Universal Primer Set for Amplification and Sequencing of HA0 Cleavage Sites of All Influenza A Viruses

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Received 9 March 2008/Returned for modification 22 April 2008/Accepted 9 June 2008

Sequence analysis of the endoproteolytic cleavage site within the hemagglutinin (HA) precursor protein HA0 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 HA0 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 HA0 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 HA0 is more rapid and reduces the risk of handling infectious material (2). The presence of multiple basic amino acids (arginine or lysine) at the HA0 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 HA0 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 HA0 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 genotypes, including integration of an IC system (9). The duplex real-time RT-PCR (9).

MATERIALS AND METHODS

Virus 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 × 10⁵ 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 IIIA-M_1for/μl, 15 pmol of primer IIIA-M_1rev/μl, 1.25 pmol of probe

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† Supplemental material for this article may be found at http://jcm.asm.org/.

† Published ahead of print on 18 June 2008.

Published ahead of print on 18 June 2008.
IVA-M-1,FAM-BHQ (Table 1), 2 μl of IJ-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) (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 45 cycles of 30 s at 94°C (denaturation), 45 s at 50°C (annealing), and 45 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 1.5 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 G7.3 and G7.4 were utilized in a concentration of 20 pmol/each of 25 μl reaction volume.

**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 HA0 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 (Ct) 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/W1781-82/03 [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 as well as H7-specific primer pair G7.3 and G7.4) recommended in the EU Diagnostic Manual for Avian Influenza (4). RNA extracted from 12 influenza A virus isolates of the H5 subtype (Ct values between 16 and 22) and 16 influenza A virus isolates of the H7 subtype (Ct 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/ADK/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.** Tenfold 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 μg/ml of carrier RNA [poly(A) homopolymer; Amer sham Biosciences, Piscataway, NJ]) and amplified using the different protocols. The results are shown in Fig. 1. The sensitivity of
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 CT 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 (CT values between 23.49 and 37.57 in M gene-specific real-time RT-PCR) (Table 2). CT values for the IC ranged between 28.50 and 31.61, indicating that no inhibitory effects were present in these samples. No CT 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 CT values for the M gene of less than 30 (except for sample R1642/07) and, in addition, sample R2619/07 (CT value of 33.10) showed positive results. Thus, influenza A virus RNA was detected in diagnostic samples with a CT value of approximately 30 or lower. Repeated testing of selected samples proved the validity of the observed results (data not shown).

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. CT 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.
Determination of HA₀ cleavage site sequence, pathotype, and HA subtype of influenza A viruses from allantoic fluids. In order to determine HA₀ 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 AM922167) exhibited 100% identity with the published sequences. In a second step, HA₀ cleavage site sequence data were also generated for 16 influenza A virus isolates for which no HA sequence data were available. However, in contrast to previously published results of studies using “pan” HA primers (11), six isolates from human hosts (four H1, one H3, and one H5), including H1N1, H3N2, and H5N1 strains, 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-

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 HA₀ 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 HA₀ 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 HA₀ 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 HA₀ 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 2. Sensitivity of PanHA RT-PCR compared to real-time RT-PCR for influenza A virus in diagnostic samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cₚ value (duplex real-time RT-PCR)</th>
<th>PanHA RT-PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2322/07</td>
<td>23.49</td>
<td>+</td>
</tr>
<tr>
<td>R2495/07</td>
<td>23.61</td>
<td>No Cₚ</td>
</tr>
<tr>
<td>R2777/07</td>
<td>26.54</td>
<td>+</td>
</tr>
<tr>
<td>R2709/07</td>
<td>27.13</td>
<td>+</td>
</tr>
<tr>
<td>R2770/07</td>
<td>27.70</td>
<td>+</td>
</tr>
<tr>
<td>R1642/07</td>
<td>27.71</td>
<td>No Cₚ</td>
</tr>
<tr>
<td>R2706/07</td>
<td>27.75</td>
<td>+</td>
</tr>
<tr>
<td>R2583/07</td>
<td>27.85</td>
<td>+</td>
</tr>
<tr>
<td>R2484/07</td>
<td>28.86</td>
<td>+</td>
</tr>
<tr>
<td>R2497/07</td>
<td>29.20</td>
<td>+</td>
</tr>
<tr>
<td>R2479/07</td>
<td>31.99</td>
<td>29.57</td>
</tr>
<tr>
<td>R2619/07</td>
<td>33.10</td>
<td>30.69</td>
</tr>
<tr>
<td>R2723/07</td>
<td>33.56</td>
<td>29.52</td>
</tr>
<tr>
<td>R2710/07</td>
<td>34.34</td>
<td>31.61</td>
</tr>
<tr>
<td>R2776/07</td>
<td>34.39</td>
<td>29.26</td>
</tr>
<tr>
<td>R2769/07</td>
<td>37.57</td>
<td>28.50</td>
</tr>
</tbody>
</table>

* FAM, 6-carboxyfluorescein.

b HEX, hexachloro-6-carboxyfluorescein.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pathotype</th>
<th>Subtype</th>
<th>Sequence of HA0 cleavage site</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Anser egypiticus/Germany/R1419/2006</td>
<td>CTG AGA AAC ATT CCT TCC ATT CAA TCT AGA GGG CTC TTC</td>
<td>(H1N1)</td>
<td>AM922139</td>
<td>LP H1 A/pintail/Shimane/324/98 (95)</td>
</tr>
<tr>
<td>A/bantam/Germany/DZ4/85</td>
<td>CCA AGA AAC GTC CCT CAG ATT GAA TCA AGG GGA TTG TTT</td>
<td>(H2N9)</td>
<td>AM922141</td>
<td>LP H2 A/herring gull/Delaware/670/1988 (98)</td>
</tr>
<tr>
<td>A/Mallard/Germany/Wv1317–21/03</td>
<td>ATA AGG AAT GTC CCT CAG ATT GAA TCA AGA GGA CTG TTT</td>
<td>(H2N3)</td>
<td>AM922142</td>
<td>LP H2 A/mallard/Sweden/S90568/03 (97)</td>
</tr>
<tr>
<td>A/Mallard/Wv677/04</td>
<td>TTA AGA AAT GTC CCT CAG ATT GAA TCA AGA GGA CTG TTT</td>
<td>(H2N3)</td>
<td>AM922143</td>
<td>LP H2 A/mallard/Sweden/S90568/03 (100)</td>
</tr>
<tr>
<td>A/Mallard/Germany/Wv943–45/04</td>
<td>TTA AGA AAT GTC CCT CAG ATT GAA TCA AGA GGA CTG TTT</td>
<td>(H2N3)</td>
<td>AM922144</td>
<td>LP H2 A/mallard/Sweden/S90568/03 (100)</td>
</tr>
<tr>
<td>A/Mallard/Germany/Wv1806–09/03</td>
<td>ATG AGA AAT ATC CCT GAA AAG GCA TCA AGA GGG CTT TTT</td>
<td>(H4N6)</td>
<td>AM922147</td>
<td>LP H4 A/duck/South Africa/1233A/2004 (H4N8) (EF041495) (97)</td>
</tr>
<tr>
<td>A/Mallard/Wv1754–57/03</td>
<td>ATG AGA AAT ATC CCT GAA AAG GCA TCA AGA GGG CTT TTT</td>
<td>(H4N6)</td>
<td>AM922148</td>
<td>LP H4 A/duck/South Africa/1233A/2004 (H4N8) (EF041495) (97)</td>
</tr>
<tr>
<td>A/Anser spec./Germany/R44/2006</td>
<td>CTT AGA AAC ACT CCT TCT ATT GAA CCC AAA GGA TTG TTT</td>
<td>(H8)</td>
<td>AM922158</td>
<td>LP H1 A/swine/Iowa/2/1987 (H1N1) (CY028171) (88)</td>
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<tr>
<td>A/chicken/Brescia/19/02</td>
<td>CCC GAA CTT CCC AAA AAA AGA AGA AAA AGA GGC CTG TTT</td>
<td>(H7N1)</td>
<td>AM922154</td>
<td>HP H7 A/fowl/Dobson/1927 (H7N7) (CY014992) (100)</td>
</tr>
<tr>
<td>A/swan/Potsdam/64/81</td>
<td>AAG AAC GTT CCT GAA ATT CCA AAA GGG AGA GGA CTA TTT</td>
<td>(H7N3)</td>
<td>AM922155</td>
<td>LP H7 A/swan/Potsdam/63/6/81 (U20467) (100)</td>
</tr>
<tr>
<td>A/Mallard/NVP/Wv1677–81/03</td>
<td>AGA AAC GTG CCA GAA ATA ATG CAA GGG AGA GGT CTA TTT</td>
<td>(H10N4)</td>
<td>AM922160</td>
<td>LP H10 A/duck/Shimane/45/1997 (H10N7) (AB296078) (97)</td>
</tr>
<tr>
<td>A/Anas platyrhynchos/Germany/R2219/2006</td>
<td>CTT AGA AAT GTC CCA GCG ATA GCA TCA AGA GGC TTG TTT</td>
<td>(H11)</td>
<td>AM922161</td>
<td>LP H11 A/duck/Siberia/700/1996 (H11N9) (AB292783) (98)</td>
</tr>
<tr>
<td>A/gull/Stralsund/Wv1136–40/03</td>
<td>CTC AGA AAT GTG CCA GCC ATA TCG AAT AGA GGA CTG TTT</td>
<td>(H13N6)</td>
<td>AM922163</td>
<td>LP H13 A/duck/Siberia/272/1998 (AB284988) (96)</td>
</tr>
<tr>
<td>A/Larus ridibundus/Germany/R2064/2006</td>
<td>CTT AGG AAT GTA CCT GCC ATA TCA AAC AGA GGG TTG TTT</td>
<td>(H13)</td>
<td>AM922164</td>
<td>LP H13 A/black-headed gull/Netherlands/1/00 (H13N8) (AY684886) (95)</td>
</tr>
</tbody>
</table>

**Notes:**
- Neuraminidase subtypes have not been determined for all isolates.
- Accession numbers are from EMBL, GenBank, and DDBJ.
- LP, low pathogenicity; HP, high pathogenicity.
- BLASTN search results.
### TABLE 4. Determination of pathotype and HA subtype of influenza A viruses from diagnostic samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequence of HA cleavage site</th>
<th>Accession no.</th>
<th>Pathotype</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Anas platyrhynchos/Germany/R1649/07 (H1N1)</td>
<td>CTG AGA AAC ATT CCT TCC ATT CAA TCT AGA GGG CTC TTC</td>
<td>AM930525</td>
<td>LP</td>
<td>H1</td>
</tr>
<tr>
<td></td>
<td>L R N I P S I Q S R G L F</td>
<td></td>
<td>H1</td>
<td></td>
</tr>
<tr>
<td>A/Anas platyrhynchos/Germany/R2322/07 (H3N8)</td>
<td>ATG CCG AAT GTA CCA GAG AAA CAA ACC AGA GOC CTA TTC</td>
<td>AM930526</td>
<td>LP</td>
<td>H3</td>
</tr>
<tr>
<td></td>
<td>M R N V P E K Q T R G L F</td>
<td></td>
<td>H3</td>
<td></td>
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<tr>
<td>A/Dendrocygna viduata/Germany/R2484/07 (H3N8)</td>
<td>ATG CCG AAT GTA CCA GAG CAA ACC AGA GOC CTA TTC</td>
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<td>LP</td>
<td>H3</td>
</tr>
<tr>
<td></td>
<td>M R N V P E K Q T R G L F</td>
<td></td>
<td>H3</td>
<td></td>
</tr>
<tr>
<td>A/Numida meleagris/Germany/R2495/07 (H7N3)</td>
<td>AAG AAT GTT CCC GAA ATC CCA AAG GGA AGA GOC CTA TTC</td>
<td>AM930528</td>
<td>LP</td>
<td>H7</td>
</tr>
<tr>
<td></td>
<td>K N V P E I P K G R G L F</td>
<td></td>
<td>H7</td>
<td></td>
</tr>
<tr>
<td>A/Numida meleagris/Germany/R2497/07 (H7)</td>
<td>AAG AAT GTT CCC GAA ATC CCA AAG GGA AGA GOC CTA TTC</td>
<td>AM930529</td>
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<td>H7</td>
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<tr>
<td>A/Numida meleagris/Germany/R2583/07</td>
<td>ATG CCG AAT GTA CCA GAG CAA ACC AGA GOC CTA TTC</td>
<td>AM930530</td>
<td>LP</td>
<td>H9</td>
</tr>
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<td></td>
<td>L R N V P A A S D R G L F</td>
<td></td>
<td>H9</td>
<td></td>
</tr>
<tr>
<td>A/Anas platyrhynchos/Germany/R2619/07</td>
<td>ATG CCG AAT GTA CCA GAG CAA ACC AGA GOC CTA TTC</td>
<td>AM930531</td>
<td>LP</td>
<td>H3</td>
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<tr>
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<td>M R N V P E K Q T R G L F</td>
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<td>H3</td>
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<tr>
<td>A/Anas platyrhynchos/Germany/R2706/07 (H2N9)</td>
<td>TTG AGA AAT GTC CCT CAG GAA GCA TCA GGA CTG TTC</td>
<td>AM930532</td>
<td>LP</td>
<td>H2</td>
</tr>
<tr>
<td></td>
<td>L R N V P Q I E S R G L F</td>
<td></td>
<td>H2</td>
<td></td>
</tr>
<tr>
<td>A/Anas platyrhynchos/Germany/R2707/07 (H2N9)</td>
<td>TTG AGA AAT GTC CCT CAG GAA GCA TCA GGA CTG TTC</td>
<td>AM930533</td>
<td>LP</td>
<td>H2</td>
</tr>
<tr>
<td></td>
<td>L R N V P Q I E S R G L F</td>
<td></td>
<td>H2</td>
<td></td>
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<tr>
<td>A/Anas platyrhynchos/Germany/R2777/07</td>
<td>CTG AGA AAC ATT CCT TCC ATT CAA TCT AGA GGG CTC TTC</td>
<td>AM930534</td>
<td>LP</td>
<td>H1</td>
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<tr>
<td></td>
<td>L R N I P S I Q S R G L F</td>
<td></td>
<td>H1</td>
<td></td>
</tr>
<tr>
<td>A/Anas platyrhynchos/Germany/R2777/07</td>
<td>TTG AGA AAC ATT CCT TCC ATT CAA TCT AGA GGG CTC TTC</td>
<td>AM930535</td>
<td>LP</td>
<td>H9</td>
</tr>
<tr>
<td></td>
<td>L R N V P A A S D R G L F</td>
<td></td>
<td>H9</td>
<td></td>
</tr>
</tbody>
</table>

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-charac-
terized strains chosen for primer design as well as for recent
isolates. Nevertheless, since influenza viruses show a high mu-
tation 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 CT 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 differ-
ence 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 char-
acterization of the HA0 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. Subse-
quently, HA0 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 influ-
enza 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 HA0 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 HA0
cleavage site of influenza A viruses from 11 cloacal swabs was
sequenced. Altogether, HA0 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 vi-
ruses from diagnostic samples of wild and domestic birds.
Nevertheless, results demonstrate that a certain amount of influ-
nza A virus genome material is necessary for successful
amplification and sequencing of the HA0 cleavage site.
Therefore, the assay described here may be especially appropriate
when utilized for diagnostic samples which yield CT 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 investiga-
tions of the molecular biology of influenza A viruses.

ACKNOWLEDGMENTS

We thank Anne Juengling and the laboratory team of the Office
International des Epizooties and German National Reference Labo-
ratory for Avian Influenza for excellent technical assistance. We are
grateful to colleagues at the Office International des Epizooties Ref-
ERENCE Laboratories for Avian Influenza in Canada, Italy, and the
United Kingdom for supplying reference virus isolates.

This work was supported by the Federal Ministry of Food, Agricul-
ture and Consumer Protection, BMELV, Germany (FSL, project no.
1.1.), and the European Union, NoE EPIZONE (Theme 4, WP3).

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