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Evaluation of a multiplex ligation-dependent probe amplification assay for the detection of respiratory pathogens in oncological patients

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ABSTRACT

Background: Respiratory tract infections are widespread and may cause significant morbidity and mortality in immunosuppressed populations such as oncological patients.
Objectives: The RealAccurate Respiratory RT PCR Kit covering 14 respiratory viruses was compared to the RespiFinder Smart22, a broad-spectrum multiplex ligation-dependent probe amplification (MLPA) test, targeting 22 viral and bacterial respiratory pathogens.
Study design: After verification of its analytical performance, the clinical performance of the RespiFinder Smart22 was evaluated by re-analysis of 96 respiratory samples from oncological patients. Additionally, the time to result (TTR) of both methods was compared.
Results: The analytical performance of the RespiFinder Smart22 fulfilled all previously specified criteria. Concordant results in both assays were achieved in 74.0% of all clinical specimens and in 91.2% when only positive results were taken into account. The RespiFinder Smart22 yielded additional results in a total of 22 (22.9% of 96) samples due to higher test sensitivity and a broader, highly multiplexed spectrum of pathogens. The TTR of a typical routine test consisting of three samples were 206 and 356 min for the RealAccurate Respiratory RT PCR Kit and the RespiFinder Smart22, respectively. However, hands-on time was reduced by 59.0% applying the MLPA method.
Conclusions: In our hands, the RespiFinder Smart22 showed excellent analytical performance while hands-on time was halved in comparison to the RT PCR method. Regarding the clinical evaluation, the MLPA method provided additional results in 22.9% (22/96) of specimens due to its comprehensive format, higher test sensitivity and the capability to detect 22 pathogens compared to 14 with the RealAccurate Respiratory RT PCR Kit.

1. Background

Respiratory tract infections are the most common infections worldwide, with an estimate of two to four infections per year in adults [1]. Especially during the colder season respiratory tract infections occur frequently and may cause significant morbidity and mortality in immunosuppressed populations such as oncological patients [2–4]. The clinical presentation of an infection with respiratory pathogens can range from asymptomatic to severe disease. Considering the broad range of respiratory pathogens it is hardly possible to assign clinical symptoms to a distinct etiology. Hence, molecular diagnostic methods have been established as gold standard and are replacing less sensitive and more

Abbreviations: RT, reverse transcriptase; PCR, polymerase chain reaction; TTR, time to result; InfA/B, influenzavirus A/B; RSVA and B, respiratory syncytical virus A and B; PIV-1, parainfluenzavirus 1; CoV-OC43, coronavirus OC43; Rhv/Entero, rhino-/enterovirus; hMPV, human metapneumovirus; Adv, adenovirus; MLPA, multiplex ligation-dependent probe amplification; Rhv, rhinovirus; HOT, hands on time; QC, quality control; RSD, relative standard deviation; HSCT, hematopoietic stem cell transplantation.

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laborious traditional diagnostic methods such as virus isolation by culture or direct immunofluorescence [1,3,5–10]. However, the comprehensive investigation of a respiratory tract infection is time- and resource-consuming when applying singleplex PCR. Therefore, multiplex-based test methods for the detection of numerous respiratory pathogens are entering routine diagnostics [8,9,11,12].

2. Objectives

In the present study a broad spectrum multiplex method, the RespiFinder Smart22 (PathoFinder, Maastricht, The Netherlands), was compared to the routinely used test (RealAccurate Respiratory RT PCR Kit, also PathoFinder) analyzing respiratory samples of oncological patients.

3. Study design

The evaluation of the RespiFinder Smart22 comprised three major parts: the verification of its analytical performance, its routine performance in comparison to the RT PCR method and the evaluation of the time to result (TTR) of both methods. The study was approved by the local ethics committee.

3.1. Molecular assays

The currently used method to detect respiratory pathogens, the RealAccurate Respiratory RT PCR Kit, allows the differentiation of 14 respiratory viruses: influenzavirus A/B (InfA/B), respiratory syncytical virus A and B (RSV A/B), parainfluenzavirus 1 (PIV-1), PIV-2/4, PIV-3, coronavirus OC43 (CoV-OC43) and CoV-229E, rhino-/enterovirus (Rhv/Entero), human metapneumovirus (hMPV) and adenovirus (Adv). The principle of the test is a one-step reverse transcription with subsequent amplification and detection via TaqMan probes on the LightCycler 2.0 platform, requiring a separate master mix for each pathogen. Hence, for analysis of all 14 pathogens in a single clinical specimen 29 capillaries, including all controls, are necessary [13]. The referring physician has the possibility to order individual pathogens as well as the whole panel.

The new method, the RespiFinder Smart22, has been available in Austria since December 2011 and allows the simultaneous differentiation of 22 pathogens. The panel consists of the same viruses as the RealAccurate Respiratory RT PCR Kit, but it covers in addition InfA(H1N1)pdm09, CoV-NL63 and CoV-HKU1, Bocavirus and four bacterial pathogens (*Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis*). The principle of the test is a multiplex ligation-dependent probe amplification (MLPA) subsequent to a reverse transcription requiring two LightCycler capillaries per specimen.

Prior to the analysis, all samples were extracted with the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the instruction for use with a specimen input volume of 200 μl and an output volume of 100 μl for both methods. All samples were stored at −20 °C prior to routine diagnostics with the RealAccurate Respiratory RT PCR Kit to minimize possible effects on the analytical performance and thawed once again for extraction and analysis with the MLPA method.

Clinical samples with discrepant results were retested at the Department of Virology of the Medical University of Vienna with singleplex real time PCRs with detection via TaqMan probes and a nested PCR was employed for the detection of Rhinovirus [14–19].

3.2. Analytical performance

The analytical performance of the RespiFinder Smart22 was verified by testing for accuracy, inter- and intra-assay variability, specificity and sensitivity based on established guidelines [20]. Known positive samples from two different external quality assessment services, QCMD and UKNEQAS that contained Adv, *Bordetella pertussis*, CoV-NL63, hMPV, InfA, InfB, PIV-2, Rhinovirus B Type 72 (Rhv-72), RSV A, and RSV B were tested for the determination of accuracy. The inter- and intra-assay variability were determined by repeatedly analyzing a sample positive for Rhv-72 and a negative sample. To test for specificity, ATCC strains of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were spiked into a previously negative tested pharyngeal wash sample. A dilution series of Rhv-72 and hMPV (undiluted to 1:10,000) was analyzed to compare the sensitivity.

3.3. Clinical performance

In this retrospective study the MLPA method was compared to the RealAccurate Respiratory RT PCR Kit by testing 96 respiratory samples (71.9% pharyngeal washes, 18.8% nasopharyngeal swabs, 9.4% lower respiratory tract samples) of 71 oncological patients treated at the 1st Internal Department of the Elisabethinen Hospital Linz received during the respiratory season of November 2011 to April 2012. After routine diagnostics with the RealAccurate Respiratory RT PCR Kit the original samples were anonymized by assigning a continuous ID number. For analysis with the RespiFinder Smart22 all samples were re-extracted as described in Section 3.1.

3.4. Time to result

The TTR of both methods was evaluated to also elucidate economical aspects on the capability of the MLPA method. It comprises the period of time from the beginning of the analysis of a sample, including the purification of the nucleic acids, to its final result. TTR consists of the hands on time (HOT) and the periods of incubation and was determined for a typical run with three samples by measurement with a stopwatch.

3.5. Data analysis

Data were analyzed with SPSS version 17.0, using descriptive statistics and contingency tables for Fisher's exact test. *P* values of <0.05 were considered significant.

4. Results

4.1. Analytical Performance of the RespiFinder Smart22

For accuracy ten different pathogens were correctly detected in accordance to the results of the external QC. A relative standard deviation (RSD) of <16% was determined for inter-assay variability and an RSD of <6% for intra-assay variability when analyzing Crossing Point-values. For specificity, no cross-reactions were seen when spiking common respiratory bacteria into a negative tested pharyngeal wash sample. Concerning sensitivity, the RespiFinder Smart22 was able to detect Rhv-72 in a 100-fold higher dilution and hMPV with equal sensitivity compared to the RealAccurate Respiratory RT PCR Kit. The results are summarized in Table 4.

4.2. Clinical performance

Demographic and clinical data are summarized in Tables 1 and 2.

Analysis with the RealAccurate Respiratory RT PCR Kit resulted in the detection of a respiratory pathogen in 30 (31.3%) of the 96 samples, whereas retesting with the RespiFinder Smart22 showed positive results in 48 (50.0%) samples ($p = 0.0123$, Fisher's exact test). The comparison of both methods showed matching results in

Table 1
Demographic data of patients.

Patient group	*n* (%)	Male	Female	Inpatients	Outpatients	Median age (range)
Hemato-oncologic	61 (85.9%)	39 (63.9%)	22 (36.1%)	45 (73.8%)	16 (26.2%)	54 (20–82)
Carcinoma	9 (12.7%)	6 (66.7%)	3 (33.3%)	8 (88.9%)	1 (11.1%)	65 (40–74)
Sarcoma	1 (1.4%)	–	1	1	–	38 (–)
Total	71	45 (63.4%)	26 (36.6%)	54	17	54 (20–82)

Table 2
Detection of respiratory pathogens in relation to underlying disease.

Underlying disease	No of Patients	Respiratory pathogen(s) detected[a]	No pathogen detected[a]
Hematologic	61	33	28
Period after HSCT			
Conditioning	3	1	2
Day 0 to days 10–30	6	3	3
Up to day 100	4	2	2
Up to 1 year	11	9	2
After 1 year	11	8	3
No HSCT	26	10	16
Carcinoma	9	2	7
Sarcoma	1	–	1
Total	71		

HSCT: hematopoietic stem cell transplantation.
[a] With RespiFinder Smart22.

Table 3
Summary of results of testing respiratory specimens of oncological patients with the RealAccurate Respiratory RT PCR Kit as well as the RespiFinder Smart22.

RealAccurate Respiratory RT PCR Kit	RespiFinder Smart22	No of specimens	Percentage
Concordant no pathogen detected		47	49.0% (47/96)
Concordant positive		24	25.0% (24/96)
Adv	Adv	3	
Adv and hMPV	Adv and hMPV	1	
CoV-OC43/-229E	CoV-OC43	3	
CoV-OC43/-229E	CoV-229E	1	
hMPV	hMPV	2	
InfA/B	InfA	7	
InfA/B and PIV-2/4	InfA and PIV-4	1	
InfA/B	InfB	2	
PIV-1	PIV-1	1	
Rhv/Entero	Rhv/Entero	3	
Discordant results		25	26.0% (25/96)
Partial panel ordered[a]		5	5.2% (5/96)
Not done[e]	CoV-229E	2	
Not done[e]	hMPV	1	
Not done[f]	Rhv/Entero	1	
Not done[e]	RSV A	1	
Extended spectrum of RespiFinder Smart22[a]		6	6.3% (6/96)
No pathogen detected	Bocavirus	2	
No pathogen detected	CoV-HKU1	3	
InfA/B	InfA and CoV-HKU1	1	
Higher test sensitivity of RespiFinder Smart22[a]		9	9.4% (9/96)
No pathogen detected	CoV-229E	1	
No pathogen detected	hMPV	1	
No pathogen detected	Rhv/Entero	5	
Adv	Adv and Rhv/Entero	1	
InfA/B and Adv	InfA, Adv and Rhv/Entero	1	
Discordant positive		2	2.1% (2/96)
Adv	Rhv/Entero[a]	1	
hMPV	No pathogen detected[a]	1	
Discordant positive[b]		3	3.1% (3/96)
Not done[f]	Rhv/Entero[c]	1	
No pathogen detected	Rhv/Entero[c]	1	
RSV A/B and hMPV	hMPV and CoV-HKU1[d]	1	

[a] Confirmed in the reference lab.
[b] Not resolved in reference lab.
[c] Not detected in reference lab.
[d] RSV detected in reference lab.
[e] Confirmed in subsequent analysis with the RT PCR method.
[f] Not confirmed in subsequent analysis with the RT PCR method.

Table 4
Analytical performance – comparison of sensitivity.

	Dilution series	RT PCR method		MLPA method[a]	
		Result day 1 (CP)	Result day 2 (CP)	Result day 1	Result day 2
Rhv-72	Undiluted	27.98	28.90	Rhv	Rhv
	1:10	30.64	30.94	Rhv	Rhv
	1:100	34.25	34.63	Rhv	Rhv
	1:1000	Not detected	Not detected	Rhv	Rhv
	1:10,000	Not detected	Not detected	Rhv	Not detected
hMPV	Undiluted	23.86	21.93	hMPV	hMPV
	1:10	25.21	25.03	hMPV	hMPV
	1:100	30.54	28.65	hMPV	hMPV
	1:1000	33.74	32.10	hMPV	hMPV
	1:10,000	35.91	36.30	hMPV	hMPV

CP, crossing point.
[a] The MLPA method is an end point test with melting curve analysis subsequent to a reverse transcription PCR. CP values of the detection step are not comparable to the CP values of the RT PCR method.

71 (74.0%) of the 96 analyzed samples, of which 47 (49.0%) samples were concordantly negative and 24 (25.0%) samples were concordantly positive. A total of 25 (26.0%) samples had discrepant results, of which 22 could be resolved by the reference laboratory. In the remaining three samples, rhino/enterovirus RNA was not detected by the reference lab twice, and in one sample with detection of multiple pathogens the reference lab detected RSV RNA. Results are presented in Table 3.

4.2.1. Additional detection

The RespiFinder Smart22 yielded 22 additional positive results due to various factors.

The RealAccurate Respiratory RT PCR Kit allows selection of individual pathogens by the referring physician. In our study, only a partial panel was ordered for 50 (52.1%) samples, mostly limiting diagnostics to influenza viruses. When analyzing these samples with the RespiFinder Smart22, an additional pathogen was detected in six (6.3% of the total 96) samples. These two additional rhino-/enterovirus infections, two coronavirus infections, and one hMPV and RSV infection each were successfully confirmed in the reference laboratory except for one Rhv/Entero positive sample. Upon retesting with the RealAccurate Respiratory RT PCR Kit all pathogens except Rhv/Entero were detected correctly (Table 3).

The higher test sensitivity of the RespiFinder Smart22 was also shown by nine additional positive results (Rhv/Entero, CoV-229E and hMPV) that were ordered but not found in routine testing but could be confirmed by the reference laboratory.

Finally, the RespiFinder Smart22 has an extended spectrum of four viral and four bacterial additional pathogens of which Bocavirus and CoV-HKU1 were detected in seven (7.3%) samples of the study collective. All but one sample containing CoV-HKU1 could be confirmed at the reference laboratory (Table 3).

4.2.2. Clinical aspects

Using routine diagnostics, a respiratory pathogen was detected at least once during the study period in 24/69 patients. However, using the comprehensive approach of RespiFinder Smart22, this result was achieved in a significantly higher proportion of patients (33/69 patients, $p < 0.001$, Fisher's exact test). Two patients of each group were excluded from the analysis because of unresolved differences in testing.

At the time of sampling, 32 (45.1%) of 71 patients had undergone hematopoietic stem cell transplantation (HSCT, median of days after HSCT: 194.5 days) and three (4.2%) patients underwent conditioning for HSCT. Over the study period, a respiratory pathogen was detected by analysis with RespiFinder Smart22 in 11/35 patients who had not received HSCT, whereas 22/34 patients shortly before or after HSCT had a pathogen detected. Thus, patients shortly before or after HSCT had a significantly higher burden of proven viral respiratory tract infection than patients without HSCT ($p = 0.0081$, Fisher's exact test).

4.3. Time to result (TTR)

First, the time requirement for the extraction of nucleic acids was analyzed. For a typical test of three samples, a TTR of 85 min (incubation 25 min, HOT 60 min) was determined for the RT PCR method, which demands the purification of a supplementary negative control, in comparison to a TTR of 77 min (incubation 25 min, HOT 52 min) for the RespiFinder Smart22.

Using the RealAccurate Respiratory RT PCR Kit, the analysis of the complete respiratory panel in three clinical samples with the corresponding negative and positive controls requires 49 LightCycler positions. A LightCycler carousel for 20 μl capillaries holds 32 capillaries, thus two runs are needed to test three samples. Due to the possibility to use two LightCyclers in parallel, the period of incubation of 123 min was counted only once. HOT was 83 min for the processing of three samples, resulting in a total TTR of 206 min.

In an analysis using the RespiFinder Smart22 two vials per sample have to be processed which have to be manually transferred between pre-amplification, hybridization, and 2-step PCR with melting curve analysis. The analysis of three specimens took a HOT of 34 min and a period of incubation of 322 min.

The total TTR for the parallel analysis of three samples was 206 min [123 min (59.7%) period of incubation and 83 min (40.3%) HOT] for the RealAccurate Respiratory RT PCR Kit, and 356 min [322 min (90.4%) period of incubation and 34 min (9.6%) HOT] for the RespiFinder Smart22 (Fig. 1).

5. Discussion

In this study we evaluated a new commercially available multiplex format for the molecular biological diagnosis of respiratory pathogens, the RespiFinder Smart22, in comparison to the routinely used RealAccurate Respiratory RT PCR Kit.

The analytical performance of the RespiFinder Smart22 fulfilled all preset criteria; of note the MLPA method was more sensitive in the detection of Rhv/Entero. Results from other groups indicate that the analytical sensitivity of the RespiFinder Smart22 ranges from 5 to 50 copies per reaction for most targets using commercially available quantitated DNA/RNA PCR controls [12], which equals singleplex PCR sensitivity [8]. However, absolute limits of detection for the individual targets are not available for either method.

The results of the clinical performance revealed a higher test sensitivity of the MLPA test principle in comparison to the RT PCR method as the positivity rate rose from 31.3% to 50.0% in the

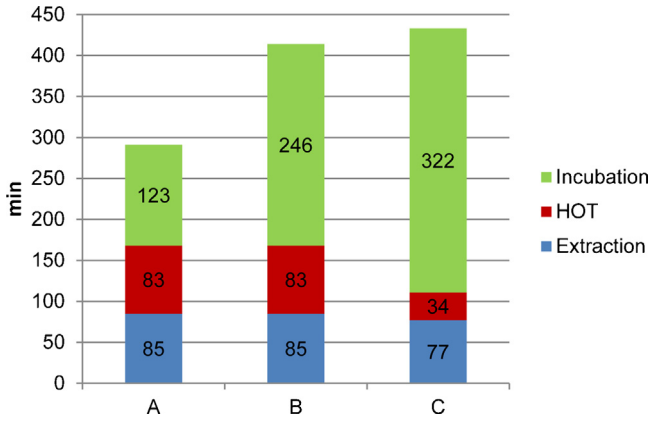

Fig. 1. Time to result – RealAccurate Respiratory RT PCR Kit vs. RespiFinder Smart22. Column A: 3 samples with RT PCR method, 2 LigthCyclers in parallel. Column B: 3 samples with RT PCR method, 1 LightCycler. Column C: 3 samples with RespiFinder Smart22. HOT: Hands on Time (colored online).

analyzed collective. Besides higher test sensitivity the extended spectrum of the RespiFinder Smart22 contributed to the higher positivity rate as Bocavirus and CoV-HKU1 were detected in 7.3% of the samples. A further reason for the difference in the positivity rate is the selective ordering in routine diagnosis. In six (6.3% of the total 96) samples the MLPA method detected an additional pathogen, which is actually included in the range of the RealAccurate Respiratory RT PCR Kit but had not been ordered. The results of five of these samples were confirmed by the reference laboratory. Retesting with the RT PCR method yielded a result in four of the six samples, confirming the higher test sensitivity of the RespiFinder Smart22 for Rhv/Entero. Narrow spectrum diagnostics of respiratory tract infections is considered problematic because of their nonspecific clinical presentation [3]. Therefore, PCR detection of InfA/B, RSV, PIV and in second line hMPV, Rhv/Entero, CoV and Adv is recommended for at-risk patients [21,22]. Especially in HSCT recipients it is important to accurately and swiftly diagnose respiratory infections because of potential progression to severe complications such as pneumonia and also for hospital hygiene reasons [23–27].

Accordingly, HSCT recipients in our sample collective had a significantly higher burden of respiratory tract infections than other oncological patients. In a similar study with 122 HSCT recipients, a lower number of 26.2% of positive cases was reported but only seven viral pathogens (InfA and B, PIV 1, 2 and 3, hMPV) were targeted [28]. Brittain-Long et al. used a multiplex real-time PCR with a similar spectrum of pathogens as the RespiFinder Smart22 in a patient collective with no underlying disease. They reported a positivity rate of 48% out of 954 samples, with InfA the commonest pathogen, followed by Rhv, which is comparable to our results [29].

The MLPA method has an extended pathogen spectrum compared to the RT PCR method and in our study we have detected two viral pathogens of this spectrum; Bocavirus in two samples of one patient and CoV-HKU1 in five samples of four different patients. The role of the human Bocavirus in respiratory tract infections is not yet fully understood, a pathogenic potential is only attributed to infections with high viral loads [22,30] or to the function of a helper virus which may trigger the replication of another respiratory virus [30]. Unlike the Bocavirus, CoV-HKU1 has a clear clinical relevance in respiratory tract infections as reported by Milano et al. in a sample collective of 215 HSCT recipients [25]. In our study collective a Coronavirus was detected in 12 (12.5%) of the samples of which 5 (5.2%) were CoV-HKU1.

Timely reporting of molecular diagnostic tests for respiratory infections is of importance for correct treatment of the patients and adequate hospital hygiene measures. The RespiFinder Smart22 showed a TTR of 5 h 56 min for three clinical samples which is consistent with the 6 h indicated by the manufacturer. Since 90.4% of the TTR consist of incubation time the manufacturer recently introduced an updated version of the kit with a reduction of incubation time by 2 h [31]. Thus, reporting results within a working day of 8 h should easily be possible. The TTR of the RealAccurate Respiratory RT PCR Kit was 3 h 26 min which is significantly longer than the TTR of 2 h stated by the manufacturer since independent of the numbers of patient samples already the LC-run lasts 123 min [32]. When analyzing the complete respiratory panel of three samples in parallel, two LightCyclers are needed or, if there is only one machine, incubation time doubles and the receipt of the results is delayed by 123 min. However, if a 96-well Real Time PCR instrument is available three samples can be processed in a single run. The multiplex format of the RespiFinder Smart22 allows analysis of up to 15 patient samples in parallel, underscoring the advantage of multiplex-based methods in comparison to singleplex PCR concerning the factor time [5]. Most important, hands on time (HOT) was more than halved by the MLPA method, which is a significant improvement regarding lab staff time commitment.

In conclusion, the RespiFinder Smart22 provided reliable results in the diagnosis of respiratory pathogens. Due to the unmodifiable multiplex format of this test, a broad spectrum of pathogens is covered in every run, which significantly increased the positivity rate in the examined collective from 31.3% with the RealAccurate Respiratory RT PCR Kit to 50.0%. Furthermore, the use of RespiFinder Smart22 offers significant advantages concerning HOT compared to the RT PCR method.

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None.

Competing interests

None declared.

Ethical approval

Given by the Ethics Commission of Upper Austria (Reference number: C-37-12).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2014.02.010.

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