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ORIGINAL ARTICLE

Detection of respiratory viruses using a multiplex real-time PCR assay in Germany, 2009/10

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Abstract The aim of this study was to determine the prevalence of respiratory viruses and to prospectively evaluate the performance of the fast-track diagnostics (FTD) respiratory pathogens multiplex PCR assay shortly after the 2009/10 influenza pandemic. Highly sensitive monoplex real-time PCR assays served as references. Discrepant results were further analyzed by the xTAG RVP Fast assay. A total of 369 respiratory samples from children and adults were collected prospectively in Germany from December 2009 until June 2010. The sensitivity and specificity of the FTD assay after resolution of discrepant results was 92.2 % and 99.5 %, respectively. Lowest specificity of the FTD assay was observed for human bocavirus. Multiple detections were recorded in 33/369 (8.9 %) of the samples by monoplex PCR and in 43/369 (11.7 %) using the FTD assay. The most prevalent viruses were respiratory syncytial virus and human metapneumovirus. Only pandemic influenza virus A/H1N1 (2009), and not seasonal influenza virus, was detected. Viruses other than influenza virus accounted for the majority of acute respiratory infections. The FTD assay can be easily implemented in general diagnostic laboratories and facilitate the optimization of patient-management schemes.

For the CAPNETZ study group. Members of CAPNETZ study group listed in Appendix.

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Introduction

Molecular methods have significantly improved the diagnosis of acute respiratory tract infections [16, 18, 21]. These techniques offer high sensitivity and provide specific results within a shorter period of time and for a larger number of pathogens compared to classical methods such as virus isolation or direct fluorescent antibody tests. A recent study showed that multiplex PCR methods can simultaneously detect and differentiate up to 16 pathogens in one assay [12]. Various detection methods currently exist, including microsphere-based detection using Luminex platforms (xTAG RVP or ResPlex II), endpoint-melting curve analysis (FilmArray Respiratory Panel) and auto-capillary electrophoresis (Seeplex RV15 or Respi-Finder 15). The biotechnology industry has generated ready-to-use kits to standardize and ensure the quality of detection assays. However, some of these techniques require contamination-prone post-PCR processing steps as well as additional technical equipment and are not applicable for high-throughput analysis. Alternatively, in-house

multiplex real-time PCR assays have been successfully established by specialized laboratories [3, 13]. However, general laboratories, which are already equipped with real-time PCR technology, might favour commercial real-time PCR solutions because these assays can be easily implemented in routine practice without the need for additional technical equipment. Furthermore, the use of commercial solutions has increased the performance of general laboratories [7, 20]. Although numerous studies have demonstrated the excellent performance of multiplex PCR (e.g., xTAG RVP or ResPlex II), data on samples collected prospectively are rarely available for commercial multiplex real-time PCR assays. The aim of this study was to determine the prevalence of respiratory viruses shortly after the peak of the 2009 influenza pandemic in Germany and to evaluate the performance of the fast-track diagnostics (FTD) respiratory pathogens multiplex real-time PCR assay.

Materials and methods

Patients

Respiratory samples were collected prospectively from 11 study sites throughout Germany between December 1, 2009, and June 30, 2010 [2]. All patients showed clinical signs of acute respiratory tract infection with at least two of the following symptoms: cough, purulent sputum production, dyspnoea, tachypnoea accompanied by fever (temperature, >38 °C) and/or a leucocyte count of >10,000/mm^3. Informed consent was obtained from each participant or his/her legal guardian. Ethical approval was obtained from the Freiburg University Medical Center and the participating study centers. Specimens included pharyngeal samples, which were collected using flocked swabs (Copan, Brescia, Italy), nasopharyngeal aspirates, or bronchoalveolar lavage fluids. All pharyngeal samples, which were collected at 10 different study sites throughout Germany, were immediately shipped overnight in universal transport medium (Copan) at ambient temperatures according to the instructions of the manufacturer (Copan). The remaining study center was located in Freiburg, and samples were immediately sent to the laboratory in Freiburg. All specimens were processed directly without further steps of dilution (see below).

Fast-track diagnostics respiratory pathogens kit

All samples were freshly extracted using a QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (input, 200 µl; elution volume,100 µl) on a QIACube (QIAGEN). Aliquots of the specimens were stored at -70 °C. After nucleic acid extraction, samples were immediately subjected to multiplex PCR analysis using the FTD respiratory pathogens assay version 5-11/2009 (Fast-track Diagnostics, Junglinster, Luxemburg). The assay utilizes the 5′ nuclease technology using differentially labelled fluorogenic probes and is composed of five individual multiplex one-step real-time PCR assays. In tube 1, real-time PCR assays for influenza virus A, influenza virus B, and brome mosaic virus (BMV) are combined; tube 2 contains assays for coronaviruses 229E, NL63, and OC43 and enterovirus/parechovirus; tube 3 contains assays for parainfluenza viruses 2, 3, and 4; tube 4 contains assays for parainfluenza virus 1, human metapneumovirus A/B (HMPV), and human bocavirus; and tube 5 contains assays for rhinovirus, respiratory syncytial virus A/B (RSV) and adenovirus. Five microliters of the template was used for each of the five multiplex PCR assays. An internal control (BMV), which was included in the FTD kit, was added to each patient sample after nucleic acid extraction. An AgPath-ID One-Step PCR Kit (Invitrogen, Karlsruhe, Germany) was applied for PCR using an ABI 7500 real-time PCR machine (Applied Biosystems, Wiesbaden, Germany). Thermocycling conditions were as follows: 50 °C for 15 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 8 s and 60 °C for 34 s.

Reference monoplex PCR

As a reference, a panel of monoplex real-time PCR assays was used [2]. Determination of the influenza A virus subtype was done as described [19]. Primer and probe sequences are available from the corresponding author (MP) upon request. These have been extensively validated and have demonstrated ultimate sensitivity (<20 RNA or DNA copies per PCR) when using *in vitro*-transcribed RNA or DNA plasmids (data not shown). To streamline diagnostics, all assays, including those for DNA targets, were carried out in a total volume of 25 µl using a QIAGEN OneStep PCR Kit (Qiagen, Hilden, Germany). A concentration of 1 µM of each primer and 0.5 µM of the probe were used. Reverse transcription and amplification were performed using a Roche LightCycler 480 II System (Roche, Mannheim, Germany) under the following conditions: 30 min at 50 °C, 15 min at 95 °C, 45 cycles at 95 °C for 15 s and 60 °C for 30 s. The RNA bacteriophage MS2 (German collection of microorganisms and cell cultures, Göttingen, Germany) was used as an internal extraction/inhibition control as described elsewhere [6]. Briefly, 30 µl of MS2 (10^5 CFU/ml) was added to the lysis buffer before extraction. Positive (*in vitro* RNA transcripts or DNA plasmids of the respective target) and negative controls were used in each assay. Cycling threshold values <45

were considered positive. The original samples stored at -70 °C were re-extracted as described previously and immediately subjected to monoplex real-time PCR. Up to 10 patients were analyzed in parallel in one working day for all 15 pathogens by using real-time PCR in two consecutive runs.

Luminex xTAG RVP Fast

The Luminex xTAG RVP Fast version 2 (Abbott Molecular, Wiesbaden, Germany) was used to resolve discrepant samples, following the manufacturer's instructions. Amplified PCR products were analysed using a Liquichip 100 IS system (Luminex Molecular Diagnostics, Toronto, Canada). Cutoff values for positivity were set according to the recommendations of the manufacturer.

Statistics

Data were analysed using SPSS software version 19 (SPSS, Chicago, IL, USA) and the openly available statistical software environment R, version 2.10.1. Data were compared by Mann-Whitney U-test. P-values were deemed significant at the 0.05 level. Cohen's kappa was used to assess the agreement between the two methods [10].

Results

A total of 369 samples were collected prospectively throughout Germany from December 1, 2009, until June 30, 2010. Samples included 214 pharyngeal swabs, 152 nasopharyngeal aspirates and 3 bronchoalveolar lavage fluids (BAL). The median age of the patients was 6 years (95 % confidence interval [CI]: 1-59 years).

FTD assay

FTD multiplex PCR detected at least one pathogen in 194 (53 %) of 369 samples. A single pathogen was detected in 151 (41 %) of 369 samples. Dual detections were observed in 37 (10 %), triple detections in 4 (1 %), and quadruple detections in 1 (0.3 %) of 369 samples. In one sample, six different pathogens were recorded (the same result was obtained using monoplex PCR). Among the 214 pharyngeal swabs collected, 69 (32 %) tested positive for at least one pathogen. A total of 125/152 (82 %) nasopharyngeal aspirates were positive, and 3/3 (100 %) BAL samples were positive. The median age of patients with pharyngeal swab specimens (49.5 years) was significantly higher compared to patients with nasopharyngeal aspirates (2 years) ($p<0.001$, Mann-Whitney U-test).

Among the patients younger than 6 years of age, 140 (76 %) of 185 samples gave positive results, whereas among the patients older than 6 years of age, 57 (31 %) of 184 samples tested positive. The pathogen most frequently detected using the FTD assay was RSV, which was reported in 68 samples (18 %), followed by HMPV in 46 samples (12 %), and rhinovirus in 43 samples (12 %), out of a total of 369 samples (Table 1). Influenza A virus was detected in 10 samples. This specific version of the FTD assay effectively detected influenza A and B viruses, but was incapable of subtyping influenza A virus–positive samples. The internal control (BMV) showed positive results for all samples.

Reference assays

Pathogens were detected in 187 samples (51 %), including 154 samples (42 %) with a single-pathogen infection, 28 (8 %) with dual infection, 2 (0.5 %) with triple infection, and 1 (0.3 %) with quadruple infection. Moreover, monoplex PCR detected 5 and 6 different pathogens in one sample each. Among patients younger than 6 years of age, 132 (71 %) of 185 samples showed positive results, whereas among patients older than 6 years, 55 (30 %) of 184 samples tested positive. The most frequently detected pathogen was RSV, which showed positive results in 70 samples (19 %), followed by HMPV in 40 samples (11 %), and rhinovirus in 31 samples (8 %), out of a total of 369 samples. Most HMPV- and RSV-positive samples were collected in February and March 2010 (Table 2). Detection rates were >60 % in January, February, and March 2010. A total of 9 of 10 (90 %) influenza virus-positive results were obtained in patients above 6 years of age (median 36 years; 95 % CI 15.6-51.3 years). Only pandemic influenza virus A/H1N1 (2009) was detected using subtype-specific real-time PCR. MS2 phage, which served as an internal control, yielded positive results in all samples.

Comparison of the FTD assay with reference assays

The performance of the FTD assay was compared to monoplex PCR as the reference (Table 1). The overall sensitivity and specificity of the FTD assay were 84 % and 99.2 %, respectively, with good concordance with the reference monoplex PCR ($k = 0.80$). A total of 90 (24.3 %) of 369 samples yielded discrepant results. Of these, 37 (41.1 %) showed positive results using monoplex PCR, whereas negative results were obtained using the FTD assay. Another 53 (58.8 %) tested negative by monoplex PCR, but they tested positive by the FTD assay. The most discrepant results were observed in the detection assays for bocavirus, followed by that for rhinovirus. The FTD assay was incapable of distinguishing between

Table 1 Comparison of the FTD multiplex RT-PCR assay with monoplex real-time RT-PCR as the reference. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa value are shown

Pathogen	No. of samples (monoplex RT-PCR/FTD assay)				FTD PCR performance				
	+/+	+/−	−/+	−/−	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa
Adenovirus	9	2	5	353	81.8	98.6	64.3	99.4	0.71
Human bocavirus	19	7	11	332	73.1	96.8	63.3	97.9	0.65
Coronavirus NL63	0	1	1	367	na	99.7	na	99.7	na
Coronavirus OC43	5	6	1	357	45.5	99.7	83.3	98.3	0.58
Coronavirus 229E	5	2	2	360	71.4	99.4	71.4	99.4	0.71
Enterovirus/parechovirus[1]	3	1	4	361	75	98.9	42.9	99.7	0.54
Human metapneumovirus	38	2	8	321	95	97.6	82.6	99.4	0.87
Influenza virus A, universal[2]	8	2	2	357	80	99.4	80	99.4	0.79
Influenza virus B	0	0	0	369	na	100	na	100	na
Parainfluenza virus 1	4	4	0	361	50	100	100	98.9	0.66
Parainfluenza virus 2	1	3	1	364	25	99.7	50	99.2	0.33
Parainfluenza virus 3	6	1	1	361	85.7	99.7	85.7	99.7	0.85
Parainfluenza virus 4	1	0	1	367	100	99.7	50	100	0.67
Respiratory syncytial virus	65	5	3	296	92.9	99	95.6	98.3	0.93
Rhinovirus	30	1	13	325	96.8	96.2	69.8	99.7	0.79

na: not applicable

[1] FTD assay does not distinguish between enterovirus and parechovirus

[2] FTD assay does not subtype influenza virus A

enterovirus and parechovirus. In this first analysis, only an enterovirus-specific PCR was performed on all samples. Among samples with multiple detections, 16 (48.5 %) of 33 samples yielded concordant results.

Analysis of discrepant results

Discrepant samples (n = 90) were analysed by the FDA-approved xTAG RVP Fast assay and included 56 (62.2 %) nasopharyngeal aspirates and 34 (37.7 %) pharyngeal swabs. A total of 19 of 90 (21 %) FTD-positive/monoplex-PCR-negative samples were confirmed to be positive by the xTAG assay. Another 12 of 90 (13 %) FTD-negative/monoplex-PCR-positive samples were confirmed to be positive by the xTAG assay. The remaining 59 (65 %) samples tested false-positive using the FTD (n = 34) or monoplex PCR (n = 25) as adjudicated by a negative xTAG RVP Fast result. The samples that tested false-positive using the FTD assay (n = 34) included 22 nasopharyngeal aspirates and 12 pharyngeal swabs. The pathogens for which false positive results were most frequently obtained in the FTD assay were bocavirus (n = 10), adenovirus (n = 5), and rhinovirus (n = 5). In 9/10 bocavirus FTD false-positive samples, 3/5 adenovirus FTD false-positive samples, and 2/5 rhinovirus FTD false-positive samples, co-detections occurred. The median FTD cycle-threshold value was 28 (95 % confidence interval 26.8-29.4) in monoplex-PCR negative/xTAG-positive samples (n = 19), and 31 (95 % confidence interval 27.8-31.7) in monoplex-PCR-negative/xTAG-negative samples (n = 34; p = 0.039; Mann-Whitney U-test). Combination of any two of the three assays resulted in a 92.2 % and 99.5 % increase in the sensitivity and specificity, respectively, of the FTD assay (Table 3). In addition, this combination also showed excellent concordance (k = 0.89). Parechovirus-specific PCR was performed on samples (n = 8) that were determined to be enterovirus/parechovirus-positive by FTD and/or enterovirus-positive by monoplex PCR. Only 1 out of these 8 samples tested positive for parechovirus (Table 4). Notably, the FTD rhinovirus assay appeared to be more sensitive than the reference monoplex PCR. To further assess FTD rhinovirus-positive samples, sequencing part of the VP1 region of a subset of FTD multiplex-positive/monoplex-negative (n = 5) and FTD multiplex-positive/monoplex-positive (n = 8) samples detected the presence of rhinovirus C in 12 (92 %) of 13 samples, indicating restricted target specificity of the reference assay. Rhinovirus A was detected in 1 (8 %) of 13 samples. Interestingly, 4 (2 %) of the 43 FTD rhinovirus-positive samples showed cross-reaction with the FTD enterovirus/parechovirus PCR (Table 4).

Table 2 Monthly distribution of pathogens as assessed by the reference standard monoplex PCR from December 2009 until June 2010

Month	Number of samples	Percent positive	Pathogen								
			Adenovirus	Bocavirus	Coronavirus	Entero/parechovirus	HMPV	Influenza virus	Parainfluenza virus	RSV	Rhinovirus
December	46	48	1	3	1	1	2	4	2	0	8
January	55	60	5	11	3	0	2	3	2	3	4
February	91	68	1	5	6	2	17	0	4	23	4
March	96	92	1	6	7	1	15	3	7	41	7
April	37	46	1	1	0	0	3	0	3	3	6
May	27	19	2	0	0	0	1	0	0	0	2
June	17	0	0	0	0	0	0	0	0	0	0

Discussion

This prospective study showed that the FTD multiplex real-time PCR assay could detect 16 respiratory pathogens within a response time of 2.5 hours. Test costs per sample (excluding enzyme costs) of the FTD assay were 22.63 €, whereas test costs of individual monoplex PCR were 5.41 €. This unique assay also showed high specificity and sensitivity. The three most common pathogens yielded sensitivities of >94 %, indicating excellent performance. Compared to a microsphere-based assay (xTAG RVP Fast), a higher overall sensitivity (92.2 %) was reached [11]. A recent study evaluating the FTD assay found considerably lower sensitivities for selected targets (i.e., adenovirus, rhinovirus, and RSV) [22]. The authors speculated that competitive inhibition of multiple targets may have led to this finding. For some rhinovirus strains, clear dropouts were recorded, similar to our findings, as discussed below. Anderson and colleagues demonstrated good overall concordance between four multiplex PCR assays, including the FTD assay, in a recent study from New Zealand [1]. This suggests that the FTD assay is widely applicable. The results of this study highlight the importance of evaluating the performance of the assays in the epidemiological context of their intended use.

We are aware that use of monoplex PCR as a reference can confound results because variant viruses might go undetected [23]. Faux et al. recently reported a high degree of variability among different PCR assays for the detection of rhinovirus [9]. The current study showed that detection of the novel rhinovirus C did influence the performance of the reference, thus supporting the findings of Faux and colleagues. Application of a third reference test facilitated the resolution of samples that showed discrepant results. Of note, a number of false-positive results were observed for bocavirus. This finding has also been reported by Anderson et al. [1]. Further analysis with members of distinct bocavirus species and strains as well as different sample matrices is warranted. In general, nonspecific amplification using the FTD assay was associated with significantly higher cycle threshold values, confirming the results of Sakthivel et al. [22].

In our study, samples from throughout Germany were collected and shipped to our laboratory. To minimize the possible degradation of samples, we used flocked swabs in viral transport medium, which has been demonstrated to be a reliable approach [8]. A detection rate of 32 % in pharyngeal samples, which were mostly collected from adult patients, is comparable to the findings of Yang et al. [24]. This supports the notion that significant degradation did not occur, although weakly positive samples might still have gone undetected.

As this study started after the 2009 A/H1N1 pandemic in Germany, only a few influenza cases were detected in this

Table 3 Performance of the FTD assay. Samples were regarded as true positive or negative after discrepant results (n = 90) were analyzed using the xTAG RVP Fast assay and any two of the three RT-PCR assays were positive or negative. True positive (TP), false negative (FN), false positive (FP), true negative (TN), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa value are shown

Pathogen	No. of samples				FTD assay performance				
	TP	FN	FP	TN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa
Adenovirus	9	1	5	354	90.0	98.6	64.3	99.7	0.74
Human bocavirus	20	4	10	336	83.3	97.1	66.7	98.8	0.72
Coronavirus NL63	0	0	1	368	na	99.7	na	100	na
Coronavirus OC43	5	1	1	362	83.3	99.7	83.3	99.7	0.83
Coronavirus 229E	5	2	1	361	71.4	99.7	83.3	99.4	0.77
Enterovirus/parechovirus[1]	6	1	1	361	85.7	99.7	85.7	99.7	0.85
Human metapneumovirus	43	1	3	322	97.7	99.1	93.5	99.7	0.95
Influenza virus A, universal[2]	10	1	0	358	90.9	100	100	99.7	0.95
Influenza virus B	0	0	0	369	na	100	na	100	na
Parainfluenza virus 1	4	0	0	365	100	100	100	100	1
Parainfluenza virus 2	1	2	1	365	33.3	99.7	50	99.5	0.4
Parainfluenza virus 3	6	0	1	362	100	99.7	85.7	100	0.92
Parainfluenza virus 4	1	0	1	367	100	99.7	50	100	0.67
Respiratory syncytial virus	66	4	2	297	94.3	99.3	97.1	98.7	0.95
Rhinovirus	38	1	5	325	97.4	98.5	88.4	99.7	0.92

na: not applicable

[1] FTD assay does not distinguish between enterovirus and parechovirus

[2] FTD assay does not subtype influenza virus A

Table 4 Analysis of enterovirus/parechovirus-positive samples (n = 8) as assessed by the enterovirus-specific reference RT-PCR or the FTD assay. Shown are results for enterovirus (EV), rhinovirus (RV) and parechovirus (PeV) as assessed by reference monoplex RT-PCR and for EV/PeV and RV by the FTD assay and for EV/RV by the xTAG RVP Fast assay. +, positive result; −, negative result

Patient	Age	Sex	Reference RT-PCR			FTD		xTAG RVP
			EV	RV	PeV	EV/PeV	RV	EV/RV
1	4	m	+	−	−	+	−	−
2	6	m	+	−	−	−	−	+
3	5	m	+	−	−	+	−	−
4	4	m	+	−	−	+	−	nd
5	2	m	−	+	−	+	+	−
6	4	m	−	+	+	+	+	+
7	5	f	−	+	−	+	+	+
8	3	f	−	+	−	+	+	+

nd: not done

cohort; instead, a markedly high number of RSV and HMPV cases were observed. This finding is in contrast with the observations of Huck et al., who described a seasonal, inverse correlation between RSV and HMPV [15].

The uniformity of the symptoms of acute respiratory tract infections requires the use of a broad-spectrum diagnostic assay that is applicable not only during a pandemic [17]. Importantly, high detection rates of viruses other than influenza virus clearly indicate the need for rapid diagnosis to prevent nosocomial transmission. A recent study demonstrated that access to PCR may also reduce the prescription of antibiotics during a patient's initial outpatient visit [4].

To facilitate the use of novel diagnostic methods in the clinical setting, ready-to-use assays have been shown to improve the performance of general laboratories [20]. Real-time PCR technology has become well recognized in the hospital setting, and thus, this commercial assay may be easily adopted in general public-health laboratories. Finally, there is increasing evidence that bacteria play a role in co-infections [5, 14]. The commercial kit evaluated in this study has recently been upgraded to distinguish enterovirus and parechovirus and to include bacterial targets, thus increasing its value; however, this upgraded version will also warrant further evaluation. To conclude, the FTD assay evaluated here showed high sensitivity and specificity for the detection of HMPV, RSV, and rhinovirus, but it was less sensitive and specific for the detection of bocavirus.

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Conflicts of interest The authors declare no conflicts of interest.

Appendix

Members of the CAPNETZ study group, excluding the authors:

S. Krüger, D. Frechen (Aachen); W. Knüppel, I. Armari (Bad Arolsen); D. Stolz (Basel); N. Suttorp, H. Schütte, A. Tessmer, P. Martus (Berlin, Charité); T. Bauer, J. Hecht (Berlin); W. Pankow, A. Lies, D. Thiemig (Berlin-Neukölln); B. Hauptmeier, S. Ewig, D. Wehde, M. Suermann (Bochum); M. Prediger, G. Zernia (Cottbus); T. Welte, J. Rademacher, G. Barten, L. Gosman, W. Kröner (Hannover); R. Bals (Homburg/Saar); C. Kroegel, M. Pletz (Jena); K. Dalhoff, S. Schütz, R. Hörster, (Lübeck); G. Rohde (Maastricht); W. Petermann, H. Buschmann, R. Kröning, Y. Aydin (Paderborn); T. Schaberg, I. Hering (Rotenburg/Wümme); R. Marre, C. Schumann (Ulm); H. von Baum (Ulm, Med. Microbiology); T. Illmann, M. Wallner (Ulm); O. Burghuber, G. Rainer (Wien) and all study nurses.

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