL63, and 1 CP) and 2 samples from 2 patients had no viral detection requested by the submitter and so were not tested by conventional methods (Flu A from an inpatient and PIV from an out patient).

Conventional and NAT Results From Repeat Samples

Of the 290 patients, 52 had same-day samples of which 110 were BAL samples and 1 was an ETT sample. Of these 111 samples, 109 were processed by conventional methods. Forty-eight patients had no pathogens detected by conventional methods, 2 patients had the same pathogens detected from all samples (PIV from three samples and MP from two samples, respectively), and 2 patients had both negative and positive results for the same pathogen in different samples (only 1 of 2 same-day samples from each patient was positive for PIV and ADV, respectively). All 52 patients had NAT performed on their same-day samples and 42 had no

pathogens detected by NAT, 1 had pathogen detected by NAT in one sample but had insufficient volume for NAT in the other sample, 5 had the same pathogen detected by NAT in all samples, and 4 had both negative and positive results by NAT for the same pathogen on different samples (one of three samples tested positive for PIV in one patient, one of two samples tested positive for RV in one patient, and one of two samples tested positive for ADV in two patients with one of them also testing positive for PIV).

Epidemiologic Features

The monthly distribution by samples and pathogens detected is shown in Figure 2. The source of the samples is shown in Table V, with 40 of 75 positive samples (excluding 8 samples taken <10 days apart with concordant results) coming from immunocompromised hosts. There was no apparent relationship between the source of the sample and the pathogen that was detected. Isolation of the same virus weeks to months apart occurred in four solid organ transplant patients and in two children with tracheostomies.

DISCUSSION

This study demonstrated that the addition of NAT for 13 respiratory viruses, MP and CP to the conventional

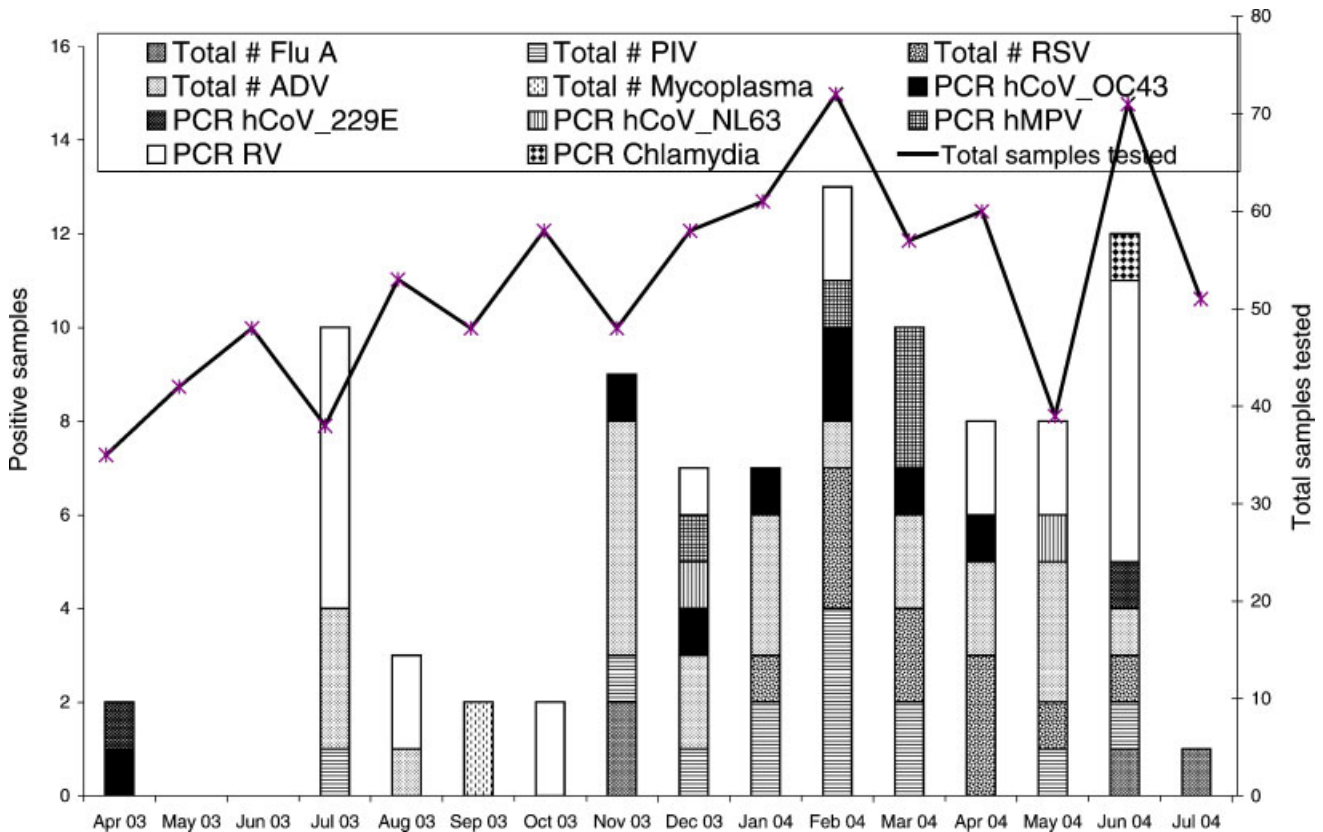


Fig. 2. Monthly distribution of bronchoscopic or endotracheal aspirate samples tested positive by antigen detection, RRC, TVC, or NATs.

TABLE V. Clinical Characteristics of 75 Patients From Which Pathogens Were Detected by NAT^a

	Inpatients /Outpatients		Flu				hCoV hCoV hCoV				Mixed	MP	CP	
	A	PIV 1-4	RSV	ADV	RV	hMPV	OC43	229E	NL63					
Solid organ transplant recipients (4 children and 29 adults)	13/20	2	7	3	7	4	1	4	1	1	3 (ADV and Flu A, ADV and RSV, ADV and RV) 1 (ADV and RV)			
Bone marrow transplant recipients (1 child and 3 adults)	2/2				2	1								
Immunocompromised adults	2/1				1			1						1
Adult ICU patients	10/0			2	1	2 ^a	1	2	1	1				
Other adults	2/2			1	1	1	1							
Pediatric patients with no solid organ or bone marrow transplant	13/8	1	1	1	3	8	1	1			4 (PIV4 and ADV, PIV4 and RV, 2 ADV and RV)		1 ^a	
Total inpatients		3	4	6	10	8	2	4	2	2		1	1	0
Total outpatients		0	4	1	5	8	2	4	0	0	7		0	1

ADV, adenovirus; CP, *Chlamydia pneumoniae*; Flu A, influenza A; hCoV, human coronavirus; hMPV, human metapneumovirus; ICU, intensive care unit; MP, *Mycoplasma pneumoniae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus.

^aFrom 83 lower respiratory tract samples. Each patient was counted only once if repeat samples were positive for the same pathogen within a 10-day period (n = 8 additional samples from 6 patients), but they were counted again if they had different viruses (n = 2 additional samples from 2 patients) or the same virus more than 10 days apart (n = 6 additional samples from 6 patients). The episodes with the same virus occurred 5 weeks apart with Flu A, 2 months apart with hCoV43, 4 months apart with ADV, and 4 months apart with RV in solid organ transplant patients, and 2 and 10 months apart with RV in pediatric patients with tracheostomies. The four patients with viruses detected only by conventional methods but negative by NAT were excluded from this table, and one patient with PIV detected by NAT on subsequent days, and a positive DFA for PIV one day and for RSV the subsequent day was classified as having PIV in this table.

^aIncludes one oncology patient.

tests performed at the submitter's request on all lower respiratory tract specimens increased the detection rate for potential pathogens from $\approx 5\%$ to $\approx 20\%$. This increased yield was mainly attributable to detection of pathogens that are not usually detected by conventional methods (RV, hMPV, hCoV OC43, hCoV 229E, hCoV NL63), but increased detection of Flu A, PIV, RSV, and especially ADV was also apparent.

Previous studies comparing NAT to conventional methods for detection of respiratory viruses have consistently demonstrated increased yield by NAT [Akhtar et al., 1999; Van Elden et al., 2002; Ison et al., 2003; Garbino et al., 2004; Legoff et al., 2005; Van Kraaij et al., 2005] but optimal conventional techniques were not used in all studies and as in the current report, viruses detected by conventional methods were occasionally not detected by NAT [Garbino et al., 2004].

Detection of MP and CP in LRTI is difficult as the sensitivity of culture is poor and yield with NAT was low in the current study. A previous study showed the yield with MP NAT to be higher for sputum than for throat swabs or NP specimens [Raty et al., 2005], suggesting that the type of sample is of importance and further studies are needed to compare NAT results on lower versus upper respiratory tract samples.

The clinical significance of viruses detected only by NAT from respiratory samples has not been established. In previous studies in normal hosts, shedding of PIV as detected by TVC usually occurred for 3 to 10 days but sometimes persisted for months [Muchmore et al., 1981] and shedding of ADV occurred for a mean of 4 days but persisted for up to 17 days [Larranaga et al., 2000]. Shedding of MP could still be documented by culture in half of patients 6 to 8 weeks after acute infection [Foy et al., 1996]. Because NAT can detect much lower concentrations of pathogens, shedding of pathogens can be detected for a longer period of time than with conventional methods [van Kraaij et al., 2005], and pathogens can potentially be detected from patients with remote infection or from patients with non-viable pathogens. Evidence that detection of respiratory viruses by NAT is not always of clinical significance is that 16% of 79 asymptomatic children had picornaviruses detected by NAT in NPS, and 19% of 84 samples from children with asthma remained positive for picornaviruses by NAT 2 weeks after an acute asthma exacerbation with no correlation between persistence of virus and symptoms [Jartti et al., 2004], but there is less evidence of asymptomatic shedding of other respiratory viruses. Even if such shedding occurs, the clinical significance of pathogens detected by NAT may be greater for lower than for upper respiratory tract samples as the expected incidence of asymptomatic shedding in the lower tract is less. NAT for multiple respiratory viral targets was negative in BAL from 50 ventilated children [Akhtar et al., 1999] and 31 adults with no evidence of LRTI [Garbino et al., 2004], suggesting that asymptomatic shedding of viruses in the lower tract is not common.

Use of NAT increases the chance of recognizing co-infection. Previous small retrospective studies have demonstrated that over half of children admitted to ICU with RSV or hMPV had co-infection [Greensill et al., 2003; Konig et al., 2004; Semple et al., 2005] but there are no prospective studies and the rate appears to be much lower in children with less severe disease [Robinson et al., 2005]. Eight patients in the current study had more than one virus identified, but a prospective study would be required to determine the clinical significance of viral co-infection as identified by NAT and the role that different respiratory viruses play as risk factors for secondary bacterial pneumonia.

The yield of respiratory viruses from NAT was higher from BAL or induced sputum than from paired nasal washes or nasal-throat swabs in four previous studies [Seemungal et al., 2000; Rohde et al., 2003; Semple et al., 2005; van Kraaij et al., 2005] and equivalent in one study [van Elden et al., 2002], and the yield of MP was higher from BAL than from throat swabs in one small study [Falguera et al., 1996]. However, the risks of an invasive procedure may outweigh the benefits of an increased yield from lower tract samples, which are especially difficult to obtain in children.

Given that viruses were detected in only 2 of 87 samples where viral detection was not requested, it is likely not cost-effective to routinely do NAT for multiple pathogens on all lower respiratory tract samples submitted for microbiologic studies. However, NAT allowed for detection of pathogens in almost 20% of samples where the submitter requested conventional viral testing but the results of these tests were negative, so it may be cost-effective to routinely perform NAT for multiple pathogens on samples where the submitter requests viral detection. For samples submitted on the same day, about 10% of total samples (all of which came from a BAL) and half of samples from which pathogen was detected had discordant results, suggesting that testing of more than one BAL sample on the same day is warranted.

One limitation of the study is that we cannot ascertain that all patients who had BAL and ETT samples submitted for viral studies had clinical evidence of a LRTI. This may explain why our rate of detecting pathogens is lower than in previous NAT studies [Akhtar et al., 1999; van Elden et al., 2002; Ison et al., 2003; Garbino et al., 2004; van Kraaij et al., 2005]. It was not possible to undertake confirmatory testing for each NAT due to limiting amounts of original material and extract available. The design of each assay (based on available sequences), the specificity checks undertaken, and (where possible) correlation with conventional methods ensured the validity of the results.

In conclusion, addition of NAT testing for 15 potential respiratory pathogens provided diagnostic information that could have altered medical management in about 15% of patients. However, it is possible that the same goal could be achieved by optimal use of conventional methods of detection of respiratory pathogens. Future

studies should compare results of lower respiratory tract samples to those obtained from upper respiratory tract samples to determine if there are advantages in obtaining the more invasive lower tract samples and should prospectively compare the outcome of patients in the presence or absence of NAT testing.

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