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Universal Primer Set for Amplification and Sequencing of HA_0 Cleavage Sites of All Influenza A Viruses▽†

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Sequence analysis of the endoproteolytic cleavage site within the hemagglutinin (HA) precursor protein HA_0 is fundamental for studies of the molecular biology of influenza A viruses, in particular, for molecular pathotyping of subtype H5 and H7 isolates. A current problem for routine diagnostics is the emergence of new strains of the H5 or H7 subtype or even other subtypes which escape detection by commonly used reverse transcription-PCR (RT-PCR) protocols. Here, the first pan-HA (PanHA) RT-PCR assay targeting the HA_0 cleavage site of influenza A viruses of all 16 HA subtypes is reported. The assay was assessed in comparison to H5 and H7 subtype-specific RT-PCRs for the HA_0 cleavage site and a real-time RT-PCR detecting the M gene. A panel of 92 influenza A viruses was used for validation. Sequence data for influenza A viruses from 32 allantoic fluid samples and 11 diagnostic swab samples of all 16 HA subtypes were generated by direct sequencing of the PanHA RT-PCR products. The results demonstrate that the new PanHA RT-PCR assay—followed by cycle sequencing—can complement existing methods and strengthen the reliability of influenza A virus diagnostics, allowing both molecular pathotyping (H5 and H7) and subtyping (non-H5 or -H7) within a single approach.

Highly pathogenic avian influenza viruses (HPAIV) cause epidemic disease with high mortality rates in both poultry and wild birds (6) and are biologically characterized by intravenous pathogenicity indices of more than 1.2 (2, 3). Molecular pathotyping by sequence analysis of the cleavage site within the hemagglutinin (HA) precursor protein HA_0 is more rapid and reduces the risk of handling infectious material (2). The presence of multiple basic amino acids (arginine or lysine) at the HA_0 cleavage site, indicating processibility by ubiquitous proprotein convertases, is a molecular indicator for pathogenicity (13, 16). These viruses cause severe systemic infections and have historically belonged to either the H5 or the H7 subtype (2). In contrast, AIV of low pathogenicity (LPAIV) as well as mammalian influenza A viruses show a monobasic composition at this site. Their HA_0 is cleaved extracellularly by tissue-specific, trypsin-like proteases; thus, LPAIV infection remains localized to the intestinal tract of birds or the respiratory tract of mammals. HPAIV consistently have a polybasic cleavage site which is cleaved intracellularly by ubiquitous subtilisin-like proteases (13, 16).

Nevertheless, current strains show high sequence heterogeneity and new strains continue to emerge. At least two isolates of the H10 subtype fulfilled the definitions of the World Organization for Animal Health (Office International des Epizooties) and the European Union (EU) for HPAIV (2, 3, 17) but lacked multiple basic amino acids at the HA_0 cleavage site. In addition, the R-S-S-R motif of H9N2 viruses from birds, swine, and humans (1, 5, 7, 20) was recently proven to have been replaced by an R-S-R-R (20) in an isolate from a quail. Although this virus showed multiple basic amino acids as a molecular determinant for identification as HPAIV, pathogenicity tests of chickens still revealed low pathogenicity. Londt et al. (12) have described a further four H5 and H7 AIV showing incongruency between molecular and biological pathotyping results. Therefore, exact molecular pathotyping, i.e., determination of HA_0 cleavage site sequences, and subtyping of all influenza A viruses are necessary for diagnostics, surveillance, and epidemiological studies as well as for investigations of the significance of the amino acid motifs for pathogenicity.

This report describes a validated one-step pan-HA (PanHA) reverse transcription-PCR (RT-PCR) amplifying a fragment encompassing the HA_0 cleavage site as a new diagnostic tool. Direct sequencing of the RT-PCR products was accomplished for molecular characterization of the HA_0 cleavage site sequences and pathotyping and subtyping of influenza A viruses of all 16 HA subtypes.

MATERIALS AND METHODS

Viruses and diagnostic samples. A panel of 92 influenza A virus isolates (see Table S1 in the supplemental material) was obtained in the form of allantoic fluid samples from the Office International des Epizooties and German National Reference Laboratory for Avian Influenza. In addition, 17 field samples, cloacal swabs from wild and domestic birds, were used.

Isolation of RNA. Viral RNA from allantoic fluid samples and cloacal swabs was purified using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with some modifications, including the addition of 5 μl of in vitro-transcribed internal control (IC) RNA (2×10^5 copies/μl) after lysis of the sample to control the efficiency of RNA isolation and RT-PCR (9).

Real-time RT-PCR. Quantitative RT-PCR detecting the matrix (M) gene of influenza A virus was performed as described previously (15) with some modifications, including integration of an IC system (9). The duplex real-time RT-PCR was performed using a 25-μl reaction volume and a SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). For one reaction, the assay was optimized to 2.5 μl of RNase-free water, 12.5 of μl reaction mix (2×), 2 μl of M-specific primer-probe mix (10 pmol of primer IVA-M_1for/μl, 15 pmol of primer IVA-M1.1rev/μl, 1.25 pmol of probe

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TABLE 1. Primers and probes used in this study

Oligonucleotide	Sequence (5′–3′)[a]	Position (nt)[b]	Reference
HA-1057.1-F	GGR GAA TGC CCC AAA TAY GT	967–986[c]	This study
HA-1057.2-F	GGR ARA TGC CCC AGR TAT GT	967–986[c]	This study
HA-1057.3-F	GGR GAA TGC CCC AAR TAY AT	967–986[c]	This study
HA-1232.1(555)-R	*CTG AGT CCG AAC ATT GAG* TTG CTA TGV TGR TAW CCA TAC CA	1142–1120[c]	This study
HA-1232.2(555)-R	*CTG AGT CCG AAC ATT GAG* TTY TGA TGY CTG AAD CCR TAC CA	1142–1120[c]	This study
IVA-M_1for	AGA TGA GTC TTC TAA CCG AGG TCG	24–47[d]	15
IVA-M1.1rev	TGC AAA AAC ATC TTC AAG TYT CTG	124–101[d]	15
IVA-M_1-FAM-BHQ	FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1[f]	74–93[d]	15
EGFP-12-F	*TCG AGG GCG ACA CCC TG*	439–456[e]	9
EGFP-10-R	*CTT GTA CAG CTC GTC CAT GC*	813–794[e]	9
EGFP-HEX	HEX-*AGC ACC CAG TCC GCC CTG AGC A*-BHQ1	703–724[e]	9

[a] Nonviral sequences are shown in italics.
[b] nt, nucleotides.
[c] Result obtained with influenza A virus RefSeq A/Goose/Guangdong/1/96 H5N1 (GenBank accession no. NC_007362) (HA).
[d] Result obtained with influenza A virus RefSeq A/Goose/Guangdong/1/96 H5N1 (GenBank accession no. NC_007363) (M).
[e] Result obtained with cloning vector pEGFP-1 (GenBank accession no. U55761).
[f] BHQ1, Black Hole Quencher 1.

IVA-M_1-FAM-BHQ/μl) (Table 1), 2 μl of IC-specific primer-probe mix (2.5 pmol/μl each of primers EGFP-12-F and EGFP-10-R and 1.25 pmol of probe EGFP-HEX/μl) (Table 1), 1 μl of SuperScript III RT/Platinum *Taq* mix, and 5 μl of template RNA. The reaction was carried out using an Mx3000P real-time PCR system (Stratagene, La Jolla, CA) with the following temperature profile: 30 min at 50°C (RT), 2 min at 94°C (inactivation of reverse transcriptase/activation of *Taq* polymerase), followed by 42 cycles of 30 s at 94°C (denaturation), 30 s at 57°C (annealing), and 30 s at 68°C (elongation). Fluorescence data were collected during the annealing step.

RT-PCR. (i) The PanHA RT-PCR was performed using a 25-μl reaction volume and a SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase. For one reaction, the assay was optimized to 4.5 μl of RNase-free water, 12.5 μl of reaction mix (2×), 2 μl of PanHA Mix 1.0 [5 pmol/μl each of primers HA-1057.1-F, HA-1057.2-F, HA-1057.3-F, HA-1232.2(555)-R, and HA-1232.1(555)-R] (Table 1), 1 μl of SuperScript III RT/Platinum *Taq* Mix, and 5 μl of template RNA. The reaction was carried out using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA) with the following temperature profile: 30 min at 50°C (RT) and 2 min at 94°C (inactivation of reverse transcriptase/activation of *Taq* polymerase), followed by 45 cycles of 30 s at 94°C (denaturation), 45 s at 50°C (annealing) and 45 s at 68°C (elongation), and 5 min at 68°C (final elongation). Amplicons with a size of 164 to 176 bp were visualized by 3% agarose gel electrophoresis for 1 h at 120 V in Tris-acetate-EDTA buffer. (ii) H5 and H7 subtype-specific RT-PCR assays were performed according to the same protocol. H5-specific primer pair H5-kha-1 and H5-kha-3 and primer pair J3 and B2a as well as H7-specific primer pair GK7.3 and GK7.4 (4) were utilized at a concentration of 20 pmol each/25 μl of reaction volume.

Direct sequencing. PanHA RT-PCR products were purified from agarose gels by use of a QIAquick gel extraction kit (Qiagen) as recommended by the manufacturer and were subjected to automated direct sequencing at Agowa, Berlin, Germany, using a 3730*xl* DNA analyzer (Applied Biosystems). The products were sequenced utilizing panHA-Mix 1.0-F (5 pmol/μl each of primers HA-1057.1-F, HA-1057.2-F, and HA-1057.3-F) and panHA-Mix 1.0-R [5 pmol/μl each of primers HA-1232.2(555)-R and HA-1232.1(555)-R] as sequencing primers.

Sequence analysis. Sequence data were analyzed and edited with BioEdit version 7.0.9.0 (8). Amino acid sequences were deduced and pathotypes were determined according to properties described previously (16). A BLASTN search was performed to identify the query sequence and to find similar sequences. All sequences generated in this study were submitted to the European Molecular Biology Laboratory (EMBL) database.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study are available from EMBL, GenBank, and DDBJ under accession numbers AM922136 to AM922167 and AM930525 to AM930535.

RESULTS

Design of the PanHA RT-PCR. A set of primers (Table 1) was designed and combined in a one-step RT-PCR to amplify a fragment of 164 to 176 bp spanning the HA_0 cleavage site of influenza A viruses of all 16 HA subtypes. A panel of 92 influenza A virus isolates (see Table S1 in the supplemental material) was used for validation. All samples were verified for their content of influenza A virus genome by real-time RT-PCR targeting the M gene (9, 15) and revealed threshold cycle (C_T) values between 15 and 28 (data not shown). With the PanHA RT-PCR protocol described here, 87 influenza A virus isolates showed clearly positive results (see Fig. S1 in the supplemental material). Only A/turkey/Wisconsin/66 (H9N2) and A/pilot whale/Maine/328/84 (H13N2) could not be detected, whereas three recent isolates of the H10 and H13 subtype (A/Mallard/Föhr/Wv1781-82/03 [H10N7], R2609/06 [H13N8], and R2622/06 [H13]) were detected with low sensitivity.

Comparison of the PanHA RT-PCR with H5 and H7 subtype-specific RT-PCR assays. The reliability of the PanHA RT-PCR in detection of influenza A virus isolates of the H5 and H7 subtypes was compared to that of subtype-specific RT-PCRs (H5-specific primer pair H5-kha-1 and H5-kha-3 and primer pair J3 and B2a and H7-specific primer pair GK7.3 and GK7.4) recommended in the EU *Diagnostic Manual for Avian Influenza* (4). RNA extracted from 12 influenza A virus isolates of the H5 subtype (C_T values between 16 and 22) and 16 influenza A virus isolates of the H7 subtype (C_T values between 17 and 24) was tested using the appropriate primer pairs. It was shown that all samples scored positive when the PanHA primers were used. In contrast, the results obtained with A/Mall/QC/2323–19/2006 (H5N2), A/duck/Alberta/48/76 (H7N3), and A/Mallard/NVP/1776–80/03 (H7N7) remained negative when the EU-recommended subtype-specific primers were used. Furthermore, A/DK/BC/26–6/05 (H5N2) was detected by only one of the two H5-specific primer pairs (data not shown).

Sensitivity of the PanHA RT-PCR for H5 and H7 detection in comparison to the sensitivity of H5 and H7 subtype-specific RT-PCR assays and M gene-specific real-time RT-PCR. Ten-fold dilution series of RNA extracted from two European (one HPAIV and one LPAIV) and two North American (LPAIV) influenza A virus isolates of both the H5 and H7 subtypes were prepared in RNA-safe buffer (0.05% Tween 20, 0.05% sodium azide, 50 ng/μl of carrier RNA [poly(A) homopolymer; Amersham Biosciences, Piscataway, NJ]) and amplified using the different protocols. The results are shown in Fig. 1. The sensitivity of

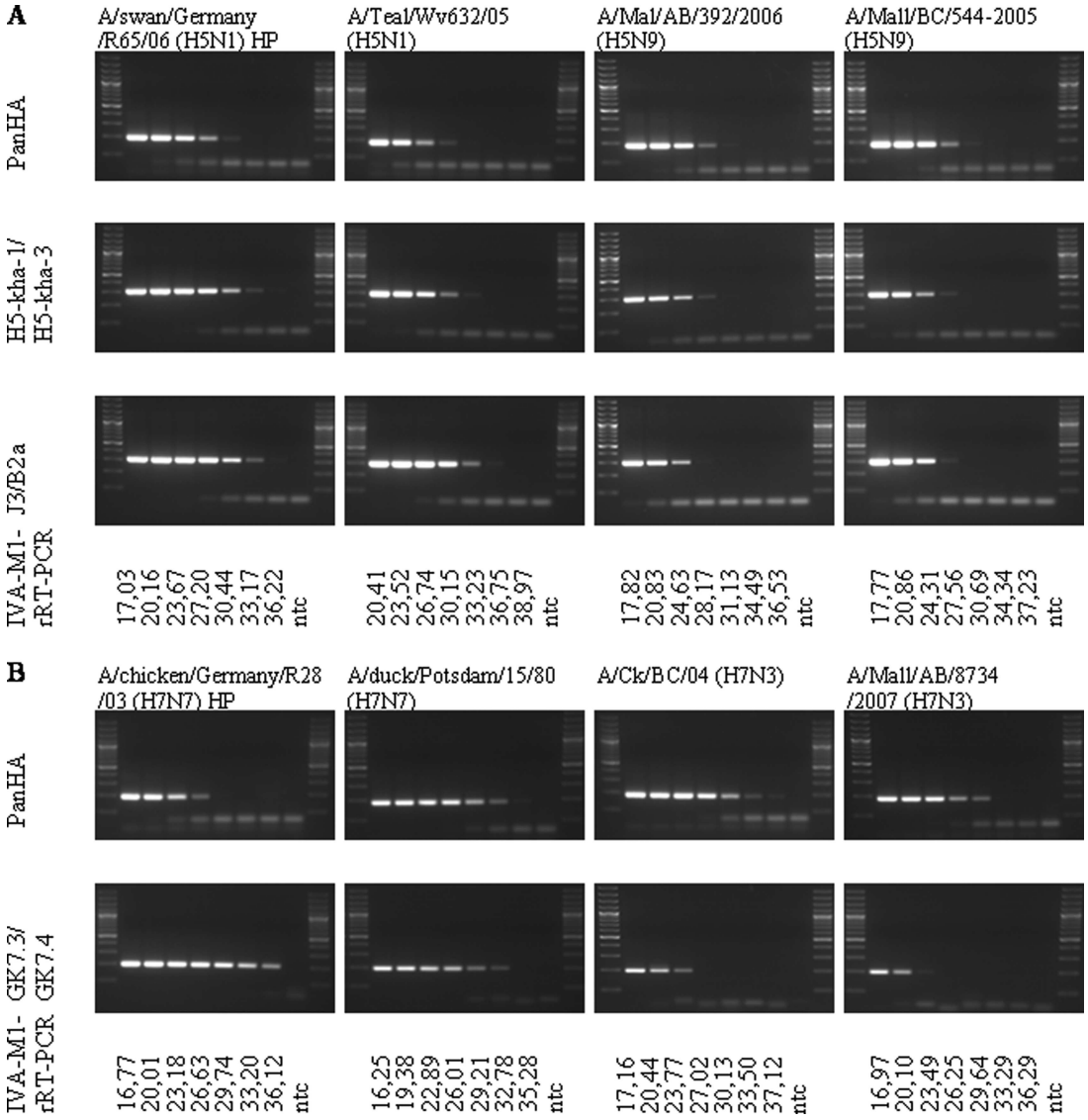

FIG. 1. Assay sensitivity for influenza A virus of subtypes H5 and H7. (A) RNA from four influenza A virus isolates of the H5 subtype was tested in 10-fold dilution series with the PanHA primers as well as H5-specific primer pair H5-kha-1 and H5-kha-3 and primer pair J3 and B2a. (B) RNA from four influenza A virus isolates of the H7 subtype was tested in 10-fold dilution series with the PanHA primers as well as H7-specific primer pair GK7.3 and GK7.4. C_T values for the M gene obtained by real-time RT-PCR are shown beneath the agarose gel image. Marker, GeneRuler 100-bp DNA Ladder Plus (Fermentas). ntc, no template control.

the PanHA RT-PCR for the selected European influenza A virus isolates was comparable to or slightly lower than that seen when the EU-recommended PCR methods were used (4). In addition, the PanHA RT-PCR protocol revealed a higher sensitivity than the subtype-specific assays for the selected North American influenza A virus isolates. In contrast to the M gene-specific real-time RT-PCR results, influenza A virus RNA from allantoic fluids with a C_T value of approximately 30 or lower was detected by the PanHA RT-PCR. Therefore, the PanHA RT-PCR protocol was approximately 100- to 1,000-fold less sensitive than the real-time RT-PCR.

Sensitivity of the PanHA RT-PCR using diagnostic samples. RNA from 17 cloacal swabs from wild and domestic birds was proven to contain influenza A virus genome (C_T values between 23.49 and 37.57 in M gene-specific real-time RT-PCR) (Table 2). C_T values for the IC ranged between 28.50 and 31.61, indicating that no inhibitory effects were present in these samples. No C_T values for the IC were observed for the two samples with the highest viral RNA loads, due to competitive inhibition by coamplification of the M product, or for sample R1642/07, due to inefficient RNA isolation or the presence of inhibitors of the RT-PCR. With the PanHA RT-PCR protocol, the influenza A virus genome could be detected in 11 out of 17 samples. All samples with C_T values for the M gene of less than 30 (except for sample R1642/07) and, in addition, sample R2619/07 (C_T value of 33.10) showed positive results. Thus, influenza A virus RNA was detected in diagnostic samples with a C_T value of approximately 30 or lower. Repeated testing of selected samples proved the validity of the observed results (data not shown).

TABLE 2. Sensitivity of PanHA RT-PCR compared to real-time RT-PCR for influenza A virus in diagnostic samples

Sample	C_T value (duplex real-time RT-PCR)		PanHA RT-PCR result
	FAM[a] (M gene)	HEX[b] (IC)	
R2322/07	23.49	No C_T	+
R2495/07	23.61	No C_T	+
R2777/07	26.54	28.92	+
R2709/07	27.13	29.39	+
R2770/07	27.70	28.91	+
R1642/07	27.71	No C_T	−
R2706/07	27.75	28.94	+
R2583/07	27.85	28.80	+
R1649/07	27.97	29.62	+
R2484/07	28.86	28.78	+
R2497/07	29.20	28.80	+
R2479/07	31.99	29.57	−
R2619/07	33.10	30.69	+
R2723/07	33.56	29.52	−
R2710/07	34.34	31.61	−
R2776/07	34.39	29.26	−
R2769/07	37.57	28.50	−

[a] FAM, 6-carboxyfluorescein.
[b] HEX, hexachloro-6-carboxyfluorescein.

Determination of HA_0 cleavage site sequence, pathotype, and HA subtype of influenza A viruses from allantoic fluids. In order to determine HA_0 cleavage site sequences, PCR primer mixes were also used as sequencing primers for subsequent direct sequencing of the purified PanHA RT-PCR products. In a first step, reliability of the novel method was ensured by generation of sequence data for 16 influenza A virus isolates and comparison to sequences deposited in EMBL, GenBank, and DDBJ. All of the sequences obtained (accession no. AM922136 to AM922138, AM922140, AM922145, AM922146, AM922150 to AM922153, AM922156, AM922157, AM922162, and AM922165 to AM922167) exhibited 100% identity with the published sequences. In a second step, HA_0 cleavage site sequence data were also generated for 16 influenza A virus isolates for which no HA sequence data were available from EMBL, GenBank, and DDBJ (Table 3). Pathotypes were determined using the amino acid sequence, and subtypes were determined by a BLASTN search. Fully concordant results were established for 15 out of 16 influenza A virus isolates in comparisons of subtyping results obtained by use of the PanHA approach with the results of molecular analyses of an HA_2 fragment according to the method published by Phipps et al. (14). In summary, sequence data for HA_0 cleavage sites were generated for 32 avian and human influenza A virus isolates of all 16 HA subtypes. Typical motifs for the different subtypes as well as for HPAIV subtypes H5 (A/HongKong/156/97 [H5N1]) and H7 (A/chicken/Brescia/19/02 [H7N1]) were detected. Moreover, some of the investigated LPAIV within the H5, H7, and H9 subtypes were found to have the capacity, following the introduction of single transversional or transitional point mutations, to become highly pathogenic and thus would not depend on insertional mutations (data not shown).

Determination of HA_0 cleavage site sequence, pathotype, and HA subtype of influenza A viruses from the diagnostic samples. HA_0 cleavage site sequence data were generated for 11 influenza A viruses from diagnostic samples which showed positive results by use of PanHA RT-PCR (Table 2 and Table 4). Pathotypes were ascertained by analysis of the amino acid sequence, and subtypes were determined by BLASTN search (Table 4). Subtypes corresponded to results obtained by sequencing of an HA_2 fragment (14) where those results were available.

DISCUSSION

Subtyping and subsequent pathotyping of influenza A viruses are of utmost significance for initiation of restrictive measures against HPAIV. Molecular methods have the advantage of accelerating diagnostics and reducing the risk of handling infectious material. Here we report a PanHA RT-PCR amplifying a fragment encompassing the HA_0 cleavage site of influenza A viruses of all 16 of the known HA subtypes. Direct sequencing of the PCR products permitted both pathotyping and subtyping within a single approach.

In order to minimize the risk of cross-contamination, a one-step RT-PCR protocol using a commercially available kit (SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase) was selected. To ensure proper detection of all 16 HA subtypes, a mix of five degenerated primers was derived and used under cycling conditions that allowed the detection of different influenza A viruses. A panel of 92 influenza A virus isolates—mainly from poultry and wild birds (subtypes H1 to H16)—originating from Eurasia, North America, and Australia was used for validation of the new assay. The panel also contained one isolate from a swine (H1) and six isolates from human hosts (four H1, one H3, and one H5), including H1N1, H3N2, and H5N1 strains. The sensitivity of the new method was sufficient, detecting 87 out of 92 influenza A virus isolates (94.6%) of all HA subtypes; negative results were obtained with only two older isolates (H9N2 and H13N2). Detection results indicating lower sensitivity were evident for three recent isolates (H10N7, H13N8, and H13) for which no HA sequence data were available. However, in contrast to previously published results of studies using "pan" HA primers for full-length amplification of the HA gene (11) or for partial amplification of the HA_2 gene for subtyping (14, 19), the amplification of a product from influenza A viruses of all 16 HA subtypes could be ensured.

All known HPAIV belong to either the H5 or the H7 subtype. Subtype-specific RT-PCR assays allowing the analysis of the HA_0 cleavage site of these subtypes have been described previously (4, 18). When the PanHA RT-PCR assay was used, all influenza A virus isolates tested (12 H5 isolates and 16 H7 isolates) yielded positive results. The results for one recent H5N2 virus from North America remained negative with each of the H5-specific primer pairs, whereas a second was detected by only one of the two primer pairs. For the H7 subtype, an older H7N3 isolate from North America as well as a recent H7N7 virus encountered during wild-bird monitoring in Germany could not be detected using the H7-specific primer pair. Moreover, the sensitivity for the selected North American influenza A virus isolates was higher when employing the PanHA primers than that seen when utilizing the EU-recommended subtype-specific primers. For European influenza A virus isolates, the use of subtype-specific primers resulted in equal or higher sensitivity. Accordingly, the implementation of the PanHA RT-PCR protocol described here is advisable, espe-

TABLE 3. Determination of pathotype and HA subtype of influenza A viruses from allantoic fluids

Isolate[a]	PanHA RT-PCR													Accession no.[b]	Pathotype[c]	Subtype[d]	Maximum identity (%) with[b,d]:
	Sequence of HA_0 cleavage site																
A/Anser egypticus/Germany/R1419/2006 (H1N1)	CTG	AGA	AAC	ATT	CCT	TCC	ATT	CAA	TCT	AGA	GGG	CTC	TTC	AM922139	LP	H1	A/pintail/Shimane/324/98 (H1N9) (AB274304) (95)
	L	R	N	I	P	S	I	Q	S	R	G	L	F				
A/bantam/Germany/DZ4/85 (H2N2)	CCA	AGA	AAC	GTC	CCT	CAG	ATT	GAA	TCA	AGG	GGA	TTG	TTT	AM922141	LP	H2	A/herring gull/Delaware/670/1988 (H2N9) (CY014556) (98)
	P	R	N	V	P	Q	I	E	S	R	G	L	F				
A/Mallard/Germany/Wv1317–21/03 (H2N3)	ATA	AGG	AAT	GTC	CCT	CAG	ATT	GAA	TCA	AGA	GGA	CTG	TTT	AM922142	LP	H2	A/mallard/Sweden/S90568/03 (H2N3) (EU057728) (97)
	I	R	N	V	P	Q	I	E	S	R	G	L	F				
A/Mallard/Germany/Wv677/04 (H2N3)	TTA	AGA	AAT	GTC	CCT	CAG	ATT	GAA	TCA	AGA	GGA	CTG	TTT	AM922143	LP	H2	A/mallard/Sweden/S90568/03 (H2N3) (EU057728) (100)
	L	R	N	V	P	Q	I	E	S	R	G	L	F				
A/Mallard/Germany/Wv943–45/04 (H2N3)	TTA	AGA	AAT	GTC	CCT	CAG	ATT	GAA	TCA	AGA	GGA	CTG	TTT	AM922144	LP	H2	A/mallard/Sweden/S90568/03 (H2N3) (EU057728) (100)
	L	R	N	V	P	Q	I	E	S	R	G	L	F				
A/Mallard/Germany/Wv1806–09/03 (H4N6)	ATG	AGA	AAT	ATC	CCT	GAA	AAG	GCA	TCA	AGA	GGG	CTT	TTT	AM922147	LP	H4	A/duck/South Africa/1233A/2004 (H4N8) (EF041495) (97)
	M	R	N	I	P	E	K	A	S	R	G	L	F				
A/Mallard/Wv1754–57/03 (H4N6)	ATG	AGA	AAT	ATC	CCT	GAA	AAG	GCA	TCA	AGA	GGG	CTT	TTT	AM922148	LP	H4	A/duck/South Africa/1233A/2004 (H4N8) (EF041495) (97)
	M	R	N	I	P	E	K	A	S	R	G	L	F				
A/Mallard/Wv1732–34/03 (H4N6)	ATG	AGA	AAT	ATC	CCT	GAA	AAG	GCA	TCA	AGA	GGG	CTT	TTT	AM922149	LP	H4	A/duck/South Africa/1233A/2004 (H4N8) (EF041495) (97)
	M	R	N	I	P	E	K	A	S	R	G	L	F				
A/chicken/Brescia/19/02 (H7N1)	CCC	GAA	CTT	CCC	AAA	AAA	AGA	AGA	AAA	AGA	GGC	CTG	TTT	AM922154	HP	H7	A/fowl/Dobson/1927 (H7N7) (CY014992) (100)
	P	E	L	P	K	K	R	R	K	R	G	L	F				
A/swan/Potsdam/64/81 (H7N3)	AAG	AAC	GTT	CCT	GAA	ATT	CCA	AAA	GGG	AGA	GGA	CTA	TTT	AM922155	LP	H7	A/swan/Potsdam/63/6/81 (H7N7) (U20467) (100)
	K	N	V	P	E	I	P	K	G	R	G	L	F				
A/Anser spec./Germany/R44/2006 (H8)	CTT	AGA	AAC	ACT	CCT	TCT	ATT	GAA	CCC	AAA	GGA	TTG	TTT	AM922158	LP	H1	A/swine/Iowa/2/1987 (H1N1) (CY028171) (88)
	L	R	N	T	P	S	I	E	P	K	G	L	F				
A/chicken/Emirates/R66/02 (H9N2)	CTG	AGG	AAC	GTG	CCT	GCT	AGA	TCA	AGT	AGA	GGA	CTA	TTT	AM922159	LP	H9	A/chicken/Dubai/463/2003 (H9N2) (EF063516) (100)
	L	R	N	V	P	A	R	S	S	R	G	L	F				
A/Mallard/NVP/Wv1677–81/03 (H10N4)	AGA	AAC	GTG	CCA	GAA	ATA	ATG	CAA	GGG	AGA	GGT	CTA	TTT	AM922160	LP	H10	A/duck/Shimane/45/1997 (H10N7) (AB296078) (97)
	R	N	V	P	E	I	M	Q	G	R	G	L	F				
A/Anas platyrhynchos/Germany/R2219/2006 (H11)	CTT	AGA	AAT	GTC	CCA	GCG	ATA	GCA	TCA	AGA	GGC	TTG	TTT	AM922161	LP	H11	A/duck/Siberia/700/1996 (H11N9) (AB292783) (98)
	L	R	N	V	P	A	I	A	S	R	G	L	F				
A/gull/Stralsund/Wv1136–40/03 (H13N6)	CTC	AGA	AAT	GTG	CCA	GCC	ATA	TCG	AAT	AGA	GGA	CTG	TTT	AM922163	LP	H13	A/duck/Siberia/272/1998 (H13N6) (AB284988) (96)
	L	R	N	V	P	A	I	S	N	R	G	L	F				
A/Larus ridibundus/Germany/R2064/2006 (H13)	CTT	AGG	AAT	GTA	CCT	GCC	ATA	TCA	AAC	AGA	GGG	TTG	TTT	AM922164	LP	H13	A/black-headed gull/Netherlands/1/00 (H13N8) (AY684886) (95)
	L	R	N	V	P	A	I	S	N	R	G	L	F				

[a] Neuraminidase subtypes have not been determined for all isolates.
[b] Accession numbers are from EMBL, GenBank, and DDBJ.
[c] LP, low pathogenicity; HP, high pathogenicity.
[d] BLASTN search results.

TABLE 4. Determination of pathotype and HA subtype of influenza A viruses from diagnostic samples

Sample[a]	PanHA RT-PCR: Sequence of HA_0 cleavage site	Accession no.[b]	Pathotype[c]	Subtype[d]	Maximum identity (%) with[b,d]:
A/Anas platyrhynchos/Germany/R1649/07 (H1N1)	CTG AGA AAC ATT CCT TCC ATT CAA TCT AGA GGG CTC TTC L R N I P S I Q S R G L F	AM930525	LP	H1	Influenza A virus (A/pintail/Shimane/324/98 (H1N9) (AB274304) (95)
A/Anas platyrhynchos/Germany/R2322/07 (H3N8)	ATG CGG AAT GTA CCA GAG AAA CAA ACC AGA GGC CTA TTC M R N V P E K Q T R G L F	AM930526	LP	H3	Influenza A virus (A/duck/Norway/1/03 (H3N8) (AJ841293) (98)
A/Dendrocygna viduata/Germany/R2484/07 (H3N8)	ATG CGG AAT GTA CCA GAG AAG CAA ACC AGA GGC CTA TTC M R N V P E K Q T R G L F	AM930527	LP	H3	A/mallard/Sweden/S90391/05 (H3N8) (EU057721) (98)
A/Numida meleagris/Germany/R2495/07 (H7N3)	AAG AAT GTT CCC GAA ATC CCA AAG GGA AGA GGC CTA TTT K N V P E I P K G R G L F	AM930528	LP	H7	A/mallard/Sweden/S90735/03 (H7N7) (EU057726) (100)
A/Numida meleagris/Germany/R2497/07 (H7)	AAG AAT GTT CCC GAA ATC CCA AAG GGA AGA GGC CTA TTT K N V P E I P K G R G L F	AM930529	LP	H7	A/mallard/Sweden/S90735/03 (H7N7) (EU057726) (100)
A/Anas domesticus/Germany/R2583/07	TTG AGG AAT GTG CCT GCT GCA TCA GAT AGA GGA TTG TTT L R N V P A A S D R G L F	AM930530	LP	H9	A/duck/Shantou/163/2004 (H9N2) (CY024016) (98)
A/Anas platyrhynchos/Germany/R2619/07	ATG CGG AAT GTA CCA GAG AAG CAG ACC AGA GGC CTA TTC M R N V P E K Q T R G L F	AM930531	LP	H3	A/mallard/Sweden/S90391/05 (H3N8) (EU057721) (97)
A/Anas platyrhynchos/Germany/R2706/07 (H2N9)	TTG AGA AAT GTC CCT CAG ATT GAA TCA AGA GGA CTG TTT L R N V P Q I E S R G L F	AM930532	LP	H2	A/mallard/Sweden/S90568/03 (H2N3) (EU057728) (97)
A/Anas platyrhynchos/Germany/R2709/07 (H2N9)	TTG AGA AAT GTC CCT CAG ATT GAA TCA AGA GGA CTG TTT L R N V P Q I E S R G L F	AM930533	LP	H2	A/mallard/Sweden/S90568/03 (H2N3) (EU057728) (96)
A/Anas platyrhynchos/Germany/R2770/07	CTG AGA AAC ATT CCT TCC ATT CAA TCT AGA GGG CTC TTC L R N I P S I Q S R G L F	AM930534	LP	H1	A/pintail/Shimane/324/98 (H1N9) (AB274304) (95)
A/Anas platyrhynchos/Germany/R2777/07	TTG AGG AAT GTG CCT GCT GCA TCA GAT AGA GGA TTG TTT L R N V P A A S D R G L F	AM930535	LP	H9	A/duck/Shantou/163/2004 (H9N2) (CY024016) (98)

[a] Subtypes have not been determined for all isolates.
[b] Accession numbers are from EMBL, GenBank, and DDBJ.
[c] LP, low pathogenicity; HP, high pathogenicity.
[d] BLASTN search results.

cially for influenza A virus isolates differing from well-characterized strains chosen for primer design as well as for recent isolates. Nevertheless, since influenza viruses show a high mutation frequency, the primer sequences for the PanHA RT-PCR will also have to be adapted continuously to currently circulating strains.

Compared to diagnostic real-time RT-PCR detecting the influenza A virus M gene (9, 15), which showed an analytical sensitivity of about 10 copies/reaction, the PanHA RT-PCR protocol was approximately 100- to 1,000-fold less sensitive. However, AIV RNA extracted from influenza A viruses from allantoic fluid samples as well as those from cloacal swabs at concentrations yielding C_T values of approximately 30 or lower in M gene real-time RT-PCR was successfully amplified by the PanHA RT-PCR. As the real-time RT-PCR used in this study is very sensitive and targets the conserved M gene, this difference was expected and is tolerable due to the different aims of the two methods. In contrast to real-time RT-PCR, which is used for rapid identification of influenza A virus, the PanHA RT-PCR products are directly sequenced for molecular characterization of the HA_0 cleavage site, allowing pathotyping and subtyping.

Sequence data generated for 16 influenza A virus isolates were compared to the sequences published in EMBL, GenBank, and DDBJ, and all were found to have 100% identity. Thus, the new protocol, utilizing primer mixes to provide sequencing primers which have different melting temperatures and nonviral tails at the reverse primers, is reliable. Subsequently, HA_0 cleavage site sequences and pathotypes were determined for 16 different influenza A virus isolates for which no HA sequence data were available from EMBL, GenBank, and DDBJ. Based on the results reported here, the feasibility of the newly developed PanHA RT-PCR, followed by cycle sequencing, for molecular pathotyping and subtyping of influenza A viruses is evident. In contrast to the method widely used with subtype-specific primers (18) or even real-time RT-PCR specific for the HA_0 cleavage site of HPAIV H5N1 of the Qinghai lineage (10) for pathotyping, the protocol is universal and can be applied to all HA subtypes.

In addition, the reported assay was further validated with 17 influenza A viruses from diagnostic samples and the HA_0 cleavage site of influenza A viruses from 11 cloacal swabs was sequenced. Altogether, HA_0 cleavage site sequence data were generated for 32 avian and human influenza A viruses of all 16 subtypes from allantoic fluid samples and 11 influenza A viruses from diagnostic samples of wild and domestic birds. Nevertheless, results demonstrate that a certain amount of influenza A virus genome material is necessary for successful amplification and sequencing of the HA_0 cleavage site. Therefore, the assay described here may be especially appropriate when utilized for diagnostic samples which yield C_T values in M gene real-time RT-PCRs (15) of approximately 30 or lower.

In conclusion, the assay described here is a useful novel tool that is supportive of rapid diagnostics as well as of investigations of the molecular biology of influenza A viruses.

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