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Development and Implementation of a Molecular Diagnostic Platform for Daily Rapid Detection of 15 Respiratory Viruses

Annika Tiveljung-Lindell,[1,2]* Maria Rotzén-Östlund,[1,2] Shawon Gupta,[1,2] Richard Ullstrand,[1,2] Lena Grillner,[1,2] Benita Zweygberg-Wirgart,[1,2] and Tobias Allander[1,2]

[1]*Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden*
[2]*Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden*

Acute respiratory tract infections are caused by a large number of viruses. Diagnostic methods have until recently been available only for a limited number of these viruses. With the objective to achieve sensitive assays for all respiratory viruses, a rational workflow in the laboratory, and a short turn-around time, a real-time PCR diagnostic platform for daily rapid detection of 15 respiratory viruses was developed. The system was evaluated on 585 stored nasopharyngeal aspirates from hospitalized children. Previous analysis by immunofluorescence and virus isolation identified viruses in 37% of the samples while the new PCR diagnostic panel detected 57% virus positive samples. The new platform was introduced in the laboratory in October 2007 and has then fully replaced the standard immunofluorescence assay for rapid detection of viruses and virus isolation. ***J. Med. Virol. 81:167–175, 2009.***

KEY WORDS: real-time PCR; respiratory tract infection; influenza; respiratory syncytial virus; human bocavirus

INTRODUCTION

Acute respiratory tract infections are the leading cause for hospitalization of infants and young children [Shay et al., 1999]. These infections are normally self-limiting, but may often require supportive care, and are occasionally life threatening, for example, in prematurely born infants and immunocompromised patients. Respiratory tract infections are a major cause of infant mortality in developing countries [Lopez et al., 2006].

A range of viruses have been associated with acute respiratory tract infections: Influenza A and B viruses, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), human coronaviruses (hCoV) NL63, 229E, OC43, and HKU-1, parainfluenza viruses 1-4 (PIV), human rhinoviruses, human enteroviruses, and human adenoviruses (AdV) [Mackie, 2003; Falsey and Walsh, 2006]. There is also gathering evidence for a pathogenic role of human bocavirus (HBoV), mainly when the virus is present at a high viral load [Allander et al., 2007; Fry et al., 2007; Kantola et al., 2008].

A clinical laboratory strives to have broad, fast, sensitive, specific, and reproducible diagnostic methods that meet the needs of patients and clinicians. Diagnosis of respiratory agents has traditionally relied on immunofluorescence (IF) and tissue culture isolation. IF is the standard rapid test that can be performed in a few hours. Virus isolation in cell culture is more sensitive than IF, but normally requires days or weeks and therefore rarely delivers results that can guide the treatment of the patient [Östlund et al., 2004]. These methods have mainly been used for the diagnosis of RSV and influenza, but also for PIV and AdV. Another diagnostic approach is the use of rapid antigen test kits based on immunochromatography [Ohm-Smith et al., 2004; Templeton et al., 2004; Gunson et al., 2005, 2006; Weinberg and Walker, 2005]. Rapid antigen test kits for RSV and influenza detection can be used at the point of care and can be performed within 30 min, but on the other hand suffer from limited sensitivity [Hurt et al., 2007; Smit et al., 2007]. However, rhinoviruses, coronaviruses, and HBoV can, at present, generally not be diagnosed by IF, virus isolation, or rapid antigen testing. The most recent addition to respiratory virus diagnostics is molecular methods such as real-time PCR. Real-time PCR combines high sensitivity with comparably short

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*Correspondence to: Annika Tiveljung-Lindell, Department of Clinical Microbiology, L2:02, Karolinska University Hospital, SE-171 76 Stockholm, Sweden.
E-mail: annika.tiveljung-lindell@karolinska.se

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analysis time. Real-time PCR generally requires a few hours longer analysis time than the standard rapid test IF, but nevertheless has the capacity to deliver same-day results. In addition, it allows some degree of quantification of the detected virus [Mackay, 2007]. Moreover, out of the methods mentioned above, real-time PCR is the only one suitable for the diagnosis of all respiratory agents.

Several real-time PCR assays for respiratory pathogens have been published in recent years, including multiplex systems targeting the majority of respiratory viruses [Templeton et al., 2004; Watzinger et al., 2004; Gunson et al., 2005, 2006; van de Pol et al., 2007; Brittain-Long et al., 2008]. High-throughput systems combining PCR with fluid microbead multiplex product detection have also been recently described [Lee et al., 2007; Mahony et al., 2007]. However, most published assays have not been adapted for large-scale diagnostic use, or lack one or more important virus targets. This prompted the development of a real-time PCR platform for 15 viruses performed in 13 reactions. This diagnostic platform has fully replaced IF and virus isolation in the laboratory. The system was designed with emphasis on efficient handling in the laboratory, minimal hands-on work, short turn-around time, and the capacity to handle many samples during the influenza and RSV epidemic seasons. The primary objective was to expand the diagnostic panel, and at the same time retain or improve the diagnostic sensitivity for influenza, RSV, PIV, and AdV compared to IF. The secondary objective was to obtain a streamlined workflow in the laboratory for respiratory samples, and keep the turn-around time to a minimum. The performance of the real-time PCR assays was evaluated by comparing retrospectively the assays to previous results from IF and tissue culture isolation of 585 stored pediatric nasopharyngeal aspirate (NPA) samples.

MATERIALS AND METHODS

The Real-Time PCR Diagnostic Panel

Nucleic acid extraction. Viral nucleic acids were extracted from 400 μl of NPA in a BioRobot M48 instrument using MagAttract Virus Mini M48 kit (Qiagen, Stockholm, Sweden) according to the manufacturer's instructions. Total nucleic acids were eluted in 100 μl of nuclease-free water.

Automated reaction setup. Reagent dispensing and sample extract transfer to 96-well plates were performed by automated pipetting using either Tecan Genesis RSP or Corbett CAS 1200 instruments.

Real-time PCR assays. New or modified assays were constructed for all respiratory viruses studied. Primers, probes, and source references are listed in Table I. Five microliters of nucleic acid template was used per reaction. Most assays were single-agent PCR assays in order to avoid target competition. Three reactions were duplex assays: RSV A with RSV B, PIV1 with PIV 3, and PIV 2 with hCoV-229E. For the RNA virus assays, a one-step real-time reverse transcription (RT)-PCR was performed in 20 μl of reaction mixture consisting of 10 μl 2× one-step RT-PCR buffer and 0.4 μl Superscript III/Platinum Taq polymerase (Superscript III Platinum one-step RT-PCR kit; Invitrogen, Stockholm, Sweden). Concentrations of primers and probes used are indicated in Table I. Amplification, detection, and data analysis were performed with the LightCycler 480 instrument using the following thermal profile: reverse transcription for 30 min at 50°C, followed by 2 min at 95°C, and 50 cycles of 5 sec at 95°C and 60 sec at 60°C. For the DNA virus assays, real-time PCR was carried out in a 20 μl reaction volume consisting of 10 μl 2× TaqMan Universal master mix (Applied Biosystems, Stockholm, Sweden) and primers and probes as indicated in Table I. The cycling conditions were 10 min at 95°C followed by 50 cycles of 1 sec at 95°C and 60 sec at 58°C.

Positive controls. Positive controls were of three types: cultured virus, high load patient samples, or plasmids. The following strains from ATCC were used: PIV 2 (Greer), PIV 3 (HA1, C243), rhinovirus (2060), and RSV A (Long). Clinical isolates were: adenovirus 2 (adenoid 6, SJV), influenza A virus (A/H3N2/Philippines, SBL), influenza B virus (USSR 31 G, SBL), RSV B (880113, SBL), hMPV (IV7450/02), enterovirus (echo 30, Bastianni, SBL), and PIV-1 (IV15196/05). RNA extracted from patient samples was used for hCoV NL63 and OC43. The positive controls for hCoV-229E, hCoV-HKU1, and HBoV were in-house cloned plasmids.

Rapid antigen testing. Very urgent requests for influenza virus or RSV were analyzed by BD Directigen EZ Flu A+B and BD Directigen EZ RSV (Becton-Dickinson, Stockholm, Sweden) according to the manufacturer's instructions.

Evaluation Procedure

Clinical specimens. A total of 585 consecutive NPA samples collected from 517 hospitalized patients at the infectious diseases ward of Astrid Lindgren Children's hospital (Karolinska University Hospital) from July 2004 to June 2005 were included in the study. Only basic demographic data of the patients providing the samples were recorded. The majority of samples, 359/585 (61.4%) were derived from children <1 year old, 172 (29.4%) 1–5 years old, 27 (4.6%) 6–10 years old, 20 (3.4%) 10–15 years old, and four (0.7%) were from patients 15–20 years old. The age of the patient was unknown for three samples (0.5%). The male to female ratio of included patients was 59/41.

The NPAs were drawn on clinical grounds and were analyzed in accordance with the diagnostic practice by IF and virus isolation when received in 2004–2005. Remaining material was stored at −70°C until retrospectively examined by molecular techniques in 2007. Samples of <400 μl were diluted twofold in nucleic acid-free water prior to extraction. The extracted nucleic acid was aliquoted and immediately stored at −70°C prior to real-time PCR analyses.

Replaced assays. The final diagnostic platform in clinical use is described above. However, some

TABLE I. Primers and Probes and Their Concentrations in the Real-Time PCR Assays

Primer/probe	Sequence (5′–3′)	Concentration (nM)	References
Adenovirus s	GCC CCA GTG GTC TTA CAT GCA CAT C	900	Heim et al. [2003] (modified)
Adenovirus as	GCC ACG GTG GGG TTT CTA AAC TT	900	Heim et al. [2003] (modified)
Adenovirus probe	6FAM-TGC ACC AGM CCS GGG CTC AGG TAC TCC GA-TAMRA	400	Heim et al. [2003] (modified)
Enterovirus s (E1)	TCC TCC GGC CCC TGA ATG CGG CTA AT	500	Glimåker et al. [1992, 1993]
Enterovirus as (E2)	ATT GTC ACC ATA AGC AGC CA	500	Glimåker et al. [1992, 1993]
Enterovirus probe	6FAM-AAC CGA CTA CTT TGG GTG TCC GTG TTT C-TAMRA	200	Lai et al. [2003]
HBoV s	GGA AGA GAC ACT GGC AGA CAA	300	Allander et al. [2007]
HBoV as	GGG TGT TCC TGA TGA TAT GAG C	300	Allander et al. [2007]
HBoV probe	6FAM-CTG CGG CTC CTG CTC CTG TGA T-TAMRA	150	Allander et al. [2007]
hCoV-229E s	TGG AAG TGC AGG TGT TGT GGC	700	New design
hCoV-229E as	TGA CTA TCA AAC AGC ATA GCA GCT G	300	New design
hCoV-229E probe	6FAM-CCA CAA TTT GCT GAG CTT GTG CCG TC-TAMRA	150	New design
hCoV-HKU-1 s	CAC TTC TAT TCC CTC CGA TGT TTC	500	New design
hCoV-HKU-1 as	TTA GAA GCA GAC CTT CCT GAG CC	300	New design
hCoV-HKU-1 probe	6FAM-CGC CTG GTA CGA TTT TGC CTC AAG GCT-TAMRA	100	New design
hCoV-NL63 s	CAG GGC TGA CAA GCC TTC TCA	700	New design
hCoV-NL63 as	GCA TCA ACA CCA TTC TGA ACA AGA	700	New design
hCoV-NL63 probe	6FAM-CGT TGG AAG CGT GTT CCT ACC AGA GAG G-TAMRA	150	New design
hCoV-OC43 s	CGA TGA GGC TAT TCC GAC TAG GT	500	van Elden et al. [2004]
hCoV-OC43 as	CCT TCC TGA GCC TTC AAT ATA GTA ACC	400	van Elden et al. [2004]
hCoV-OC43 probe	6FAM-TCC GCC TGG CAC GGT ACT CCC T-TAMRA	500	van Elden et al. [2004]
hMPV s	GAA GAR ATA GAC AAA GAR GCA AG	250	New design
hMPV as	TCC CAC TTC TAT KGT TGA TGC TAG	100	New design
hMPV probe	6FAM-TCA GCA CCA GAC ACA CC-MGB	200	New design
Influenza A s	CAT GGA ATG GCT AAA GAC AAG ACC	500	Schweiger et al. [2000]
Influenza A as	AAG TGC ACC AGC AGA ATA ACT GAG	500	Schweiger et al. [2000]
Influenza A probe	6FAM-CTG CAG CGT AGA CGC TTT GTC CAA AAT G-TAMRA	200	Schweiger et al. [2000]
Influenza B s	AGA CCA GAG GGA AAC TAT GCC C	700	Schweiger et al. [2000]
Influenza B as	CTG TCG TGC ATT ATA GGA AAG CAY	700	Schweiger et al. [2000] modified
Influenza B probe	6FAM-CCA CRT CCA GAT CTG TGC AGT TGA G-TAMRA	400	New design
PIV 1 s	ACC TAC AAG GCA ACA ACA TC	1,000	Gunson et al. [2005]
PIV 1 as	CTT CCT GCT GGT GTG TTA AT	500	Gunson et al. [2005]
PIV 1 probe	Cy5-CAA ACG ATG GCT GAA AAA GGG A-BHQ3	300	Gunson et al. [2005]
PIV 2 s	CCA TTT ACC TAA GTG ATG GAA	700	Gunson et al. [2005]
PIV 2 as	CGT GGC ATA ATC TTC TTT TT	700	Gunson et al. [2005]
PIV 2 probe	YY-AAT CGC AAA AGC TGT TCA GTC AC- BHQ1	150	Gunson et al. [2005]
PIV 3 s	CCA TCT GTT GGA CCA GGG ATA TA	700	New design
PIV 3 as	GAC ACC CAG TTG TGT TGC AGA T	700	New design
PIV 3 probe	6FAM-TGG RTG TTC AAG ACC TCC ATA YCC GAG AAA-BHQ1	300	Gunson et al. [2005]
Rhinovirus s1	YAG CCT GCG TGG CKG CC	500	New design
Rhinovirus s2	CAG GCT GCG TTG GCG GC	500	New design
Rhinovirus as	GGA CAC CCA AAG TAG TCG GTR C	500	New design
Rhinovirus probe	6FAM-TCC TCC GGC CCC TGA ATG YGG CTA A-TAMRA	300	Templeton et al. [2004]
RSV Aa	AGA TCA ACT TCT GTC ATC CAG CAA	1,000	Gunson et al. [2005]
RSV A as	TTC TGC ACA TCA TAA TTA GGA G	250	Gunson et al. [2005]
RSVA probe	6FAM-CAC CAT CCA ACG GAG CAC AGG AGA T-BHQ	300	Gunson et al. [2005]
RSV B a	AAG ATG CAA ATC ATA AAT TCA CAG GA	1,000	Gunson et al. [2005]
RSV B as	TGA TAT CCA GCA TCT TTA AGT A	1,000	Gunson et al. [2005]
RSV B probe	Cy5-TTT CCC TTC CTA ACC TGG ACA TA-BHQ	300	Gunson et al. [2005]

s, sense; as, antisense; K = G/T; M = A/C; R = A/G; S = C/G; Y = C/T; 6FAM, 6-carboxyfluorescein; Cy5, Cy5 indocarbocyanin; YY, Yakima Yellow; BHQ, black hole quencher; TAMRA, 6-carboxytetramethylrhodamine; MGB, minor groove binder with non-fluorescent quencher.

preliminary assays were used during the evaluation of 585 NPA samples. In the evaluation study, picornaviruses were detected by a SYBR green assay and melting curve analysis with the rhinovirus primers (Table I). The assay detected most picornavirus species but was optimized for rhinoviruses. In the final diagnostic panel, the assay was replaced by two separate probe assays preferentially optimized for rhinoviruses and enteroviruses, respectively (Table I), but still frequently cross-reactive. No PCR diagnostics for parainfluenzaviruses was included in the evaluation study. These viruses were only detected by IF and virus isolation.

Immunofluorescence staining. IF staining for viral antigens was performed when the sample was received in 2004–2005 according to the clinician's request and existing diagnostic practices as previously described [Östlund et al., 2004]. IF staining was performed mainly for influenza (442 requests) and/or RSV (511 requests), and occasionally also for PIV (37 requests) and AdV (31 requests).

Virus isolation. In accordance with existing diagnostic practices 2004–2005, the samples were inoculated on HeLa cells, Green Monkey kidney cells, Fetal Rhesus Monkey kidney (Ma-104) cells and, during the influenza season (October–May), Madin-Darby Canine Kidney (Mdck) cells as described previously [Östlund et al., 2004].

RESULTS

Evaluation

Virus isolation and immunofluorescence staining. Among the 585 evaluation samples from 2004 to 2005, 178 viral infections were diagnosed by virus isolation and 160 were detected by IF staining. Taken together, these techniques resulted in 222 viral findings in 214 samples. The virus species identified in this way were RSV, influenza A and B, PIV 1-3, enteroviruses and AdV (Table II). In addition, herpes simplex virus type 1 was isolated from one NPA, but not considered a pathogen of acute respiratory tract infections. The most prevalent finding was RSV, present in 148 (69%) of the 214 positive samples.

Real-time PCR. Real-time PCR analyses resulted in improved diagnostic sensitivity as another 120 patient samples, negative by IF and virus isolation, were identified as containing viral pathogens. One important improvement was that PCR had superior sensitivity to IF for all agents tested. PCR also had superior sensitivity to virus isolation for all agents except influenza A (Table II). The main improvement was, however, the identification of virus species not targeted by the previous diagnostic approach: 6 cases with hMPV, 20 with HBoV, 17 with hCoV-NL63, 17 with hCoV-OC43, 1 with hCoV-HKU1, and 55 with picornaviruses, likely predominantly rhinoviruses, were identified (Table II).

Viral load. Real-time PCR analysis allows an approximate estimation of the viral load from the cycle threshold (Ct) value, which is inversely correlated to the logarithmic viral load [Mackay, 2007]. The mean Ct value for most viruses was around 30, corresponding to approximately 10^3 viral genomes/ml NPA (Table III). Only hMPV contrasted to this pattern, with Ct 17–18 (corresponding to approximately 10^7 copies/ml) in all six positive samples. Values from the picornavirus assay should not be directly compared with the other results, as they were produced with a different product detection system (SYBR green) in the evaluation study.

Multiple infections. Two viruses were found in 37 of the assessed samples (Table IV). In addition, five triple virus detections were observed (HBoV, AdV, and picornavirus in three cases, and one case each of HBoV, AdV, hCoV and RSV, AdV and hCoV, respectively). Picornaviruses (rhinoviruses) and AdV were the viruses detected most commonly together with another agent. HBoV was detected at significantly higher viral loads as a sole infection as compared to when detected in the context of a multiple infection (Table III).

Nucleic acid extraction and sample volume. Due to a limited volume of stored NPA available for molecular diagnostics, 113 of the 585 of the samples in the evaluation panel were diluted twofold prior to nucleic acid extraction. Fifty-six (49.6%) of the 113 diluted samples had a positive result compared to 243 (51.5%) of the 472 undiluted samples ($P = 0.71$, Chi square).

Thus, the twofold dilution did not result in a significant reduction of total virus findings compared to the undiluted samples.

TABLE II. Number of Viral Findings in the Evaluation Study of 585 Respiratory Samples From 2004 to 2005 Detected by Virus Isolation, IF, and Real-Time PCR

Virus	Total no. of viral findings	Virus isolation	IF	Real-time PCR
RSV	168	107 (64%)	137 (82%)	166 (99%)
Influenza A	33	27 (82%)	20 (61%)	23 (70%)
Influenza B	7	4 (57%)	2 (29%)	7 (100%)
Picornavirus	59	4[a]	nd	58
hCoV	35	nd	nd	35
PIV	25	25	1	nd
Adenovirus	24	8	nd	24
HBoV	20	nd	nd	20
hMPV	9	3	nd	6

nd, not done.
[a]Rhinovirus isolation not performed.

TABLE III. Distribution of Cycle Threshold (Ct) Values For Single and Double Infections Per Virus Species (Evaluation Study of Samples From 2004 to 2005)

	CT range	Mean Ct value	Mean Ct value double infections	Mean Ct value single infections
RSV	20–39	28.3	28	28.4
Influenza A	27–33	30.5	30.5	30.6
Influenza B	22–33	30.4	—	30.4
Picornavirus	17–32	25.4	25.3	25.6
hCoV	27–38	30.8	32.3	29.4
Adenovirus	23–40	31.8	30.3	31.6
HBoV	22–37	29.7	31.4	25.7
hMPV	17–18	18.3	—	18.3

Quality control for molecular diagnostics (QCMD) proficiency programmes 2006. The assays for influenza A and B viruses, RSV, PIV, hMPV, hCoV, and rhinoviruses were evaluated in the various proficiency programmes distributed by QCMD, to determine the sensitivities and specificities of the assays. All real-time PCR assays yielded satisfactory results in the proficiency panels.

The Real-Time PCR Diagnostic Panel in Clinical Diagnosis

In a pre-implementation survey, the major customers were asked to define a daily cut-off reporting time, before which results must be available to support decision making and the workflow in the ward. Most clinicians agreed that 16.00 hr was an adequate deadline for receiving results. Clinicians considered RSV and influenza reports during the epidemic season of higher priority for decision making, and a rapid separate service for these agents was requested. These requirements led to the daily workflow as shown in Figure 1. Analyses are offered as two diagnostic packages: a basic package including only RSV and influenza, and a complete package including all 15 tested agents. The choice of analysis package is at the discretion of the clinician, but the extracted material is kept at -70°C, enabling retrospective supplemental testing. This setup resulted in turn-around times between 4 and 8 hr for RSV and influenza analysis during day-time and reports once daily for the other agents. The maximum capacity per run is limited by the 96-well PCR reaction plates as well as the time required for extraction, and is nine samples three times daily for RSV and influenza and nine samples once daily for the complete panel. Every sixth sample is a water negative control. A positive extraction control containing cultured RSV, influenza A and B is included with each run. A pooled RNA control and a pooled DNA control are used for controlling the PCR reactions for each of the remaining agents. In order to aid the clinician's interpretation of the results, separate comments are added to positive results regarding rhinoviruses and enteroviruses describing cross-reactivity. Comments are also added to HBoV-positive results as these findings, in particular at low viral loads, are considered more likely to reflect clinically irrelevant viral shedding and should be interpreted with caution.

Rapid antigen testing is used for very urgent or after-hours requests. Samples with negative results are re-analyzed by real-time PCR on the following day.

Diagnostic results 2007–2008. The PCR diagnostic platform came into operation on October 1, 2007, and fully replaced IF and virus isolation from the first day. Until March 31, 2008, 1,322 samples (1,129 NPAs and 193 bronchoalveolar lavage fluids or bronchial/tracheal secretions) were analyzed. Of the 1,129 NPAs, 791 (70%) were only tested for influenza A and B and RSV (basic package) while 338 (30%) samples were tested for all 15 viruses in the platform (complete package). In total, 618 viral findings were made (243 RSV, 132 influenza B, 92 picornavirus (by lowest Ct value tentatively classified as 71 rhinovirus and 21 enterovirus), 65 influenza A, 25 AdV, 20 HBoV, 17 hCoV HKU1, 7 PIV1, 6 hCoV 229E, 3 hMPV, 3 hCoV OC43, 3 hCoV NL63, 1 PIV2, and 1 PIV3). The diagnostic yield for the basic package was 40%. Respiratory viruses were detected in 192 of the 338 nasopharyngeal samples

TABLE IV. Number of Patients With Double Virus Infections (Evaluation Study of samples From 2004 to 2005)

	RSV	Adenovirus	HBoV	hCoV	Picornavirus
Adenovirus	6				
HBoV	5	0			
hCoV	4	2	1		
Influenza A	4	1	0	0	
Picornavirus	3	2	2	5	
hMPV	0	0	0	1	0
PIV	0	0	0	0	1

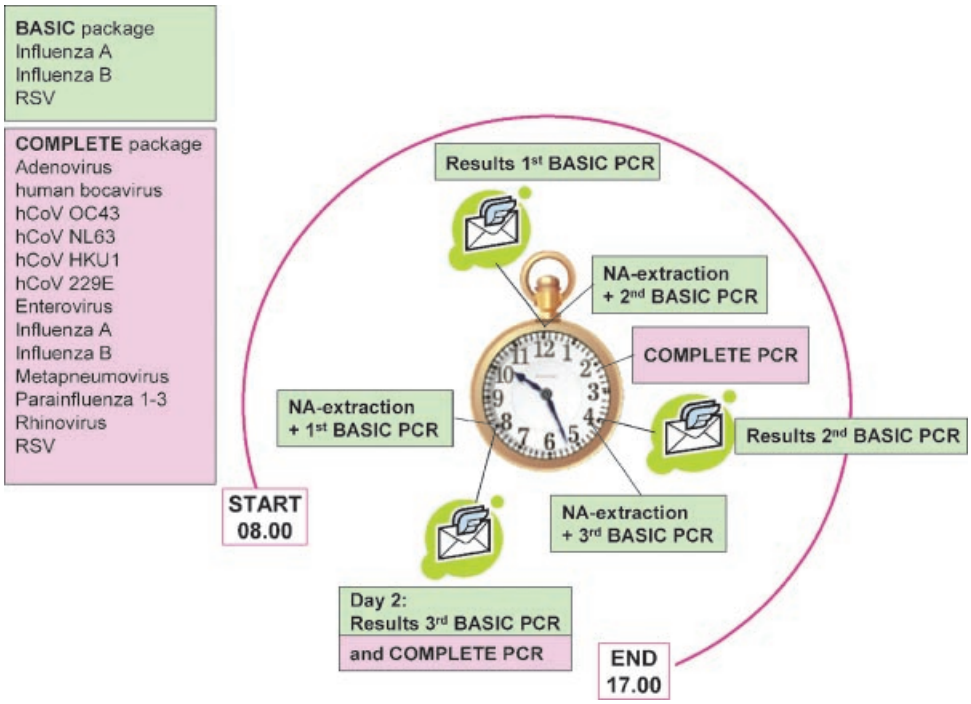

Fig. 1. Schematic illustration of the daily workflow. RSV and influenza viruses are analyzed 3 times daily and the other agents are analyzed once a day. DNA and RNA viruses are analyzed separately due to different thermocycling conditions.

assessed by the complete diagnostic package, that is, a diagnostic yield of 57%. As expected, only 47 (24%) of the 193 investigated tracheal/bronchial/bronchoalveolar samples (all analyzed by the complete package) gave a positive result.

DISCUSSION

Many diagnostic PCR assays for respiratory agents have been described in the literature, but only a limited number are complete diagnostic platforms [Templeton et al., 2004; Watzinger et al., 2004; Gunson et al., 2005, 2006; Oosterheert et al., 2005; van de Pol et al., 2007; Brittain-Long et al., 2008]. The present molecular respiratory virus diagnostic platform has fully replaced traditional IF and virus isolation diagnostics in the laboratory.

The system analyzes 15 viruses in 13 separate PCR reactions. This solution represents a trade-off between costs and assay sensitivity. Multiplex assays are more cost effective, but require substantial efforts in design and optimization, and there is a risk of sensitivity loss due to target competition. Individual real-time PCR assays, on the other hand, avoid the problem of target competition, but are associated with higher costs. However, reagent costs are minor compared to labor costs. In the present analysis platform, reagent costs were, in spite of the multiple assay format, estimated to approximately one-third of the total analysis cost. Thus, replacing the very labor-intensive cell culture and IF procedures by PCR did in this case lead to lowered total costs for respiratory virus diagnosis. Another, somewhat unexpected, advantage was reduced after-hours work with IF. An automated, streamlined, and robust system was hence considered more important for cost-effectiveness than minimizing reagent costs. However, future improvements could include transfer to a 384-well format as well as combining the assays to multiplex reactions.

The time required for extraction, PCR, and data analysis is 4 hr, which is more than for IF. However, in a large laboratory with a continuous inflow of samples, actual time from sampling to result report depends on a number of factors other than analysis time. Additional important factors are transport time to the laboratory, how frequently analyses are performed, and how analysis results are handled and released from the laboratory. Despite a theoretical analysis time of 90 min for IF, an investigation of the laboratory workflow showed that IF results were very often read and reported only twice daily, resulting in turn-around times of several hours. Moreover, nearly all IF requests concerned diagnosis for RSV or influenza, which occur in the epidemic season. Therefore, the platform was designed for high capacity and rapid results for RSV and influenza, which would at least match the turn-around time of the existing service. Analysis for the other viruses was less frequently requested, and previously diagnosed mainly by virus isolation or not at all, so the new platform could only be a major improvement. For that reason, the analyses come in two diagnostic packages: a basic package containing

influenza A, influenza B, and RSV, and a complete package containing all 15 agents. The basic package analyses are performed three times a day and the complete package once daily (Fig. 1). Having two fixed diagnostic packages streamline pre-analytical logistics and simplify ordering for the clinicians.

In order to meet the need for particularly urgent analysis in selected cases, which was previously met by IF, rapid immunochromatography tests were applied, similar to the approach described by Nilsson et al. [2008]. Such tests are increasingly being used at the point of care. Compared to IF, the sensitivity of rapid antigen tests is lower but analysis time is shorter [Ohm-Smith et al., 2004; Weinberg and Walker, 2005]. Because of the limited sensitivity, a more accurate second-line analysis will be still necessary, and a broad and sensitive PCR system will be a very suitable second-line test [Nilsson et al., 2008]. Thus, the rapid antigen tests match diagnosis by PCR, and may form a new standard diagnostic combination, like that of IF and virus isolation.

A main advantage of the molecular assays is the inclusion of previously undiagnosed agents, such as rhinoviruses, coronaviruses, hMPV, and HBoV. These agents are currently of interest for the care of particularly severe cases, immunosuppressed patients, and for clinical studies [Ljungman et al., 2001; Watzinger et al., 2004]. For example, recent studies suggest that mild respiratory tract infections are a substantial risk factor for pulmonary complications after hematopoietic stem cell or lung transplantation [Kaiser et al., 2006; Rossen et al., 2006].

The diagnostic yield of the platform during the first months in clinical use was 57% for the complete package applied to NPAs. This is comparable to the results of the evaluation study. A low diagnostic yield in bronchoalveolar lavage fluid and broncheal/tracheal secretions was expected. These samples are to a large extent drawn from patients in whom previous diagnostic efforts have failed. Patients with long-standing pulmonary disease of unknown origin are also overrepresented in this group.

In the evaluation study on samples from 2004 to 2005, IF and virus isolation gave positive results in 214 of 585 samples (37%). The real-time PCR diagnostic panel was positive for a virus in another 120 samples (20%), increasing the total proportion of positive findings to 57%. Improved sensitivity for RSV, influenza B, and AdV is notable, but the main proportion of the improvement came from detection of agents for which there were previously no diagnostic methods. The exact sensitivity in genome equivalents for each assay was not determined. It was considered of limited clinical relevance as the detection sensitivity in genome equivalents was not determined for the alternative diagnostic methods IF and virus isolation. Comparing analysis results with other diagnostic methods on clinical samples was considered the most relevant performance parameter in this case. The total positive diagnostic outcome of 57% of the 2004–2005 study is comparable to other prevalence studies of respiratory viruses in non-selected patient materials [Bellau-Pujol et al., 2005; Mahony et al., 2007]. Because the material is not clinically well defined, a large proportion of the diagnostic gap may be due to reasons such as patients sampled late in the course of the disease, patients tested for reasons other than viral respiratory tract disease, or inadequate sampling technique. Thus, 100% positive diagnostic results cannot be expected in a study of this type. In a more strictly defined clinical material, the number of positive findings is usually higher [Jartti et al., 2004].

Multiple infections were found in 42 samples, including five cases with triple infection (Table IV). Establishing the relevance of multiple virus findings is difficult. Possible interpretations of such findings include concomitant infections, sequential infections, or long-term, post-infection virus shedding [Allander et al., 2007; Brittain-Long et al., 2008]. Viral load, estimated from the Ct value, may sometimes help the interpretation. However, the present study indicated that Ct values are informative only for some agents. HBoV has in other studies been co-detected with other viruses in up to 80% of samples [Allander et al., 2007; Fry et al., 2007]. In the present study, 60% of HBoV findings were co-infections. HBoV Ct values were significantly lower (viral load higher) in sole HBoV infections than in co-infections (Table III). This is in agreement with earlier results suggesting that HBoV infections with high viral load in the respiratory tract represent symptomatic primary infection, and those with low viral load represent prolonged virus shedding [Allander et al., 2007; Kantola et al., 2008]. The same tendency was observed for coronaviruses (Table III). For other viruses, Ct values were generally very similar whether the virus was found alone or in association with other agents (Table III). Picornaviruses and AdV were the most frequent findings in double infections, mainly in combination with RSV. One may speculate that RSV is usually the main pathogen in these cases, but the present study could not provide evidence for this assumption.

For retrospective studies, sufficient sample volume is a frequent problem. However, viral loads in the nasopharynx vary over several logs. In the present study, a twofold dilution of the samples did not have a major impact on the diagnostic yield. Thus, sensitivity loss due to moderate sample dilution appears to be of minor importance for the diagnosis of respiratory tract infections.

All molecular diagnostics should be under regular surveillance and update of primers and probes, due to the genetic variability and constant genetic drift of the targeted viruses. Severe consequences of "diagnostic escape" mutations have been described for *Chlamydia trachomatis* [Ripa and Nilsson, 2007]. This aspect is even more important for RNA viruses. For some agents, such as influenza, monitoring can be managed by virus isolation in selected laboratories, but for many unculturable agents, a DNA-sequence-based monitoring system is recommended. A principal approach for such a system has been described [Allander et al., 2005]. If such

a monitoring system is systematically applied, monitoring based on virus isolation may be unnecessary.

In conclusion, a manageable and practical platform for real-time PCR diagnostics of 15 respiratory viruses was developed and implemented in a clinical laboratory. The system has been complemented with rapid immunochromatography antigen tests for urgent requests concerning RSV and influenza. The improvements compared to traditional diagnostics include increased diagnostic sensitivity, possibilities for quantitative analysis, and a rational workflow in the laboratory. The main advantage is the large spectrum of detectable viruses, of particular importance for the seriously ill patients and scientific activities of a university hospital.

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