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Short communication

Rapid detection of foot-and-mouth disease virus, influenza A virus and classical swine fever virus by high-speed real-time RT-PCR☆

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A B S T R A C T

High sensitivity, minor risk of cross-contamination and in particular the rapid reaction time make quantitative real-time polymerase chain reaction (qPCR) assays well suited for outbreak investigations as well as for monitoring epidemics of pathogens.

In this study qPCR assays for three highly contagious animal diseases, namely foot-and-mouth-disease (FMD), influenza A (IA) and classical swine fever (CSF) have been developed. Furthermore, an amplification control targeting 18S ribosomal RNA was included. Each assay was validated with samples from infected animals using three different standard qPCR-machines in two thermal profiles: one standard and one high-speed approach, respectively. The high-speed PCR assays allowed the reliable diagnosis of FMD, influenza A and CSF in less than 28 min with an analytical sensitivity of at least 200 genome copies/μl in every case, with slight differences regarding reaction time and sensitivity for the individual PCR-cycler instruments. Therefore, the newly established rapid RT-PCR systems will be a valuable method for the monitoring and control of these three important viruses and will be a robust option for the development of novel molecular pen-side tests.

Foot-and-mouth disease (FMD), influenza A as well as classical swine fever (CSF) are highly contagious diseases causing high economic losses in animal production worldwide. To prevent the spread of these diseases into huge geographic areas, rapid laboratory diagnosis is crucial (Belak, 2007). Due to the combination of a high sensitivity, the reduced risk of cross-contamination and the possibility of quantitative analysis, real-time PCR (qPCR) is a valuable tool for the detection of viruses (Mackay, 2004; Mackay et al., 2002). However, used commonly PCR-protocols require about 90–120 min. To decrease the time taken for diagnosis high-speed qPCR assays for detection of e.g. influenza or adenoviruses have been developed recently (Fujimoto et al., 2010; Sakurai et al., 2011). The described PCR systems provided a result in less than 30 min, but a specialized qPCR-machine was used. In this study, high-speed PCR assays for the detection of foot-and-mouth disease virus (FMDV), influenza A viruses (IAV) and classical swine fever virus (CSFV) have been developed and validated using three different common standard qPCR-machines.

A variety of different sample materials obtained from animals infected experimentally with FMDV, IAV and CSFV respectively, was used in this study. All samples were provided by the corresponding German National Reference Laboratories. RNA was extracted with the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and eluted in 50 μl kit elution buffer.

Primer and probes specific for CSFV and IAV have been described previously (Hoffmann et al., 2005, 2010). To select FMDV-specific primers and probes published sequence information (NCBI database) was used. In addition to the pathogen-specific assays each sample was tested by an internal amplification control (IC) specific PCR system which targets 18S ribosomal RNA. Sequences of all primers and probes are shown in Table 1.

The RT-qPCRs were carried out using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq in a total reaction of 12.5 μl, the amount of master mixture components for the FMDV-, IAV-, CSFV- and IC-specific PCR systems is given in Table 2. Concentrations of primers and probes necessary in a single reaction are listed in Table 1. The merging of the single master mixture components was executed at room temperature. Finally, 2.5 μl RNA template was added and qPCR was carried out using a Bio-Rad CFX 96 Real-Time Detection System (software version Bio-Rad CFX Manager 2.0; Bio-Rad, Hercules, CA, USA), an Eco™ Real-Time PCR system (Eco™ Software v3.0.16.0; Illumina, Inc., San Diego, CA, USA) as well as a LightCycler® 480 Real-Time PCR System (LCS480

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Table 1
Sequences of primers and probes used in this study.

Name	Sequence 5′–3′	Concentration (pmol/reaction)	Amplicon (base pair)	Reference
FMD-IRES-3F	ACC TGG WGR CAG GCT AAG GA	10	78	This study
FMD-IRES-3.2R	CCY RGT CCC CTT CTC AGA T	10		
FMD-IRES-3FAM	FAM-CCC TTC AGG TAC CCC GAG GTA ACA-BHQ1	3.125		
CSF100-F	ATG CCC AYA GTA GGA CTA GCA	10	93	Hoffmann et al. (2005)
CSF192-R	CTA CTG ACG ACT GTC CTG TAC	10		
CSF-Probe 1	FAM-TGG CGA GCT CCC TGG GTG GTC TAA GT-BHQ1	1.25		
IAV-M1-F	AGA TGA GTC TTC TAA CCG AGG TCG	10	99	Hoffmann et al. (2010)
IAV-M1.1-R	TGC AAA AAC ATC TTC AAG TYT CTG	7.5		
IAV-M1.2-R	TGC AAA GAC ACT TTC CAG TCT CTG	7.5		
IAV-M1-FAM	FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1	1.25		
18sRNA-1F	GCG GGTAAC CCG TTG AAC C	4	156	This study
18sRNA-1R	CCA TCC AAT CGG TAG TAG CG	4		
18sRNA-1FAM	FAM-ATT CCC CAT GAA CGA GGA ATT CCC AGT A-BHQ1	5		

1.5.0.39; Roche Diagnostics Deutschland, Mannheim, Germany). The following thermal profile was used: PCR initial activation step at 95 °C for 1 min; 45 cycles of a two-step cycling consisting of denaturation at 98 °C for 1 s and annealing and extension at 54 °C for 1 s. A separate reverse transcription step was not performed. The short time between PCR mix preparation and Taq polymerase activation by the initial denaturation step was sufficient for an effective cDNA generation. In order to compare the results with a standard method, an identical reverse transcription qPCR (RT-qPCR) set-up was carried out in the three qPCR-machines mentioned above using the following thermal profile: reverse transcription step at 50 °C for 15 min, PCR initial activation step at 95 °C for 2 min; 45 cycles of a three step cycling consisting of denaturation at 95 °C for 15 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s. All samples were tested in duplicates with an appropriate external standard.

The two different thermal profiles resulted in a total reaction time of 1 h and 42 min (standard protocol), and 40 min, 23 s (high-speed PCR) when using the Bio-Rad CFX 96 Real-Time Detection System. With the Eco™ Real-Time PCR system the time required to get a final diagnosis was 1 h, 29 min, and 27 min and 55 s, respectively; and with the LightCycler® 480 Real-Time PCR System 1 h and 37 min or 33 min and 44 s were necessary (Fig. 1).

The analytical sensitivity of the FMDV-, IAV- and CSFV-specific PCR systems was determined using series of 10-fold dilutions of an appropriate standard (Fig. 2). The FMD assay amplified the RNA in a linear fashion from 2.0E+06 copies down to 2 copies/μl using both thermal profiles in every cycler (Figs. 1 and 2). Different sample types obtained from cattle, goats, sheep or swine infected experimentally with FMDV strains of serotypes A, C, O and Asia were tested both with the standard and the high-speed profile. The FMD- as well as the IC-specific PCR system gave a positive result in each case (Table 3).

When using the standard protocol the IAV assay amplified RNA down to 2 genome copies/μl, and identical result was obtained with the high-speed PCR in the Bio-Rad CFX 96 Real-Time Detection System. Using the Eco™ Real-Time PCR system or the LightCycler® 480 Real-Time PCR System in combination with the high-speed approach, 20 genome copies/μl could be detected (Figs. 1 and 2). With exception of one throat swab (subtype H1N1) tested in the LightCycler® 480 every diagnostic sample showed a positive result for IAV and IC in the rapid thermal profile (see Table 3).

Two CSFV genome copies were detected using the standard protocol in each used PCR-machine; 20 genome copies were the detection limit with the high-speed approach in the cyclers from Bio-Rad and Illumina, and 200 genome copies were the limit in the instrument from Roche (Figs. 1 and 2). Serum samples, tonsils and lymph nodes of swine and wild boar infected with different genotypes were tested likewise. Every sample was found positive when using the Bio-Rad CFX 96 Real-Time Detection System and the Eco™ Real-Time PCR system. However, the high-speed protocol in combination with the LightCycler® 480 failed to detect a 2.3 strain in tonsils of a wild boar, in the standard protocol this sample scored positive with a quantification cycle value of 30.2 (1.2E+03 genome copies/μl). In addition, 5 out of 14 samples were positive in only one of the tested duplicates. In every case the IC-specific assay gave a positive result (Table 3).

In summary, all virus positive diagnostic samples representing a broad range of viral genome loads were detected by the high-speed PCR both on the CFX96 system from Bio-Rad and the Eco™ Real-Time PCR system from Illumina (100% diagnostic sensitivity). With

Table 2
Composition of master mixtures for each of the targets.

Reagent	Volume (μl)			
	FMDV	CSFV	IAV	IC
2× reaction mix	6.25	6.25	6.25	6.25
5 mM magnesium sulfate	1	–	1	1
RT/Platinum® Taq Mix	0.25	0.25	0.25	0.25
RNase-free water	1.5	2.5	1.5	–
Primer-probe-mix	1	1	1	1
Negative RNA swine	–	–	–	1.5
Template RNA	2.5	2.5	2.5	2.5
Total reaction volume	12.5	12.5	12.5	12.5

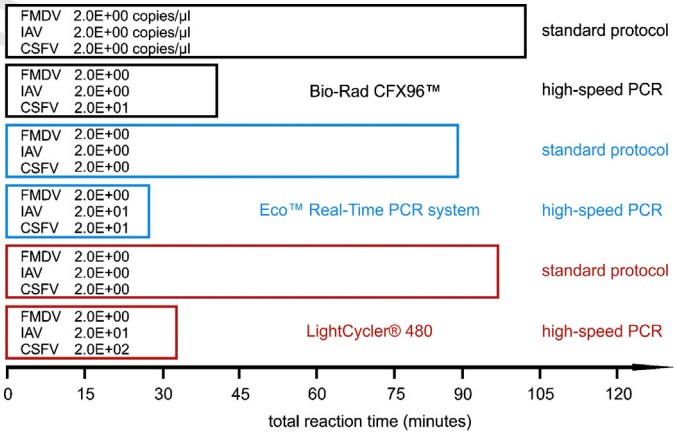

Fig. 1. Total reaction time in the standard and the high-speed thermal profile using a Bio-Rad CFX 96 Real-Time Detection System (black bars), an Eco™ Real-Time PCR system (blue bars) as well as a LightCycler® 480 Real-Time PCR System (red bars). The analytical sensitivity of each approach (in copies/μl) is indicated in the respective bar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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