

A multiplex one-step real-time RT-PCR assay for influenza surveillance

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For surveillance purposes real-time PCR assays for influenza viruses had to be adapted to the pandemic influenza A(H1N1)2009 strain. We combined published primers and probes for influenza A, influenza B and an internal amplification control with a detection system for influenza A(H1N1)2009 to set up a rapid, reliable, simple and cost-effective high-throughput multiplex one-step real-time RT-PCR. The workflow also includes automated sample preparation for high-throughput screening. The lower limit of detection of the multiplex assay was 3.5×10^2 RNA copies per PCR reaction. The diagnostic sensitivity of the multiplex assay was 87.7%, but increased to 99.4% for influenza-positive samples yielding C_t values of less than 34 cycles in the respective diagnostic assay. High specificity was confirmed by sequencing and correct detection of 15 reference samples from two quality assurance studies. The multiplex PCR was introduced for surveillance of samples from a network of general practitioners and paediatricians in Bavaria, Germany during the influenza pandemic of 2009. Comparison with surveillance data from reported cases proved the reliability of the multiplex assay for influenza surveillance programmes.

Introduction

In April 2009, a novel influenza A(H1N1) virus emerged [1] that could not be detected by routine diagnostic assays for subtyping seasonal influenza A(H1N1) viruses. Therefore, accurate and reliable diagnostic tests for the new influenza A strain had to be established to screen patients with influenza-like illness (ILI) for the 2009 pandemic influenza virus [2-9]. At the onset of the pandemic, public health control measures, namely the isolation of patients and suspected cases to limit the spread of the virus, were guided by the results of these tests [10].

In October 2009, mass vaccination programmes with different pandemic influenza vaccines were implemented globally. In Germany, about 6 million people were vaccinated from the end of October to the end of December 2009. At that stage of the pandemic the World Health Organization (WHO), the European Centre for Disease Prevention and Control (ECDC) and the

Robert Koch Institute in Germany (RKI) recommended strengthening the influenza surveillance. This surveillance should persist throughout the whole year and include the new influenza strain as well as seasonal influenza strains, because co-circulation was reported and also expected in the future. At that time, no multiplex real-time RT-PCR assay was available for the simultaneous detection of seasonal influenza A, influenza B and pandemic influenza A(H1N1)2009 viruses. Published diagnostic assays focused more on subtyping of influenza viruses using microarrays and sequencing [11-14]. However, these tests are not suitable for high-throughput routine diagnostic screening.

For large scale surveillance of ILI patients cost effective and time-saving methods for the detection of influenza viruses are needed. The multiplex real-time RT-PCR assay described here provides a diagnostic tool for the fast, simultaneous and reliable diagnosis of influenza A and B viruses with validated and well established real-time PCR protocols with minor modifications, and includes an officially recommended real-time PCR protocol for simultaneous subtyping of the pandemic influenza A(H1N1)2009 virus.

Methods

Specimen collection

For specificity and sensitivity testing as well as for the evaluation of the multiplex assay different panels of clinical samples and reference material were used in this study:

The specificity of the PCR protocol for subtyping pandemic influenza A(H1N1)2009 virus was assessed by sequencing 50 PCR products from clinical samples collected in the beginning of the pandemic in May 2009.

We tested the specificity of the multiplex assay with the following samples: influenza A/Bavaria/63/2009 (a pandemic influenza (H1N1)2009 virus) in six consecutive dilutions, influenza A/Brisbane/59/2007 (H1N1) in four dilutions, influenza A/Brisbane/10/2007 (H3N2), influenza A/chicken/Germany/R3294/2007 (H5N1) in two dilutions, influenza A/whooper swan/Germany/R65-2/2006 (H5N1) and influenza B/Brisbane/60/2008.

TABLE 1

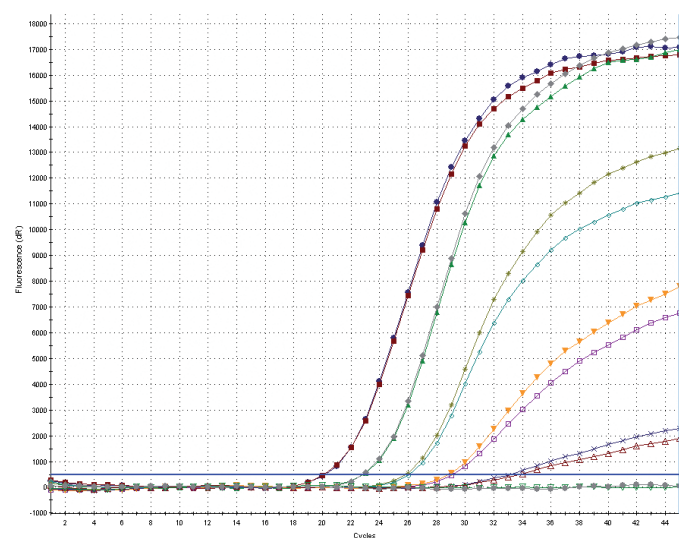
Primers and probes used in the multiplex one-step real-time RT-PCR assay for the detection of different influenza virus strains

	Primers and probes	Sequence (5'→3')	Working concentration	Reference
Influenza A	InfA M+25	AGATGAGTCTTCTAACCGAGGTCG	400 nM	15
	InfA M-124	TGCAAAAACATCTTCAAGTCTCTG	400 nM	
	InfA M-124-mod	TGCAAGACACTTTCCAGTCTCTG	400 nM	
	InfA M + 64-FAM	6FAM-TCAGGCCCTCAAAGCCGA-BBQ	200 nM	
Influenza A(H1N1)2009	Flu Sw H1 F236	TGGGAAATCCAGAGTGGAATCACT	400 nM	9
	Flu Sw H1R318	CGTTCATTGTCTGAAGTACTAGTGT	400 nM	
	Flu Sw H1 TM298-TEX	TEX-CCACAATGTAGGACCATGAGCTTGCTGT-BBQ	200 nM	
Influenza B	InfB BP-13	GAGCACAATTGCCTACCTGC	400 nM	16
	InfB BMP102	CCACCGAACCAACAGTGTAAAT	400 nM	
	InfB BMP-72-CY5	CY5-AGATGGAGAGGCAGCGAAGTACTAGC-BBQ	200 nM	
Internal amplification control	IAC EGFP-12-F	TCGAGGGGACACCCCTG	400 nM	18
	IAC EGFP-10-R	CTGTACAGCTCGTCCATGC	400 nM	
	IAC EGFP-HEX	HEX-AGCACCCAGTCCGCCTGAGCA-BBQ	200 nM	

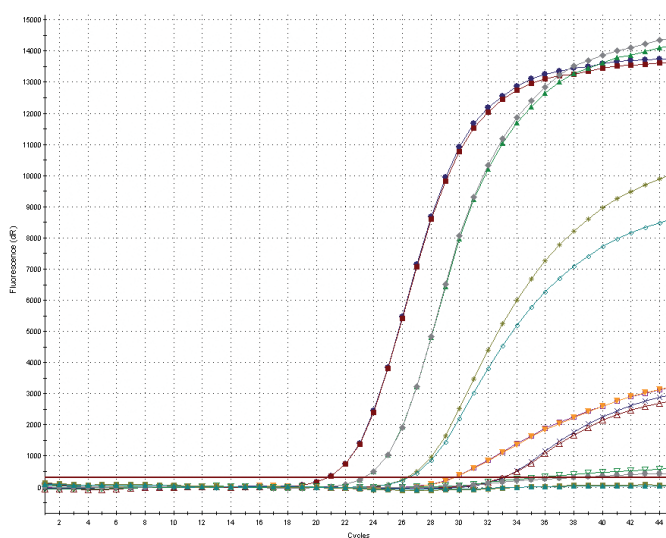
FIGURE 1

Typical RT-PCR amplification curves for influenza viruses

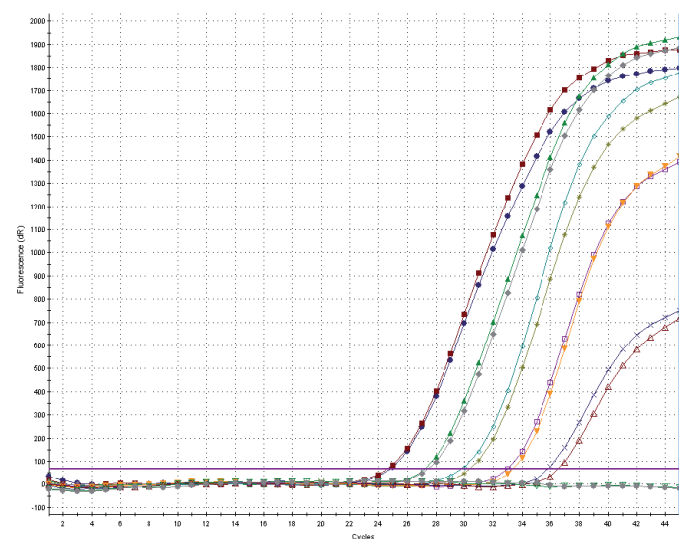
A: Seasonal influenza A virus detected in the FAM channel



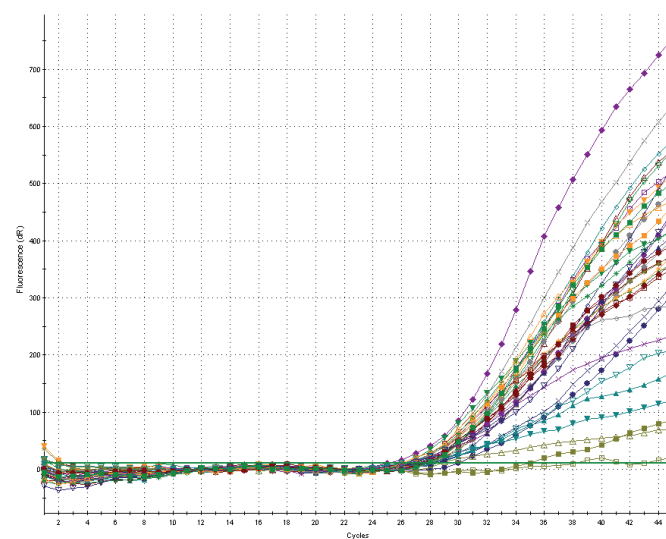
B: Pandemic influenza A(H1N1)2009 virus detected in the TEX channel



C: Influenza B virus detected in the CY5 channel



D: HEX channel showing the internal amplification control



All viruses in dilution series of 10 ng RNA to 1 pg RNA.

The samples were provided for two external quality assurance studies (organised by INSTAND e.V., Germany in 2009/10. In addition, the oseltamivir-resistant strain influenza A/Berlin/58/2008 (H1N1) was provided by the national reference centre for influenza at the RKI in Berlin.

The analytical sensitivity (limit of detection) of the multiplex real-time RT-PCR assay was determined using plaque-quantified influenza A/Hamburg/05/2009 (H1N1) virus with a concentration of 3.5×10^5 PFU/ml [15]. A 10-fold dilution series of extracted RNA was generated from 3.5×10^5 to 3.5 plaque-forming units per ml (PFU/ml) and analysed in triplicate in the FAM-channel (matrix gene) as well as the ROX channel (HA gene) of the multiplex PCR assay. To compare the sensitivity of the multiplex and each single assay, RNA was prepared from egg cultures of an early case of pandemic influenza A(H1N1)2009 in Bavaria, detected on 29 April 2009 and confirmed by the national reference centre for influenza at the RKI, as well as from cell cultures of reference material: influenza A/Bayern/89/2007 (H1N1), influenza A/Sydney/5/1997 (H3N2) and influenza B/Brisbane/60/2008. RNA was analysed in 10-fold dilution series in nuclease-free water containing background calf thymus DNA (Type I fibres, Sigma-Aldrich) in a concentration of 100 ng/ μ l. RNA dilutions were prepared from 100 ng to 1 pg per PCR reaction.

For evaluation of the multiplex one-step real-time RT-PCR assay and to determine diagnostic sensitivity,

TABLE 2
PCR efficiencies of the single assays compared to the multiplex assay

	E single	E multiplex
Influenza A	112.9%	112.9%
Influenza A(H1N1)2009	103.1%	105.5%
Influenza B	92.0%	120.0%

E: PCR efficiency

FIGURE 2
Detection rate of the multiplex PCR for influenza viruses in samples with different C_t values in the respective diagnostic assay

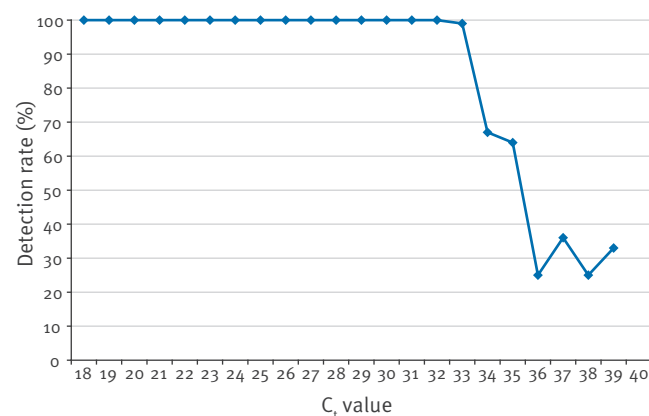


TABLE 3
Sensitivity, specificity, positive predictive value and negative predictive value of the multiplex assay

	All single assays				$C_t < 34$ in the single assays				$C_t > 34$ in the single assays						
	Samples	Sensitivity	Specificity	PPV	NPV	Samples	Sensitivity	Specificity	PPV	NPV	Samples	Sensitivity	Specificity	PPV	NPV
Seasonal influenza A	47	78.7	100	100	90.0	31	100	100	100	100	16	37.5	100	100	90.0
Seasonal influenza B	50	76.0	100	100	88.2	28	96.4	100	100	98.9	22	50	100	100	89.1
Pandemic influenza A(H1N1)2009	130	95.4	98.9	99.2	93.7	116	100	98.9	99.1	100	14	57.1	98.9	88.9	93.7
Overall	227	87.7	99.6	99.5	90.6	175	99.4	98.9	99.4	98.9	52	48.1	98.9	96.2	76.7

NPV: negative predictive value; PPV: positive predictive value. Numbers are also shown for samples with C_t -values below and above 34.

we used clinical samples obtained from ILI patients during the influenza season 2008/09 and the 2009 influenza pandemic in Bavaria. ILI was defined by sudden onset with fever ($>38.5^{\circ}\text{C}$), cough, sore throat and myalgia and/or headache. We had previously tested the samples with diagnostic real-time RT-PCR assays for seasonal influenza A and B [16,17] and for influenza A(H1N1)2009 [2,9]. The panel consisted of 317 samples: 90 influenza-negative samples, 47 samples positive for seasonal influenza A(H3N2) and A(H1N1), 50 samples positive for influenza B viruses as well as 130 samples positive for influenza A(H1N1)2009 virus. Original specimens included nasopharyngeal and throat swabs in viral transport medium. After screening for influenza, the remaining RNA was stored at -80°C until testing with the multiplex assay.

Nucleic acid extraction

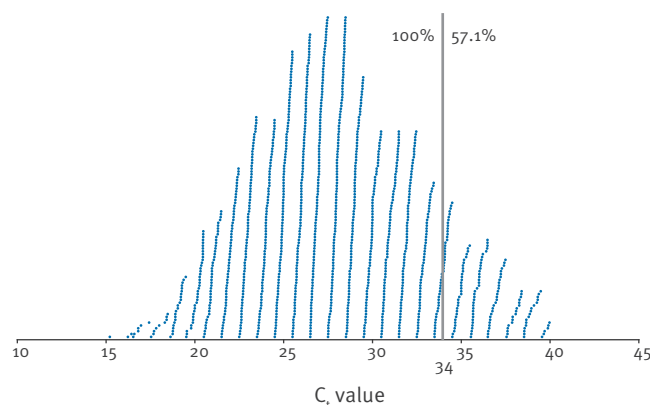
Viral nucleic acid was extracted using the QIAamp Virus Bio Robot 9604 kit (Qiagen) adapted to the robot Hamilton Microlab Star (Hamilton) for large numbers of samples or the Viral RNA mini kit (Qiagen) for small numbers of samples. From our routine diagnostic analyses we know that the extraction method has no influence on the results.

Internal amplification control

Commercially available heterologous *in vitro*-transcribed RNA (INTYPE IC-RNA Labordiagnostik, Leipzig, Germany) was used as PCR inhibition control. This *in vitro* transcript has proven its robustness in previous multiplex real-time RT-PCR assays [18]. The stock solution (8×10^5 copies/ μl) of the *in vitro*-transcribed RNA was stored at -80°C , and the working dilutions of 1×10^5 copies/ μl were stored at -20°C .

FIGURE 3

C_t values of samples positive for pandemic influenza A(H1N1)2009 in the diagnostic PCR, Bavaria, 27 April–9 November 2009 (n=1,322)



Each dot represents one sample. The performance of the multiplex assay was retrospectively calculated for the first wave of the 2009 influenza pandemic. The resulting detection rates of the multiplex assay for influenza A(H1N1)2009 in the validation study for samples below (100%) and above (57.1%) a C_t value of 34 are shown. The multiplex assay would have detected at least 93.6% of the positive samples.

Multiplex real-time RT-PCR assay

Different published primers and probes of real-time RT-PCR assays specific for influenza A, influenza B and pandemic influenza A(H1N1)2009 viruses were tested to determine whether they could be used together in a multiplex assay. We show here only those primer and probe sets that performed well when combined in preliminary tests.

In order to minimise the risk of PCR product contamination, we introduced one-step RT-PCR protocols using the commercially available QuantiTect Virus +ROX Vial kit (Qiagen) including QuantiTect Virus No Rox (NR) Mastermix and QuantiTect virus RT. For specific detection of influenza A, influenza A(H1N1)2009, influenza B and the internal amplification control (IAC), we used primers and probes of published or officially recommended real-time PCR systems (Table 1): a previously published real-time RT-PCR assay [16] for influenza A viruses targeting the matrix gene, with an optimised reverse primer (InfA M-124-mod) for reliable detection of pandemic influenza A(H1N1)2009 (recommended by the national reference centre for influenza at the RKI); an officially recommended real-time PCR system with primers and a TaqMan probe for the specific detection of influenza A(H1N1)2009 [9] targeting the HA gene, and a real-time RT-PCR assay for the detection of influenza B [17] targeting the matrix gene, with a slightly modified reverse primer that has been routinely applied for years for routine diagnosis in our laboratory. The detection system for the internal amplification control has been described previously for multiplex real-time PCR assays [18].

All primers and probes were synthesised by TIB Molbiol. For the three influenza single target real-time RT-PCR assays a 25 μl PCR reaction was prepared containing: 400 nM of each forward and reverse primer (see Table 1), 100 nM of TaqMan probe, 1x QuantiTect virus reverse transcription mix, 1x QuantiTect virus NR mastermix, 4U RNase inhibitor (Invitrogen) and 5 μl RNA extract.

For optimisation of the multiplex assay all primer concentrations were titrated from 100 to 500 nM and all probe concentrations from 100 to 300 nM. Fluorescence filter sets for 6-carboxyfluorescein (FAM), hexachloro-6-carboxy-fluorescein (HEX/VIC), Texas Red (TEX/ROX) and a cyanine dye (CY5) were used simultaneously. The influenza A- and B-specific probes were labelled with FAM and CY5, respectively. The probe specific for pandemic influenza A(H1N1)2009 was labelled with TEX. The IAC probe was labelled with HEX. All four TaqMan probes were labelled with Black Berry Quencher (BBQ) as quencher dye. For the multiplex real-time PCR assay optimised probe concentrations were applied (see Table 1).

For single and multiplex real time PCR thermal cycling was performed on MX3000P and MX3005P real-time PCR instruments (Agilent Technologies) under the fol-

lowing conditions: 20 min at 50°C; 10 min at 95°C; 45 cycles of 15 s at 95°C and 45 s at 60°C.

Efficiency of the multiplex assay

PCR efficiencies were determined for influenza A, influenza B and pandemic influenza A(H1N1)2009 in each single assay as well as the individual channels of the multiplex assay. PCR efficiency was calculated according to the PCR amplification formula $E = 10^{(1/\text{slope})} - 1 \times 100\%$, E being the PCR efficiency.

Results

Optimisation of the multiplex assay

Primer titration from 100 to 500 nM as well as probe titration from 100 to 300 nM indicated an optimal primer concentration of 400 nM and an optimal probe concentration of 200 nM for all four assays in the multiplex real-time RT-PCR (see Table 1). Higher or lower concentrations did not alter the sensitivity of the multiplex assay significantly (results not shown).

The optimised multiplex real-time RT-PCR assay in a 25 µl PCR reaction volume was composed as follows: 400 nM of all primers and 200 nM of each of the four TaqMan probes, 1x QuantiTect virus RT mix, 1x QuantiTect virus NR mastermix, 4U RNase inhibitor, 0.25 µl IAC RNA (2.5×10^4 copies) and 5 µl RNA extract. Thermal cycling was performed on MX3000P and MX3005P under the same conditions as the individual single assays. The optimised multiplex real-time RT-PCR assay is shown in Figure 1 for 10-fold dilution series of viral RNA from 10 ng to 1 pg RNA.

Specificity of the multiplex assay

The specificity of the diagnostic assays for influenza A and influenza B has previously been tested and confirmed [16,17]. Therefore it was not further tested during multiplex optimisation. We checked the specificity of the PCR for pandemic influenza A(H1N1)2009 virus that was unpublished at the time [9] by sequencing the 80 bp amplicons (HA gene) of positive pandemic influenza A(H1N1)2009 samples. All fifty sequenced PCR products were 100% identical to published sequences of pandemic influenza A(H1N1)2009 proving the high specificity of the assay. The specificity of the multiplex assay was confirmed in two official external quality assurance studies (INSTAND e.V., Germany) comprising 15 samples of six different influenza strains, which were tested in duplicate. No cross-reactivity was observed in any of the 15 samples, and all specific targets showed strong positive signals. Furthermore the oseltamivir-resistant strain influenza A/Berlin/58/2008 (H1N1) was tested and correctly identified by the multiplex real-time RT-PCR assay.

Analytical sensitivity of the multiplex assay

With plaque-quantified influenza A/Hamburg/05/2009 (H1N1) we found a linear dynamic range from 10^5 to 10^2 genome equivalents. The detection limit was below 3.5×10^2 PFU/ml for the matrix gene as well as the HA gene. Testing of each RNA concentration of the influenza

A (Sydney/5/1997 (H3N2) and B (Brisbane/60/2008) reference material in triplicates yielded a sensitivity of 10 pg per PCR reaction for each detection system in the single assays as well as in the multiplex assay and detected RNA extracted from influenza-infected cell cultures (seasonal influenza A(H3N2) and B) and from egg cultures (influenza A(H1N1)2009) with equal sensitivity.

Efficiency of the multiplex assay

The real-time PCR runs of the sensitivity tests for influenza A (Sydney/5/1997 (H3N2), influenza A/Hamburg/05/2009 (H1N1) and influenza B (Brisbane/60/2008) were applied for the determination of the PCR efficiencies in the multiplex real-time PCR compared to the individual single real time PCR assays. The PCR efficiencies of the single real-time PCR assays in comparison to the individual channels of the multiplex PCR assay are shown in Table 2. The PCR efficiency of each individual assay was determined as between 92% to 120% for the individual assays. The PCR efficiencies of the respective single assay were comparable to the PCR efficiency in the multiplex assay. The influenza B assay had a PCR efficiency of 92% in the single assay while in the multiplex assay the PCR efficiency was 120%, which was considered as acceptable for a screening assay.

Evaluation of the multiplex assay with samples of ILI patients

A total of 317 stored RNA samples from the respiratory tract of ILI patients that had previously been tested with diagnostic real-time RT-PCR assays, were retrospectively tested with the multiplex assay. The overall diagnostic sensitivity of the multiplex assay was 87.7%, specificity was 99.6% and positive (PPV) and negative predictive values (NPV) 99.5% and 90.6%, respectively, compared to the respective diagnostic assay. Ninety samples had been negative in all diagnostic assays. Of those 90, 89 were also negative when we tested them in the multiplex assay, but one sample yielded a positive result for pandemic influenza A(H1N1)2009 in the multiplex assay (C_t value 35).

Of 175 influenza-positive samples with C_t values under 34 in the respective diagnostic assay, 174 were confirmed by the multiplex assay, with positive signals for seasonal influenza A (31/31), influenza B (27/28) and pandemic influenza A(H1N1)2009 (116/116) viruses. The influenza B-positive sample that was missed in the multiplex PCR had had a C_t value of 34 in the diagnostic PCR. In samples that had C_t values above 34 in the respective diagnostic assay, the reliability of detection with the multiplex assay was lower: 25 of 52 influenza samples overall, with 6 of 16 seasonal influenza A, 11 of 22 influenza B, and 8 of 14 influenza A(H1N1)2009 (Figure 2 and Table 3). The sensitivity of detection of influenza A(H1N1)2009 was slightly lower with the primers targeting the matrix gene (116/130; 89.2%) than with primers targeting the HA gene (124/130; 95.4%) especially in samples that had been only weakly

positive in the respective diagnostic PCR (C_t values >34). The IAC was positive in all influenza-negative samples, indicating that failure to detect influenza virus was not due to inhibition.

Based on the detection rates of this evaluation we calculated that the multiplex assay would have correctly identified at least 1,238 of the 1,322 (93.6%) influenza A(H1N1)2009-positive samples (Figure 3), which were analysed at the Bavarian Health and Food Safety Authority between 27 April and 9 November 2009 using the diagnostic assays. The C_t values were between 20 and 32 for 1,025 of these samples.

The multiplex assay was introduced as the sole screening test into laboratory influenza surveillance in Bavaria on 10 November 2009. Until 16 April 2010, 310 of 1,228 nasopharyngeal and throat swabs of ILI patients tested positive for influenza A(H1N1)2009 using this assay. The results reflected the epidemic curve of reported cases of influenza A(H1N1)2009 in November 2009 in Bavaria.

The IAC was negative in five throat swabs which all tested negative for influenza viruses. After 10-fold dilution of the sample, the IAC was positive in all five samples. The negative results of three of these samples were confirmed negative when retested in dilution in the multiplex assay, while two were positive for pandemic influenza A(H1N1)2009.

Discussion

We report on a multiplex one-step real-time RT-PCR assay for the simultaneous detection of seasonal influenza A and B as well as influenza A(H1N1)2009 viruses. The assay was optimised for multiplex real-time PCR from published, validated and well established PCR protocols with minor modifications. The multiplex assay proved to be as specific as the respective diagnostic PCR assay. Only one sample tested negative in the diagnostic assays but positive for influenza A(H1N1)2009 in the multiplex assay in two replicates. We ran out of patient material and could not retest the sample with the diagnostic assay. As we detected only a low positive signal, neither a false positive result of the multiplex assay due to contamination, nor a false negative result of the diagnostic assay could be ruled out. A PCR inhibition control was successfully integrated into the assay for accurate interpretation of negative results. Interestingly, two samples positive for pandemic influenza A(H1N1)2009 would have been missed without the IAC. Dilution of the RNA before PCR successfully abolished the inhibitory effect. As we used the *in vitro*-transcribed RNA as an amplification control we could not control for inhibitory effects due to the extraction protocol.

We consider our multiplex assay that has shown its functionality in a high number of patient samples a useful tool for general public health laboratories. In contrast to the evaluation of other published assays

[5,7,19], we have tested our multiplex assay on a very large number of clinical samples, including a high number of positive samples. In our analysis of patient samples, the diagnostic sensitivity of the multiplex PCR was slightly lower than that of the respective diagnostic assays, even if RNA dilution series of reference material showed equal sensitivity when determining the detection limit of the multiplex assay in comparison to the single assays. This might be explained by degradation due to storage of weakly positive patient RNA samples for up to one year, whereas dilution series were performed with freshly isolated RNA from reference material for the single as well as the multiplex assays. The overall sensitivity was 87.7%, but was 99.4% for samples with moderate and high viral loads (C_t value >34). In a situation with population-wide screening in which patients with acute ILI yielding high viral loads are tested, we consider the slightly lower sensitivity acceptable. The assay has been validated for routine diagnosis of influenza and is used for large scale surveillance of influenza activity. While the pandemic subtype was reliably recognised during the 2009 pandemic, specificity and sensitivity of the multiplex assay was also shown for seasonal, avian and an oseltamivir-resistant virus. The assay is used to monitor influenza viruses throughout the whole year. By introducing the multiplex assay we were able to lower costs by saving reagents and working time. Furthermore we reduced sample turnaround time in comparison to the diagnostic PCR assays.

Diagnostic tools for surveillance are applied for the general identification of influenza viruses. Although mutation of the pandemic influenza A(H1N1)2009 virus was rare in the 2009 pandemic [20], we also addressed this possibility by including conserved regions (matrix genes) as PCR target. Our multiplex assay is capable to both identify the circulating pandemic strain (HA gene) and screen for other influenza A and B viruses (matrix genes). These should be further subtyped to confirm other seasonal influenza A subtypes or to detect changes in the circulating strain.

Chen *et al.* [21] also published a multiplex real-time RT-PCR assay for the simultaneous detection and subtyping of influenza viruses including the pandemic influenza AH1N1(2009), that has been evaluated on a high number of patient samples. Compared with our one-step real-time RT-PCR assay, this assay is based on a two-step real-time RT-PCR.

The 2009 pandemic is a reminder for public health laboratories to monitor influenza activity not only during the season of influenza circulation, but during the whole year. Our assay proved to be a convenient, rapid, reliable and cost effective way to meet this requirement.

Acknowledgements

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