Nucleic acid-based detection of influenza A virus subtypes H7 and N9 with a special emphasis on the avian H7N9 virus

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In 2013, a novel influenza A virus of subtype H7N9 was transmitted from avian sources to humans in China, causing severe illness and substantial mortality. Rapid and sensitive diagnostic approaches are the basis of epidemiological studies and of utmost importance for the detection of infected humans and animals. We developed various quantitative reverse transcriptase PCR (RT-qPCR) assays for (i) the generic detection of the haemagglutinin (HA) gene of H7 viruses or the neuraminidase (NA) gene of N9 viruses, and (ii) the specific detection of HA and NA of the novel avian H7N9/2013 virus. The sensitivity of the newly developed assays was compared with previously published PCRs, and the specificity of all RT-qPCRs was examined using a panel of 42 different H7 and 16 different N9 isolates. Furthermore, we analysed the performance of the RT-qPCR assays with dilution series and diagnostic samples obtained from animal experiments. Our study provides a comprehensive set of RT-qPCR assays for the reliable detection of the novel avian H7N9 virus, with high sensitivity and improved and tailored specificity values compared with published assays. Finally, we also present data about the robustness of a duplex assay for the simultaneous detection of HA and NA of the avian influenza H7N9/2013 virus.

Introduction

Multiple reassortment events, trans-species transmissions, and viral adaptation of influenza A viruses (IAV) in non-human host species shaped the latest human pandemic influenza virus that emerged in 2009 [1]. Most recently, another animal influenza virus, this time of purely avian origin, was introduced into the human population in the east of China: influenza A subtype H7N9 [2], hereafter referred to as avian H7N9 virus. At least 354 people were infected, most probably after contact with infected poultry, other avian species, or contaminated environment [3,4]. A total of 113 deaths ensued (last revised on 18 February 2014, WHO) [5].

The avian reservoir of this virus has remained obscure. In contrast to highly pathogenic influenza A(H5N1) virus, avian H7N9 virus is of low pathogenicity in tested avian host species. Experimentally infected birds did not develop any overt clinical signs, and a natural infection with avian H7N9 virus did not induce disease [2,6]. This severely impedes syndromic surveillance as an early warning measure for the spread of this virus in poultry and wild birds. The risk of the disease spreading to Europe is considered low [7].

Experiments using the ferret model, demonstrated that avian H7N9 virus could easily be transmitted via close contact, while air-borne transmission between the ferrets was limited [8]. Avian H7N9 virus is transmitted between birds [9], and probably continues to circulate in poultry and/or wild birds in China. As a consequence, surveillance systems based on rapid, highly specific, and sensitive molecular-diagnostic approaches are mandatory for the verification of clinical cases in humans, but also for monitoring and surveillance of poultry and wild bird populations. For this purpose, we developed a set of real-time quantitative reverse transcriptase polymerase chain reactions (RT-qPCR), which target different fragments of the haemagglutinin (HA) and the neuraminidase (NA) genes of influenza A viruses, with special emphasis on the novel avian H7N9/2013 virus and with explicit advantages.

Methods

Viruses and RNA samples

The avian influenza A(H7N9) virus (A/Anhui/1/2013) used in this study was kindly provided by the World Health Organization (WHO) Collaborating Centre London, United Kingdom. For virus propagation, embryonated chicken eggs or Madin Darby canine kidney (MDCK) cell cultures were inoculated at different multiplicities of infection (MOIs) and incubated at 37 °C for five or three days, respectively. In addition to this H7N9 virus, RNA samples from 458 influenza virus strains representing 16 HA and nine NA subtypes from the German National Reference Laboratory for Avian Influenza at the Friedrich-Loeffler-Institute,

Insel Riems, were used for the analytical validation of the newly developed assays. This panel includes viruses of 42 different H7 and 16 different N9 subtypes. All viruses were of avian origin, except the H7 strain A/equi/Prague/1/56, which was of equine origin. Furthermore, a dilution series of avian H7N9 virus as well as swabs and tissue samples originating from animal experiments with chickens, pigeons and ferrets inoculated with influenza A/Anhui/1/2013 virus were used for the validation of the different assays. The animal trials gained governmental approval under the registration number LVL MV/TSD/7221.3-1.1-021/13.

RNA isolation

Viral RNA was extracted from supernatants of infected cell cultures or allantoic fluids of embryonated chicken eggs using the QIAamp viral RNA kit (Qiagen, Hilden, Germany). Swab samples as well as organ samples were extracted using the MagAttract Virus Mini M48 kit (Qiagen) on a Biosprint 96 platform (Qiagen).

Primers and probes

Primers and probe of the pan-influenza A IAV-M1.2 assay [10] were used to determine the quantification cycle of all samples tested. For comparative analyses, the recently published assays from Corman et al. [11] and Wong et al. [12] were tested in parallel, accompanied by a further assay (FLI-H7-CODA), which uses the so-called CODA primers from the Belgian National Reference Laboratory for Avian Influenza at the Veterinary and Agrochemical Research Centre (CODA-CERVA) in combination with the FLI probe (published by the OIE/FAO Network of expertise on animal influenza (OFFLU) on the website www.offlu.net) [13]. In addition, for the in silico identification of primers and probes specific for the avian H7N9 virus, consensus sequences for the HA gene as well as the NA gene were generated from sequences published within the Global Initiative on Sharing All Influenza Data (GISAID) database (www.gisaid.org). Assays for each segment were designed for broad detection of all published H7 (FLI-H7generic-2) and N9 (FLI-N9generic-11) sequences. In addition, assays for H7 (FLI-H7anhui-8) and for N9 (FLI-N9anhui-1) of avian H7N9 A/Anhui/1/2013 and related strains were designed. For the selection of the primers and probes of the generic H7 and N9 assays, we used 910 nearly complete H7 gene sequences as well as 181 nearly complete N9 gene sequences available at GenBank (www.ncbi.nlm.nih.gov/genbank). All oligonucleotides were synthesised by Metabion GmbH (Martinsried, Germany) and stored at -20 °C until use. Sequences of primer and probe sets used in this study are summarised in Table 1. A schematic of the relative location of the target regions of various sets of primer and probe along the HA and NA genes is given in the Figure. In silico analysis of primer and probe binding properties was carried out with the software Primer 3 [14].

Internal extraction control

For IAV screening investigations, the IAV-M1.2 assay was combined with an internal control system in a duplex assay [15]. Therefore, an in vitro transcript of the enhanced green fluorescent protein (EGFP) gene was used in a duplex PCR set-up and the specific fragment was detected using a HEX-labelled probe to exclude false negative results.

Real-time RT-PCR (RT-qPCR)

A one-step RT-qPCR protocol was chosen in order to minimise the risk of cross-contamination. The composition of a 12.5 µL total reaction using the RNA UltraSense One-Step qRT-PCR kit was as follows: 5.875 µL RNase-free water, 2.5 µL 5x RT-PCR buffer, 0.625 µL RT-PCR enzyme mix, and 1 µL primer–probe mix, and 2.5 µL RNA template. All RT-qPCR runs were performed on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) using the following temperature profile: 15 min at 50 °C, 2 min at 95 °C, 45 cycles of 15 sec at 95 °C, 15 sec at 60 °C and 30 sec at 72 °C. Fluorescence values (FAM, HEX) were collected during the annealing step. All analyses were done in triplicate (development and comparison of the RT-qPCR) or duplicate (animal trial), and mean values are presented. Reactions with a quantification cycle (Cq) value of less than 42 scored negative.

Generation of in vitro-transcribed standard RNA

The HA and the NA sequences of the H7N9 A/Anhui/1/2013 virus (accession numbers: EPI439507, EPI439509) were ordered as synthetic genes flanked by EcoRI and XhoI cloning sites in a pUC-derived plasmid backbone (GeneArt, Regensburg, Germany). Synthesis of RNA run-off transcripts from a T7 promoter site upstream of the 5' EcoRI cloning site was performed as recommended by the manufacturer (T7 RiboMAX, Promega, Germany). RNA copies were calculated according to the formula:

(X g/µL RNA / [transcript length in nucleotides x 340]) x 6.022 x 10^{23} = Y molecules/µL.

PCR reactions for quantification of the in vitro-transcribed standard RNA were performed using the RNA UltraSense One-Step qRT-PCR kit with its specific parameters.

Results

In order to develop primer and probes for the generic detection of the HA of H7 viruses (assay FLI-H7generic-2) or the NA of N9 viruses (assay FLI-N9generic-11), 910 nearly complete H7 sequences as well as 181 nearly complete N9 sequences were analysed in silico. Furthermore, primer and probes for the specific detection of the HA (assay FLI-H7anhui-8) or NA (assay FLI-N9anhui-1) of the novel avian H7N9/2013 virus were designed based on consensus sequences published on www.gisaid.org (Table 1). In the present study, we analysed the performance of the newly developed

Table 1

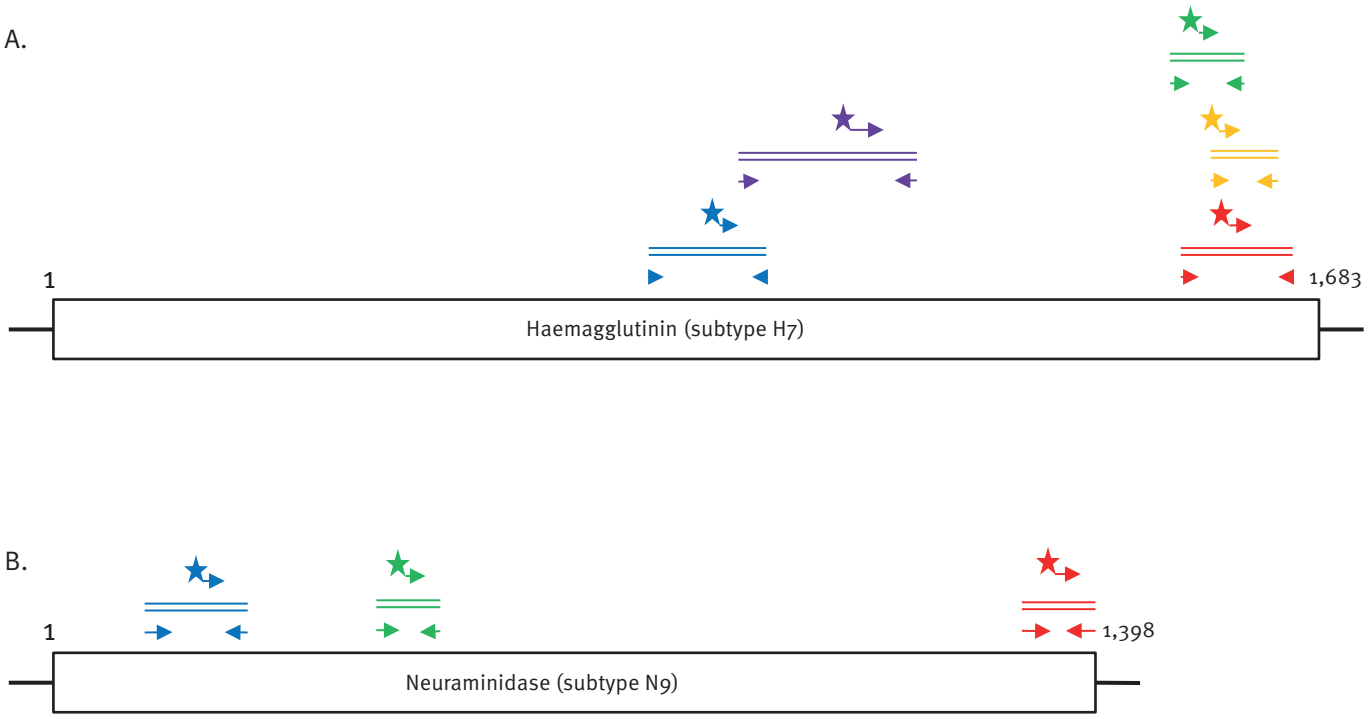
Primers and probes used in this study for nucleic acid-based detection of influenza A virus subtypes H7 and N9

Designation	Sequence 5'→3'	Genome position[a]	Concentration of primer and probes in the primer–probe mix
Pan-IAV assay			
IAV-M1.2 [10]			
IAV-M1-F	AGA TGA GTC TTC TAA CCG AGG TCG	1–24	20 µM
IAV-M1.1-R	TGC AAA AAC ATC TTC AAG TYT CTG	99–76	15 µM
IAV-M1.2-R	TGC AAA GAC ACT TTC CAG TCT CTG	99–76	15 µM
IAV-M1-FAM	FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1	49–68	2.5 µM
H7 assays			
FLI-H7generic-2 [This study]			
IAV-HA7-1593-F	AYA GAA TAC AGA TWG ACC CAG T	1,523–1,544	20 µM
IAV-HA7-1740-R	TAG TGC ACY GCA TGT TTC CA	1,653–1,672	20 µM
AIV-HA7-1649-FAM	FAM-TGG TTT AGC TTC GGG GCA TCA TG –BHQ1	1,579–1,601	2.5 µM
FLI-H7anhui-8 [This study]			
IVA-H7anhui-830F	TGA GAG GAA AAT CTA TGG GAA TC	806–828	15 µM
IVA-H7anhui-981R	CTT AAC ATA TCT CGG ACA TTT TCC A	933–957	15 µM
IVA-H7anhui-951FAM_as	FAM-CCT GCT ATC TAT GTT CTG AAA TGG CAA GT-BHQ1	899–927	5 µM
FLI-H7-CODA [13]			
IAV-HA7-CODA-F	GYA GYG GYT ACA AAG ATG TG	1,553–1,572	20 µM
IAV-HA7-CODA-R	GAA GAC AAG GCC CAT TGC AA	1,619–1,638	20 µM
IAV-HA7-CODA-FAM	FAM-TGG TTT AGC TTC GGG GCA TCA TG-BHQ1	1,579–1,595	2.5 µM
Wong-H7 [12]			
H7-anhui-916F	ATA GAT AGC AGG GCA GTT GG	916–935	5 µM
H7-anhui-1156R	GAT CAA TTG CCG ATT GAG TG	1,137–1,156	5 µM
H7-anhui-1096FAM	FAM-CCY TCY CCY TGT GCR TTY TG-BHQ1	1,096–11,15	5 µM
Corman-H7 [11]			
HA7_1_2013rtF	TAC AGG GAA GAG GCA ATG CA	1,501–1,520	10 µM
HA7_1_2013rtR	AAC ATG ATG CCC CGA AGC TA	1,584–1,603	10 µM
HA7_1-2013rtFAM	FAM-ACCCAGTCAAACTAAGCAGCGGCTA-TAMRA	1,538–1,562	5 µM
N9 assays			
FLI-N9generic-11 [This study]			
IVA-N9-1363F	AGY ATA GTA TCR ATG TGT TCC AG	1,315–1,337	20 µM
IVA-N9-1439R	AAG TAC TCT ATT TTA GCC CCA TC	1,369–1,391	20 µM
IVA-N9-1393FAM	FAM-TTC CTB GGA CAA TGG AAC TGG CC-BHQ1	1,345–1,367	5 µM
FLI-N9anhui-1 [This study]			
IVA-N9anhui-173F	AAC CTG AAA CAA CCA ACA CAA G	140–161	15 µM
IVA-N9anhui-299R	GTT AAG TTA TTG AAA TTC CTG CTT G	227–251	15 µM
IVA-N9anhui-227HEX	HEX-CAA ACA TCA CCA ACA TCC AAA TGG AAG AG-BHQ1	194–222	5 µM
Corman-N9 [11]			
NA9_2013rtF	CCAGTATCGCGCCCTGATA	447–465	10 µM
NA9_2013rtR	GCATTCCACCCTGCTGTTGT	497–516	10 µM
NA9_2013rtFAM	FAM-CTGGCCACTATCATCACCGCCCA-TAMRA	468–490	5 µM

[a] Genome position according to influenza A/Anhui/1/13 (H7N9); GISAID accession numbers: HA: EPI439507, NA: EPI439509, M: EPI439506.

Figure

Relative location of primer and probe sets for the detection of the haemagglutinin (A) or neuraminidase gene (B) of influenza A virus subtypes H7 and N9



A. red: FLI-H7generic-2; blue: FLI-H7anhui-8; orange: FLI-H7-CODA; violet: Wong-H7; green: Corman-H7.
B. red: FLI-N9generic-11; blue: FLI-N9anhui-1; green: Corman-N9.

RT-qPCRs as well as recently published assays with regards to the specific detection of the HA or NA of influenza A/Anhui/1/13 (H7N9), the broad generic detection of H7 or N9 viruses, and the diagnostic sensitivity for the detection of avian influenza A(H7N9) virus in samples from experimentally infected animals. All samples were tested by the pan-influenza IAV-M1.2-assay to verify the viral genome load by generic amplification of conserved parts of the M segment of all IAV [10]. Amplification plots and the calculation of the efficiency, linearity, and precision of the newly developed and recently published assays are available from authors upon request.

RT-qPCR systems for the haemagglutinin genes of influenza A(H7) viruses and the avian influenza A(H7N9) virus

We compared the sensitivity of the generic H7 assay FLI-H7generic-2 with the FLI-H7-CODA assay which used the primers previously developed by CODA-CERVA [16] and has been published by the World Organisation for Animal Health/Food and Agriculture Organization (OIE/FAO) Network of expertise on animal influenza (OFFLU) in combination with a FLI probe [13]. In addition, the newly developed avian H7N9-specific H7 assay FLI-H7anhui-8 was compared with the published assays Wong-H7 and Corman-H7 [11,12]. To this end, we tested a dilution series of viral RNA from influenza A/Anhui/1/13 (H7N9) as well as a dilution series of an RNA run-off transcript of the H7 gene of influenza A/Anhui/1/13 (H7N9) (see Tables 2 and 3).

All assays detected the HA gene of the avian influenza A (H7N9) virus with similar high analytical sensitivity (only the Wong-H7 assay reacted with slightly higher Cq-values; Tables 2 and 3). Based on the Cq-values of the dilution series, the two generic assays had a 10-fold lower detection limit compared with the three H7N9-specific assay, which performed nearly identically (Table 2). The generic assays, FLI-H7generic-2 and FLI-H7-CODA produced similar Cq-values, whereas the H7N9-specific assays FLI-H7anhui-8 and Corman-H7 produced slightly lower Cq-values. The Wong-H7 assay exhibited the highest Cq-values. However, based on the optimal setting of an in vitro transcript, a detection limit of less than 10 genome copies per PCR reaction could be observed for all three influenza A/Anhui/1/13 HA assays (Table 3).

In order to verify both the inclusiveness and the exclusiveness of the newly developed assays in comparison with previously published H7 assays, 42 available H7 IAV isolates were tested (Table 4). The FLI-H7generic-2 assay detected all 42 different H7-isolates, whereas

Table 2

Comparative analytical sensitivity for the detection of haemagglutinin sequences of avian influenza A(H7N9) based on RNA dilution series

Dilution series of A/Anhui/1/2013[a]	IAV-M1.2[b]	Generic H7 RT-qPCRs[b]		Avian H7N9-specific H7 RT-qPCRs[b]		
		FLI-H7generic-2	FLI-H7-CODA	FLI-H7anhui-8	Corman-H7	Wong-H7
H7N9_10-3	19.4	20.3	19.5	21.8	21.5	23.7
H7N9_10-4	23.3	23.7	22.9	25.2	25.0	27.6
H7N9_10-5	26.2	27.0	26.5	28.6	28.5	30.9
H7N9_10-6	29.5	30.5	29.7	31.9	31.3	34.1
H7N9_10-7	33.5	33.7	33.2	34.9	34.6	37.7
H7N9_10-8	No Cq	No Cq	No Cq	37.8	37.9	39.0
H7N9_10-9	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq

No Cq: no value obtained >42.

[a] RNA extracted from allantoic fluid of embryonated chicken eggs infected with the isolate A/Anhui/1/13 (H7N9) was used.

[b] Numerals in columns represent Cq values.

the FLI-H7-CODA protocol detected 37 samples. We also tested 416 non-H7 isolates with the FLI-H7generic-2 assay. Here, six of 31 subtype H10 isolates showed weak cross-reactivity (Cq-values of 20–25 for the IAV-M1.2 assay and Cq >35 with the FLI-H7generic-2 assay) and therefore a specificity of 98.6% (data not shown). Based on the alignments, the primer set designated for the FLI-H7-CODA assay is not optimised for the broad detection of H7-viruses, but performed very well for the detection of A/Anhui/1/13 H7N9. Both the Corman-H7 assay and the Wong-H7 assay were initially designed for the sensitive detection of avian H7N9 virus [11,12], but showed cross-reactivity with several other H7-strains. Therefore, both assays were not specific for the recent avian H7N9 virus. In contrast, despite high viral loads (low Cq values in the IAV-M1.2 assay), none of the analysed H7 isolates showed any reactivity with the FLI-H7anhui-8 assay, which is highly specific for the HA of the recently emerged Chinese lineage of the avian H7N9 virus.

Viruses of the American lineages of subtypes H7 and N9 were underrepresented in our set of RNAs available for PCR validation. RNA from only one American strain was available (A/chicken/Jalisco/12283/12 (H7N3)). This Mexican strain was detected by the FLI-H7generic-2 assay, while all other H7 assays failed to detect it. In order to obtain an impression of the amplification potential for a larger set of American strains, an in silico analysis of primer and probe binding properties was carried out. This was based on an alignment of 548 H7 and 228 N9 sequences extracted from GenBank. The alignment of AIV strains from North and South America was then analysed with Primer3 [14]. Successful amplification was assumed, when not more than a single mismatch was detected in the respective sequence (one mismatch per primer/probe) (Table 5). When at least one primer or the probe harboured two or more mismatches, amplification was assumed to be unlikely. On the basis of this crude assessment, the newly developed FLI-N9generic-11 and FLI-H7generic-2 PCRs were expected to be able to amplify the majority of American H7 and N9 strains, since 164 of 199 N9 sequences and 448 of 547 H7 sequences revealed a perfect match with both primers and probe (Table 5). Nevertheless, a thorough wet-lab characterisation and validation is an indispensable prerequisite for use of these RTqPCRs on the American continent. Primers and probes of other PCRs listed in Table 5 analysed showed different grades of mismatches, and the generation of specific amplicons with cDNA of American H7 and N9 strains is expected to be less likely.

Table 3

Comparative analytical sensitivity for the detection of haemagglutinin sequences of avian influenza A(H7N9) based on an in vitro transcript of the H7 segment

T7 in vitro transcript of the HA of A/Anhui/1/2013[a]	Generic H7 RT-qPCRs		FLI-H7anhui-8
	FLI H7generic-2	FLI-H7-CODA	
200,000	18.7[b]	19.9	19.9
20,000	22.2	23.6	23.4
2,000	25.6	26.9	26.8
200	29.0	30.3	30.2
20	31.9	33.7	33.7
2	36.6	37.1	36.9
0.2	No Cq	No Cq	No Cq

HA: haemagglutinin; No Cq: no value obtained >42.

[a] RNA copies per µL of template.

[b] Numerals in columns represent Cq values.

Table 4

Comparative analytical specificity for the detection of haemagglutinin sequences of H7 influenza A viruses (n=42)

Strain (influenza subtype, biotype)	IAV-M1.2[a]	Generic H7 RT-qPCRs[a]		Avian H7N9-specific H7 RT-qPCRs[a]		
		FLIH7generic-2	FLI-H7-CODA	FLI-H7anhui-8	Corman-H7	Wong-H7
A/equi/Prague/1/56 (H7N7, LP)	22.4	24.6	34.7	No Cq	No Cq	No Cq
A/Turkey/Ontario/18-1/2000 (H7N1, LP)	21.7	24.6	No Cq	No Cq	No Cq	No Cq
A/Teal/Föhr/Wv180/05 (H7N2, LP)	20.3	20.6	19.6	No Cq	21.5	No Cq
A/mallard/Alberta/8734/2007 (H7N3, LP)	23.1	24.6	No Cq	No Cq	No Cq	No Cq
A/swan/Germany/736/06 (H7N4, LP)	20.5	20.7	19.8	No Cq	21.5	No Cq
A/chicken/Italy/473/99 (H7N1, LP)	18.6	18.8	18.0	No Cq	19.9	39.4
A/duck/Alberta/48/76 (H7N3, LP)	22.6	23.4	No Cq	No Cq	No Cq	No Cq
A/swan/Potsdam/62/81 (H7N3, LP)	22.7	25.5	23.1	No Cq	No Cq	No Cq
A/swan/Potsdam/64/81 (H7N3, LP)	25.6	27.9	25.6	No Cq	No Cq	No Cq
A/duck/Potsdam/13/80 (H7N7, LP)	19.8	21.8	20.8	No Cq	No Cq	36.0
R87/99 (H7N7, LP)	17.5	23.6	17.2	No Cq	No Cq	No Cq
A/duck/Potsdam/15/80 (H7N7, LP)	18.9	22.5	19.8	No Cq	No Cq	36.5
A/Avian/R224/10 (H7N7, LP)	18.2	20.9	19.8	No Cq	No Cq	36.1
A/Mallard/NVP/41/04 (H7N1, LP)	21.1	21.3	20.3	No Cq	22.9	40.8
A/turkey/Italy/472/99 (H7N1, LP)	21.2	20.7	19.6	No Cq	21.9	No Cq
A/Alexandria tyrode/T145 (H7N1, LP)	18.4	23.6	17.4	No Cq	No Cq	41.8
A/turkey/Ireland/PV8/98 (H7N7, LP)	21.8	21.4	20.3	No Cq	29.7	No Cq
A/ch/Dgania/Israel/1980_R709/09 (H7N2, LP)	24.4	27.2	24.9	No Cq	29.3	35.8
A/turkey/Germany/R655-5/09 (H7N7, LP)	20.8	20.5	19.7	No Cq	21.7	No Cq
A/Mallard/Germany/R192/09 (H7N7, LP)	21.7	28.7	20.9	No Cq	22.8	No Cq
A/mallard/Sko212-219K/07 (H7N3, LP)	23.7	23.7	22.9	No Cq	25.1	No Cq
A/guinea fowl/Germany/R2495/07 (H7N3, LP)	25.1	25.4	24.5	No Cq	26.6	No Cq
A/ch/Ger/79 “Taucha” (H7N7, HP)	16.4	20.0	17.8	No Cq	No Cq	37.7
A/FPV/Rostock/45/36 (H7N1, HP)	18.8	30.7	18.4	No Cq	No Cq	29.7
A/FPV/Rostock/45/34 (H7N1, HP)	19.5	30.5	18.5	No Cq	No Cq	30.5
A/FPV/dutch/27 (H7N1, HP)	19.1	29.8	17.9	No Cq	No Cq	36.8
A/chicken/Brescia/19/02 (H7N1, HP)	19.2	30.3	18.1	No Cq	No Cq	37.4
A/hen/Italy/444/99 (H7N1, HP)	19.2	19.5	18.5	No Cq	20.7	No Cq
A/chicken/Britsh Columbia/CN-07/2004 (H7N3, HP)	21.2	20.7	No Cq	No Cq	No Cq	No Cq
A/broiler/Itlay/445/99 (H7N1, HP)	18.7	18.8	17.7	No Cq	19.7	39.5
A/chicken/Germany/R28/03 (H7N7, HP)	18.7	18.7	17.5	No Cq	21.0	39.9
A/Mallard/Germany/R756/06 (H7N4, LP)	22.5	22.3	20.9	No Cq	23.0	No Cq
A/Mallard/Germany/R721/06 (H7N7, LP)	28.5	23.6	21.8	No Cq	24.4	No Cq
A/Greylag goose/Germany/R752/06 (H7N7, LP)	26.5	21.2	20.0	No Cq	23.5	No Cq
A/Mute swan/Germany/R901/06 (H7N1, LP)	20.5	20.4	18.9	No Cq	21.2	No Cq
A/Mallard/Föhr/Wv190/05 (H7N7, LP)	24.3	23.4	22.5	No Cq	24.5	No Cq
A/Teal/Föhr/Wv177/05 (H7N7, LP)	22.4	27.6	26.8	No Cq	28.6	No Cq
A/sentinel-duck/Germany/SK207R/07 (H7N3, LP)	25.4	26.1	24.7	No Cq	27.5	No Cq
A/Mute swan/Germany/R57/06 (H7N7, LP)	24.4	24.7	23.4	No Cq	29.9	No Cq
A/duck/Italy/636/03 (H7N3, LP)	20.0	20.7	19.6	No Cq	21.6	No Cq
A/turkey/Italy/2043/03 (H7N3, LP)	21.3	22.6	20.7	No Cq	23.3	No Cq
A/chicken/Jalisco/12283/12 (H7N3, HP)	21.8	22.8	No Cq	No Cq	No Cq	No Cq

HP: highly pathogenic; LP: low pathogenic; No Cq: no value obtained >42.

[a] Numerals in columns represent Cq values.

Table 5

In silico analysis of primer and probe binding properties with avian influenza subtype H7 (n=548) and N9 (n=228) sequences of North and South American origin

Assay	Number of sequences tested	Primer/probe match 100%	Primer/probe single mismatch[a]	Primer/probe double mismatch[a]	Primer/probe more than two mismatches[b]	Expected detection
FLI-N9generic-11	199[c]	164	34	1	0	Yes
Corman-N9	228	0	0	154	74	Doubtful/poor
FLI-N9anhui-1	228	0	0	0	228	Unlikely
FLI-H7generic-2	547[c]	448	99	0	0	Yes
FLI-H7anhui-8	548	0	0	0	548	Unlikely
FLI-H7-CODA	548	0	0	0	548	Unlikely
Wong-H7	548	0	0	0	548	Unlikely
Corman-H7	548	0	0	0	548	Unlikely

[a] None of the mismatches were located within the last three nucleotides at the 3' end of the primers except for Corman-N9 (NA9_2013rtF) were one mismatch in the last two nucleotides were identified.

[b] At least one primer or the probe harboured more than two mismatches, rendering stable binding unlikely.

[c] 29 sequences of subtype N9 and one sequence of subtype H7 had to be excluded from analysis due to lack of sequence information for the specific site (sequences too short).

RT-qPCR systems for the neuraminidase genes of influenza A (N9) viruses and the avian influenza A (H7N9) virus

An RNA dilution series of viral RNA from the avian influenza A (H7N9) virus was analysed using two newly developed N9 assays (FLI-N9generic-11 and FLI-N9anhui-1) in comparison with the N9 assay specific for the avian H7N9 virus published by Corman et al. (Corman-N9, [11]) (Table 6). Furthermore, the results for the two newly developed N9 assays were independently confirmed by the use of RNA run-off transcripts of the N9 gene of influenza A/Anhui/1/13 (Table 7).

Based on the Cq-values, the performance of all NA assays was nearly identical. Analysing the dilution series of viral RNA, the FLI-N9generic-11 assay was more sensitive than the FLI-N9anhui-1 assay or the Corman-N9 assay, which exhibited identical detection limits. The analysis of the in vitro transcript revealed a lower detection limit for the FLI-N9generic-11 assay (less than 10 genome copies per reaction) than the FLI-N9anhui-1 assay (less than 100 genome copies per reaction).

Furthermore, we verified the analytical specificity of the three PCR systems by analysing 16 IAV isolates of subtype N9 (Table 8). Since all N9 strains were detected by the newly developed FLI-N9generic-11 assay, but none of 442 tested non-N9 subtypes (data not shown), this assay was confirmed to be suitable for the specific and sensitive generic detection of N9 viruses. Despite very high viral genome loads (low Cq values in the

Table 6

Comparative analytical sensitivity for the detection of neuraminidase sequences of avian influenza A (H7N9) based on RNA dilution series

Dilution series of A/Anhui/1/2013[a]	IAV-M1.2[b]	FLI-N9generic-11[b]	Avain H7N9-specific N9 RT-qPCRs[b]	
			FLI-N9anhui-1	Corman-N9
H7N9_10-3	19.4	20.9	22.0	20.6
H7N9_10-4	23.3	24.7	25.4	23.7
H7N9_10-5	26.2	27.8	28.7	26.9
H7N9_10-6	29.5	31.1	31.4	29.7
H7N9_10-7	33.5	34.1	35.1	33.1
H7N9_10-8	No Cq	36.0	No Cq	No Cq
H7N9_10-9	No Cq	No Cq	No Cq	No Cq

No Cq: no value obtained >42.

[a] RNA extracted from allantoic fluid of embryonated chicken eggs infected with the isolate A/Anhui/1/13 (H7N9) was used.

[b] Numerals in columns represent Cq values.

Table 7

Comparative analytical sensitivity for the detection of neuraminidase sequences of avian influenza A(H7N9) based on an in vitro transcript of the N9 segment

T7 in vitro transcript of the NA of A/Anhui/1/2013[a]	FLI-N9generic-11[b]	FLI-N9anhui-1[b]
200,000	19.8	19.9
20,000	23.3	23.6
2,000	26.7	26.8
200	30.1	29.9
20	33.1	32.5
2	35.7	No Cq
0.2	No Cq	No Cq

NA: neuraminidase; No Cq: no value obtained >42.

[a] RNA copies per µL of template

[b] Numerals in columns represent Cq values.

IAV-M1.2 assay), only three of the 16 tested N9 isolates showed positive cross-reactivity using the newly developed FLI-N9anhui-1 assay, indicating a good specificity of this assay for the specific detection of influenza A/Anhui/1/13 (H7N9). Interestingly, although designed to specifically detect the NA of this avian H7N9 virus, the Corman-N9 assay detected 15 of the 16 tested N9 strains. Therefore, compared to the Corman-N9 assay, the FLI-N9anhui-1 test is more specific.

Analysis of the combined influenza A(H7N9) RT-qPCR

The use of different fluorescent tags in the newly developed assays allowed us to specifically detect the HA (FLI-H7anhui-8, FAM) and NA (FLI-N9anhui-1, HEX) of the novel avian H7N9 virus simultaneously in a duplex approach (Tables 9 and 10). Based on the in vitro transcripts, the detection limit of the duplex RT-qPCR was less than 10 genome copies per reaction for both HA and NA, and therefore corresponds with the results of the uniplex FLI-H7anhui-8 and FLI-N9anhui-1 assays.

We also used the 42 different H7 and the 16 different N9 isolates of our IAV panel for determining the specificity of the duplex assay. Like the single RT-qPCRs of the FLI-H7anhui-8 assay, none of the tested H7 viruses was detected with the combined assay (data not shown). However, the N9 gene of influenza A/wigeon/Germany/R636/07 (H11N9) and A/Anas platyrhynchos/Germany/R2219/2006 (H11N9), which were shown to be detected by the FLI-N9anhui-1 assay (Table 8), were also detected with the duplex assay (data not shown). Taken together, the duplex assay allowed the sensitive and specific detection of HA and NA of avian H7N9 in a single PCR run.

Validation of the diagnostic sensitivity of the RT-qPCR systems for the haemagglutinin and neuraminidase genes of the avian influenza A(H7N9) virus

Finally, we analysed the diagnostic performance of the newly developed assays for the generic detection of H7

Table 8

Comparative analytical specificity for the detection of neuraminidase sequences of N9 viruses influenza A viruses (n=16)

Strain(influenza subtype)	IAV-M1.2[a]	FLI-N9generic-11[a]	Avain H7N9-specific N9 RT-qPCRs[a]	
			FLI-N9anhui-1	Corman-N9
A/wild duck/Germany/R3111/07 (H2N9)	21.1	21.9	No Cq	20.5
A/mallard/Alberta/329/2006 (H5N9)	20.3	21.4	No Cq	35.5
A/mallard/British Columbia/544/2005 (H5N9)	24.7	27.8	No Cq	37.1
A/Mallard duck/Germany/R2711/07 (H2N9)	24.0	24.5	No Cq	23.5
A/wigeon/Germany/R636/07 (H11N9)	20.9	20.8	26.3	20.7
A/shearwater/West Australia/2567/79 (H15N9)	19.1	23.0	No Cq	No Cq
A/Ostrich/Germany/R48/10 (H6N9)	24.9	25.5	No Cq	22.5
A/duck/Germany/R3349/09 (H11N9)	22.2	22.4	No Cq	21.8
A/Graylag goose/Germany/R1416/08 (H2N9)	25.8	26.3	No Cq	25.2
A/Graylag goose/Germany/R1485/08 (H2N9)	25.6	26.5	No Cq	24.6
A/Graylag goose/Germany/R1486/08 (H2N9)	20.5	20.6	No Cq	19.5
A/Graylag goose/Germany/R1487/08 (H2N9)	26.3	27.6	No Cq	24.4
A/mallard/Germany/R3108/07 (H11N9)	22.9	22.7	36.8	21.5
A/tk/Ontario/7732/66 (H5N9)	18.4	19.4	No Cq	32.0
A/Mallard/Föhr/Wv1499-1503/03 (H11N9)	19.4	19.6	No Cq	19.1
A/Anas platyrhynchos/Germany/R2219/2006 (H11N9)	23.5	23.7	29.9	22.6

No Cq: no value obtained >42.

[a] Numerals in columns represent Cq values.

Table 9

Analytical sensitivity of the combined primer and probe sets for avian influenza A(H7N9) viruses based on RNA dilution series

Diluion series of A/Anhui/1/2013[a]	IAV-M1.2[b]	FLI-H7N9-Combi-FAM[b,c]	FLI-H7N9-Combi-HEX[b,d]
H7N9_10-3	19.4	20.7	20.9
H7N9_10-4	23.3	24.2	24.7
H7N9_10-5	26.2	28.1	28.4
H7N9_10-6	29.5	31.0	31.2
H7N9_10-7	33.5	33.2	34.2
H7N9_10-8	No Cq	34.3	No Cq
H7N9_10-9	No Cq	No Cq	No Cq

No Cq: no value obtained >42.

[a] RNA extracted from allantoic fluid of embryonated chicken eggs infected with the isolate A/Anhui/1/13 (H7N9) was used.
[b] Numerals in columns represent Cq values.
[c] FAM channel: FLI-H7anhui-8.
[d] HEX channel: FLI-N9anhui-1.

and N9 viruses (FLI-H7generic-2; FLI-N9generic-11), and for the specific detection of avian influenza A(H7N9) (duplex assay FLI-H7anhui-8/FAM; FLI-N9anhui-1/HEX) in comparison with previously published assays (FLI-H7-CODA, Corman-H7 assay, Wong-H7 assay, Corman-N9 assay) [11-13] on sample material from an animal experiment (Table 11). Ten samples per species (ferrets, chickens and pigeons) were randomly chosen and included both different individuals and several time points after infection. The ferret samples included nasal washings and organ samples, whereas chicken samples represented pharyngeal and cloacal swabs, and organs. Pigeon samples included pharyngeal and cloacal swabs (Table 11). Generally, the newly developed assays detected viral RNA robustly and irrespective of the sample matrix (swab, tissue, etc.). Compared with the most sensitive assay, IAV-M1.2, the generic assays performed almost equally well. The test systems specific for avian influenza A(H7N9) demonstrated an at least 10-fold drop in sensitivity. Sample material exhibiting low viral RNA loads, such as the pigeon cloacal swab samples, was identified as positive less frequently with all assays. Therefore, all primer and probe sets represent useful assays for the sensitive and specific diagnosis of the avian influenza A(H7N9) virus, however, the FLI-H7-CODA assay demonstrated a qualified alternative with better overall results based on the Cq-values and the signal strength.

Discussion

With the availability of RT-PCR, numerous assays for the subtyping of influenza viruses have been developed, and multiplex approaches have frequently been proposed [17]. Continuous progress is achieved regarding the signal detection of the PCR products, e. g. by PCR-ELISAs [18,19] and in particular by RT-qPCR technologies. Due to a seemingly constant increase in the number of outbreaks of highly pathogenic avian influenza (HPAI) caused by infections with subtype H5 or H7 viruses in many countries, RT-PCR and RT-qPCRs were especially designed for the broad detection and differentiation of these HA genes [18,20-22] and their pathotypes on the basis of the HA cleavage site motif [23-25]. Often these assays are combined with a differentiation of the NA subtypes N1, N2, or N7 [26]. So far, only few studies have focused on the generic detection of the N9 gene [27] or the H7 gene [16,28]. In addition, a one-step H7-specific reverse transcription loop-mediated isothermal amplification (LAMP) assay has been described for the identification of H7 viruses and shown to be more sensitive than conventional RT-PCR systems [29]. Another recent study uses the LAMP approach together with hydroxynaphthol blue dye for a colorimetric detection of the novel avian influenza A(H7N9) virus [30].

Within a few months of the appearance of the novel avian influenza A(H7N9) virus, different RT-qPCR assays were designed and validated for its detection, as a rapid response to its emergence in humans. They are therefore preferentially tailored for use with human samples [11,12]. Nevertheless, generic H7 assays are useful and needed, especially in places where different lineages of subtype H7 may be circulating in avian reservoir hosts. In our study, the assay by Wong et al. [12] detected the H7 of avian influenza A(H7N9) viruses with good sensitivity, but also detected further H7 viruses with high Cq values. This cross-reactivity has been observed with the use of high-titre allantoic fluids and may not play a role with clinical samples of avian or human origin that generally bear lower viral loads. Nevertheless, we characterise the Wong H7 assay as not specific for the avian influenza A(H7N9) virus. A similar result was obtained for the assay reported by

Table 10

Analytical sensitivity of the combined primer and probe sets for avian influenza A(H7N9) viruses based on in vitro transcripts of the haemagglutinin and neuraminidase segments

T7 in vitro transcript of the HA of A/Anhui/1/2013[a]	FLI-H7N9-Combi-FAM[b,d]	FLI-H7N9-Combi-HEX[c,d]
200,000	18.8	20.4
20,000	22.0	24.2
2,000	24.9	27.2
200	28.7	30.5
20	32.1	33.6
2	35.9	37.2
0.2	No Cq	No Cq

HA: haemagglutinin; No Cq: no value obtained >42.
[a] RNA copies per µL of template.
[b] FAM channel: FLI-H7anhui-8.
[c] HEX channel: FLI-N9anhui-1.
[d] Numerals in columns represent Cq values.

Table 11

Comparative diagnostic sensitivity for the detection of haemagglutinin and neuraminidase sequences of avian influenza A(H7N9) in samples obtained during experimental animal infections

Sample[a]	IAV-M1.2[d]	Generic H7 RT qPCRs[d]		Avian H7N9-specific H7 RT-qPCRs[d]			Generic N9 RT-qPCR[d]	Avian H7N9-specific N9 RT-qPCRs[d]	
		FLI-H7 generic-2	FLI-H7-CODA	FLI-H7N9-Combi-FAM[b]	Corman-H7	Wong-H7	FLI-N9 generic-11	FLI H7N9 Combi-HEX[c]	Corman-N9
Ferret, n.w. A	17.0	18.4	16.6	21.9	22.0	23.7	17.9	20.0	20.9
Ferret, n.w. B	17.0	18.3	17.1	21.4	22.4	23.8	18.2	20.0	21.7
Ferret, n.w. C	18.3	20.8	18.7	24.4	24.3	26.4	19.7	22.0	22.6
Ferret, n.w. D	31.7	34.9	32.1	39.8	37.6	39.7e	33.9	38.3	37.8
Ferret, n.w. E	27.8	29.0	28.0	32.1	33.5	35.4	29.1	31.5	32.9
Ferret, organ A	23.3	25.5	23.8	29.2	29.7	31.6	24.3	27.6	27.9
Ferret, organ B	22.6	24.8	22.6	28.4	28.6	30.8	23.3	25.5	26.8
Ferret, organ C	22.0	23.5	22.4	26.6	27.9	29.1	23.0	25.9	27.0
Ferret, organ D	26.7	27.9	26.4	33.4	32.8	35.3	28.4	31.0	33.4
Ferret, organ E	27.3	29.0	27.1	35.3	33.2	36.9	28.7	32.0	33.1
Chicken, ph.s. A	20.3	21.7	20.7	26.3	26.2	27.8	21.8	24.5	25.0
Chicken, ph.s..B	23.3	25.0	23.7	28.7	29.3	31.7	35.1	27.7	27.6
Chicken, ph.s. C	23.1	24.3	22.9	28.1	28.4	29.8	24.2	27.0	28.0
Chicken, ph.s. D	22.9	24.7	22.7	29.2	28.6	31.0	23.5	28.0	27.0
Chicken, c.s. A	30.0	31.5	30.4	36.5	36.1	37.5	30.7	34.7	34.5
Chicken, c.s. B	24.2	26.2	25.5	29.7	30.6	33.2	25.6	28.7	29.0
Chicken, c.s. C	24.3	26.5	25.1	30.2	30.4	32.4	25.4	29.0	29.1
Chicken, c.s. D	23.3	24.2	23.1	28.1	28.8	29.8	24.2	27.0	28.1
Chicken, organ A	30.7	35.2	32.2	41.7[e]	38.9	No Cq	30.2	No Cq	36.7
Chicken, organ B	26.2	27.2	25.6	32.4	32.1	35.5	26.6	29.7	32.2
Pigeon, ph.s. A	29.4	31.2	29.5	36.6	35.2	38.0	30.0	33.0	33.5
Pigeon, ph.s. B	24.7	26.3	23.8	29.8	30.5	31.5	26.7	28.7	29.8
Pigeon, ph.s. C	30.7	32.3	30.2	37.2	36.9	38.3	31.4	34.7	35.1
Pigeon, ph.s. D	31.3	33.8	32.1	38.2	38.0	40.2	32.1	36.1	35.9
Pigeon, ph.s. E	32.3	35.0	33.6	37.9	38.9	39.6[g]	34.5	40.1[e]	37.0
Pigeon, c.s. A	33.2	36.3[e]	34.6	No Cq	39.7	No Cq	35.5	38.0[e]	38.9
Pigeon, c.s. B	33.7	36.1[e]	33.4	39.1[e]	38.8	39.5[e]	34.4	37.5[e]	37.7
Pigeon, c.s. C	No Cq	36.4[e]	35.8[e]	40.8[e]	No Cq	No Cq	35.6	No Cq	No Cq
Pigeon, c.s. D	36.3[e]	No Cq	33.7	No Cq	40.7	No Cq	34.3	37.5	39.8
Pigeon, c.s. E	36.4[e]	No Cq	No Cq	No Cq	40.6	No Cq	35.7	No Cq	39.2

No Cq: no value obtained >42.

[a] RNA extracted from samples of different origin (nasal washing (n.w.), organs, pharyngeal swabs (ph.s.), or cloacal swabs (c.s.)) obtained at different days post infection with the isolate A/Anhui/1/13 was used.

[b] FAM channel: FLI-H7anhui-8.

[c] HEX channel: FLI-N9anhui-1.

[d] Numerals in columns represent Cq values.

[e] Only one of the duplicates was positive.

Corman et al. [11]. Lack of analytical specificity of these assays is likely to be due to the limited range of lineages selected during design of the primer and probe sets. Previous validation studies of these PCRs were limited to exclude cross-reactions with other human IAV subtypes (and other respiratory viruses of human origin). In addition to the H7 assay, Corman et al. also provided a primer and probe set for the detection of N9. This assay has a high sensitivity for the avian influenza A(H7N9) virus, but also detects all except one of the other N9-viruses tested, and, therefore, should be considered as a generic rather than a lineage-specific assay.

In order to improve the specificity for diagnostic purposes, focusing on animal (i.e. avian) swabs, we intended to define primer and probe sets for (i) the generic detection of the HA of H7 viruses, (ii) the

specific detection of the HA of the avian influenza A(H7N9) virus, (iii) the generic detection of the NA of N9 viruses, (iv) the specific detection of NA of the avian influenza A(H7N9) virus, and (v) the simultaneous detection of HA and NA of the avian influenza A(H7N9) virus. These assays proved to offer excellent sensitivities and specificities on the tested sample preparations. The definition of the new oligonucleotides was facilitated by the fact that a considerable number of new sequence records had accumulated in the EpiFlu database. The HA and NA sequences of the novel avian influenza A(H7N9) isolates form distinct phylogenetic lineages which can be discerned from other Eurasian H7 and N9 sequences [31]. The emerging zoonotic avian influenza A(H7N9) viruses in China, which display low pathogenicity in poultry, but still cause severe clinical disease in humans, pose, in contrast to the HPAI H5N1 epizootic, a particular problem for monitoring in poultry and other avian populations. Since no clinical symptoms are induced in infected poultry, the virus can be transmitted and spread by healthy poultry. According to results of experimental infections, chickens and quails are suspected to be particularly efficient transmitters because these species excreted the largest amounts of virus. The occurrence of a second wave of human infections with A(H7N9) since October 2013 [32] confirmed that pockets of presumably avian reservoirs of this virus continue to exist. This situation emphasises the relevance of surveillance programmes that are based on molecular diagnostic tools for rapid and sensitive but also highly discriminatory detection of influenza A(H7N9) viruses.

Although the reverse primer of the FLI-H7-CODA assay contained a mismatch to the HA sequence of influenza A/Anhui/1/13 (H7N9) virus, it performed well on the detection of the novel avian influenza A(H7N9) virus. A slightly reduced sensitivity could be observed for the combined HA and NA duplex assay, which is due to limitations in the primer design in favour of a high specificity.

In routine settings of diagnostic laboratories, qualitative determination is of primary importance. RT-qPCRs provide the added advantage of a semiquantitative impression, assuming that, as in our assays, inhibitory effects are excluded. Full quantitative analysis based on RNA copy number would be reserved for research purposes, in cases when the amount of excreted virus needs to be analysed comparatively. The newly developed assays would be suitable for each of these fields.

A further advantage of the newly developed RT-qPCRs is that all newly developed assays (FLI-H7generic-2, FLI-N9generic-11, FLI-H7anhui-8, FLI-N9anhui-1, and the FLI-H7anhui-8/FLI-N9anhui-1 combination), but also the previously published assays (IAV-M1.2, FLI-H7-CODA, Corman-H7, Wong-H7, Corman-N9) can be run with the same chemistry and the same amplification protocol, allowing easy handling in diagnostic laboratories.

Previously established assays such as the FLI-H7-CODA and Corman-H7 assay displayed a high sensitivity for the avian influenza A(H7N9) virus, but failed to detect several other isolates of subtype H7. When a panel of 459 viruses (including the avian H7N9 virus) was tested with the FLI-H7generic-2 assay, all 39 tested strains of Eurasian origin, and in addition even one H7 strains of the North American lineage as well as the avian H7N9 virus, were detected. A weak cross reaction was observed with only six viruses of non-H7 subtypes, all six belonging to the closely related subtype H10. The FLI-N9generic-11 showed a high sensitivity for the avian influenza A(H7N9) virus, detected all tested isolates of subtype N9, but none of the 442 strains of the other eight non-N9-NA subtypes.

While this manuscript was in preparation, Hackett et al. published three RT-qPCR assays for the specific detection of the influenza A(H7N9) virus based on the conserved M gene, that can be adopted into established protocols for the detection of human influenza viruses [33]. Furthermore, Li et al. developed an assay for the simultaneous detection of the HA and NA genes of influenza A(H7N9) viruses, but again focussing on samples of human origin and the diagnostic differentiation from other human respiratory viruses [34].

In light of the high mutation rate of IAV, we recommend different combinations of the introduced assays to be used in a diagnostic cascade system: If poultry is screened for the presence of IAV of subtype H7, the IAV-M1.2 assay should be applied first, and in case of a positive result, samples should subsequently be examined with the FLI-H7generic-2 assay, as this assay showed the broadest diagnostic inclusiveness of H7 viruses. Screening, especially of human samples, for IAV of subtype H7N9 should also start with the IAV-M1.2 assay. Positive samples should then be examined by either the FLI-H7-CODA or the Corman-H7 assay, as well as the FLI-N9generic-11 test. Finally, for a direct confirmation of avian influenza A(H7N9) virus in samples which are positive in the generic pan-IAV assays, the combination of the FLI-H7anhui-8/FLI-N9anhui-1 RT-qPCRs in a duplex assay is recommended. Notably, in our study, the newly developed assays have not been validated on human samples. Therefore, a validation of the performance of these assays should be performed on human clinical samples before they are implemented in diagnostic laboratories.

Taken together, suitable RT-qPCR assays with high sensitivity and considerably improved specificity for the generic detection of H7 and N9 subtypes, and for the specific detection of the avian influenzaA (H7N9) virus are provided in this study. Notably, the robustness of a specific duplex assay to confirm the avian influenza A(H7N9) virus was proven.

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Conflict of interest

None declared.

Authors' contributions

Designed the study: MB, BH, TH. Collected and synthesized the data: DK, TH, CG, AP, BH. Interpreted the data: DK, JB, TH, CG, AP, MB, BH. Prepared the first draft of this manuscript: DK, JB. All authors reviewed and revised the first and final drafts of this manuscript.

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