JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2007, p. 600–603 Vol. 45, No. 2
0095-1137/07/$08.00+0 doi:10.1128/JCM.01681-06

Rapid and Highly Sensitive Pathotyping of Avian Influenza A H5N1 Virus by Using Real-Time Reverse Transcription-PCR▽

Bernd Hoffmann, Timm Harder, Elke Starick, Klaus Depner, Ortrud Werner, and Martin Beer*

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel Riems, Germany

Received 15 August 2006/Returned for modification 9 October 2006/Accepted 5 December 2006

Rapid typing of the pathogenicity of avian influenza A viruses (AIV) of subtypes H5 and H7 is crucial to initiate adequate protective measures preventing the spread of highly pathogenic AIV (HPAIV). Here, a new real-time reverse transcription-PCR assay which enables sensitive and specific detection and cleavage site analysis of HPAIV H5N1 of the Qinghai lineage is described.

Avian influenza viruses (AIV) characterized by intravenous pathogenicity indices of greater than 1.2 are termed highly pathogenic (1). Only representatives of subtypes H5 and H7 have been shown to exhibit highly pathogenic AIV (HPAIV) characteristics and to cause disastrous epidemic disease in poultry (2). The presence of a polybasic, subtilisin-sensitive endoproteolytic cleavage site (CS) within the hemagglutinin (HA) precursor protein (HA_0) has been identified as a reliable marker for HPAIV (7, 9). AIV strains of low pathogenicity, in contrast, reveal a monobasic composition at this site which is targeted by tissue-specific, trypsin-like proteases (10). Therefore, a molecular pathotyping of AIV isolates is also feasible by determining the sequence encoding this cleavage site by using conventional sequencing techniques.

With the occurrence of HPAIV H5N1 of the Qinghai lineage in wild birds in Germany and other European countries since February 2006, a high risk of transmission to poultry holdings became evident. In order to determine the prevalence of H5N1 in the wild-bird population, rapid analysis, including cleavage site sequence determination, of sample material from hundreds of sometimes decomposed carcasses of wild birds was required. For virus detection in routine diagnostics, real-time reverse transcription-PCR (rRT-PCR) protocols are widely used (3, 4, 5, 8). However, nucleotide sequencing for further analysis of PCR-positive samples is comparatively time- and labor-intensive and is therefore unsuitable for high-throughput demands. Also, a substantial amount of PCR product is required for sequence-based methods, which may be difficult to obtain with many of the wild-bird samples that still yield positive results with the widely used and highly sensitive rRT-PCR test systems. Therefore, an rRT-PCR was developed for the direct, fast, and highly sensitive analysis of the HPAIV H5N1/Qinghai-like HA cleavage site sequence representative of the AIV H5N1 strains currently occurring in Europe.

A set of primers (FliH5_1028F and FliH5_1190R) and two probes were designed for the amplification and detection of a fragment spanning the cleavage site sequence of the H5 HA gene (Table 1). The hexachloro-6-carboxyfluorescein (HEX)-labeled probe (FliH5-1148-HEX) was designed to target to a sequence reasonably conserved among various H5 strains. The 6-carboxyfluorescein (FAM)-labeled probe (FliH5-CS-FAM) was specific for the cleavage site sequence of H5N1 isolates of the Qinghai lineage. Viral RNA was extracted from tracheal or cloacal swabs or allantoic fluid by use of a viral RNA mini kit (QIAGEN). One-step rRT-PCR was accomplished with an ABI 7500 (Applied Biosystems) or an MX3000p (Stratagene) cycler by use of a QuantiTect probe RT-PCR kit (QIAGEN). A total of 5 μl of RNA extract was amplified in a volume of 25

TABLE 1. Primers and probes used in this study

Primer/probe	Sequence of primer/fluorescence labeling probe(s) (5′–3′)[a]	Nucleotide position[b]	Reference or source
EuH5LH1	ACA TAT GAC TAC CCA CAR TAT TCA G	1504–1528	8[c]
EuH5RH1	AGA CCA GCT AYC ATG ATT GC	1655–1636	8[c]
EuH5-FAM	FAM-TCA ACA GTG GCG AGT TCC CTA GCA-BHQ1	1609–1632	8
FliH5-1028F	GGG GAA TGC CCC AAA TAT GT	946–965	This study
FliH5-1190R	TCT ACC ATT CCC TGC CAT CC	1075–1094	This study
FliH5-CS-FAM	FAM-AGA GAG AAG AAG AAA AAA GAG AGG ACT A-TAMRA	1017–1044	This study
FliH5-1148-HEX	HEX-TTG GAG CTA TAG CAG GTT TTA TAG AGG-BHQ1	1046–1072	This study

[a] BHQ1, Black Hole Quencher 1; TAMRA, 6-carboxytetramethylrhodamine.
[b] Based on GenBank accession number DQ458992 (A/mallard/Bavaria/1/2006 [H5N1]).
[c] Primers modified by VLA Weybridge.

* Corresponding author. Mailing address: Friedrich-Loeffler-Institut Insel Riems, Boddenblick 5a, 17493 Greifswald-Insel Riems, D-17493, Germany. Phone: 0049-38351-7-200. Fax: 0049-38351-7-151. E-mail: martin.beer@fli.bund.de.

▽ Published ahead of print on 20 December 2006.

TABLE 2. Sensitivity of the H5 cleavage site assay Fli-CS-FAM compared to that of a standard H5-specific diagnostic assay and that of the included control assay FliH5-HEX

H5N1 dilution series	C_T value											
	EuH5-FAM rRT-PCR[a]				FliH5-CS-FAM rRT-PCR				FliH5-HEX rRT-PCR			
	Replicate no.			Mean ± SD	Replicate no.			Mean ± SD	Replicate no.			Mean ± SD
	1	2	3		1	2	3		1	2	3	
10^{-2}	20.52	20.31	20.08	20.3 ± 0.22	22.99	22.96	22.23	22.7 ± 0.43	21.77	21.47	21.97	21.7 ± 0.25
10^{-3}	23.95	23.81	23.83	23.9 ± 0.08	25.58	24.49	23.92	24.7 ± 0.84	24.77	24.53	24.88	24.7 ± 0.18
10^{-4}	27.17	27.36	27.07	27.2 ± 0.15	29.14	30.22	29.23	29.5 ± 0.60	28.36	29.40	28.69	28.8 ± 0.53
10^{-5}	30.60	30.92	30.76	30.8 ± 0.16	32.79	32.69	32.45	32.6 ± 0.17	31.21	31.76	31.43	31.5 ± 0.28
10^{-6}	34.32	35.17	34.87	34.8 ± 0.43	39.44	36.13	35.39	37.0 ± 2.16	36.63	34.96	34.27	35.3 ± 1.21
10^{-7}	>42	38.00	>42	40.7 ± 2.31	>42	41.78	39.78	41.2 ± 1.22	>42	>42	37.80	40.6 ± 2.42
10^{-8}	>42	>42	>42	42.0 ± 0.00	>42	>42	>42	42.0 ± 0.00	>42	>42	>42	42.0 ± 0.00

[a] Based on the method of Spackman et al. (8), with primers modified by VLA Weybridge.

μl by employing the following temperature profile: 30 min at 50°C, 15 min at 95°C, and 42 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. FAM- and HEX-specific emission data were collected during the annealing step. Cycle threshold (C_T) values of <40 were considered indicative of the presence of H5-specific RNA when all negative controls revealed C_T values of ≥40. Performance characteristics of this duplex assay were compared to those of a generic H5-specific rRT-PCR (8) modified by the Community Reference Laboratory for Avian Influenza (Table 1), referred to as the EuH5-FAM rRT-PCR, by using dilution series of the egg-derived HPAIV H5N1 isolate A/duck/Vietnam/TG24-01/05. When comparing the C_T values as depicted in Table 2, the EuH5-FAM rRT-PCR appeared to be slightly more sensitive, although, on a qualitative basis, no differences among the three assays were evident.

Further in-depth analysis involved a panel of 22 different H5 isolates (Table 3). The EuH5-FAM rRT-PCR detected all 22 isolates. The FliH5-HEX assay detected 18 strains, including all recent HPAIV of Asian origin. In contrast, only HPAI Qinghai-like viruses or very closely related isolates of Asian origin gave a specific signal in the FliH5-CS-FAM assay. The results clearly confirm that detection with the FliH5-CS-FAM assay was directly linked to the nucleotide sequence of the cleavage site (Table 4). Already, the substitution of two nucleotides in the cleavage site region of A/chicken/GXLA/1204/05 completely abolished the signal generation. Similarly, negative results with the FliH5-CS-FAM assay were obtained when one or more triplets were deleted in the probe region. Since all viruses which gave a signal in the FliH5-CS-FAM assay were also positive with the HEX probe, the FliH5-HEX assay can be

TABLE 3. Specificities of the new H5 gene cleavage site assay Fli-CS-FAM for different AIV H5 isolates

Isolate no.	Strain identification[a]	HA/NA subtype	Mean C_T value (±SD)		
			EuH5-FAM	FliH5-CS-FAM	FliH5-HEX
1	A/chicken/Scotland/59	H5N1	28.94 ± 0.03	>42 ± 0.00	>42 ± 0.00
2	A/chicken/Italy/22/98	H5N9	28.57 ± 0.12	>42 ± 0.00	>42 ± 0.00
3	A/teal/Germany/Wv1310-13K/03	H5N2	28.45 ± 0.03	>42 ± 0.00	>42 ± 0.00
4	A/mallard/Germany/Wv474-77K/04	H5N?[b]	26.95 ± 0.07	>42 ± 0.00	>42 ± 0.00
5	A/tern/South Africa/61	H5N3	26.05 ± 0.01	>42 ± 0.00	30.32 ± 0.15
6	A/duck/Potsdam/2216/84	H5N6	29.33 ± 0.15	>42 ± 0.00	33.14 ± 0.23
7	A/chicken/Italy/8/98	H5N2	28.00 ± 0.36	>42 ± 0.00	35.73 ± 0.18
8	A/duck/Vietnam/TG24-O1/05	H5N1	31.57 ± 0.11	>42 ± 0.00	31.16 ± 0.26
9	A/chicken/Vietnam/P41/05	H5N1	29.40 ± 0.16	>42 ± 0.00	28.39 ± 0.06
10	A/chicken/Vietnam/P78/05	H5N1	28.74 ± 0.08	>42 ± 0.00	29.14 ± 0.27
11	A/chicken/Vietnam/P22/05	H5N1	29.17 ± 0.14	>42 ± 0.00	28.14 ± 0.12
12	A/chicken/GXLA/1204/05	H5N1	26.65 ± 0.08	>42 ± 0.00	28.46 ± 0.07
13	A/Hongkong/156/97	H5N1	28.76 ± 0.05	30.56 ± 0.19	29.61 ± 0.08
14	**A/chicken/Indonesia/R132-134/03**	H5N1	27.68 ± 0.08	29.76 ± 0.12	28.65 ± 0.14
15	**A/falco cherugg/Saudi Arabia/R324/05**	H5N1	27.10 ± 0.03	31.07 ± 0.32	29.82 ± 0.27
16	**A/turkey/Turkey/R11/06**	H5N1	29.32 ± 0.08	32.59 ± 0.20	31.42 ± 0.04
17	**A/chicken/Turkey/R12/06**	H5N1	30.78 ± 0.17	33.29 ± 0.27	32.42 ± 0.17
18	**A/x/Romania/2910/06**	H5N1	29.73 ± 0.05	32.36 ± 0.22	31.37 ± 0.16
19	**A/x/Romania/3076/06**	H5N1	29.81 ± 0.08	32.55 ± 0.14	31.55 ± 0.19
20	**A/whooper swan/Germany/R65/06**	H5N1	31.63 ± 0.51	32.55 ± 0.15	31.53 ± 0.11
21	**A/coot/Germany/R822/06**	H5N1	29.01 ± 0.15	31.29 ± 0.19	30.20 ± 0.52
22	**A/cat/Germany/R606/06**	H5N1	30.96 ± 0.17	32.53 ± 0.13	31.61 ± 0.09

[a] HPAIV H5N1 of the Qinghai lineage are indicated by bold type. x, unknown species.
[b] ?, unknown NA subtype.

TABLE 4. Determination of the nucleotide and amino acid cleavage site sequences of different AIV H5 strains tested

Strain no.	H5 cleavage site sequence (5′–3′) (nucleotides)[a,b]	H5 cleavage site sequence (amino acids)[a]	Pathotype[b]	Origin[c]
1	CAA AGG----AAG AAA AGA*GGT CTA TTT	QR----KKR*GLF	HP	This study
2	CAA AAG----GAG ACA AGA*GGA CTA TTT	QK----ETR*GLF	LP	This study
3	CAG AGA----GAA ACA AGA*GGA CTA TTT	QR----ETR*GLF	LP	This study
4	CAA AAA----GAA ACA AGA*GGA CTA TTT	QK----ETR*GLF	LP	This study
5	AGG GAG ACG CGC AGG CAG AAA AGA*GGT CTA TTT	RETRRQKR*GLF	HP	This study
6	CAA AGA----GAG ACA AGA*GGT CTA TTT	QR----ETR*GLF	LP	This study
7	CAA AGA--AGA AGA AAG AAA AGA*GGA CTA TTT	QR--RRKKR*GLF	HP	This study
8	AGA GAG AGA-AGG AAA AAG AGA*GGA TTA TTT	RE-RRKKR*GLF	HP	AM183677
9	AGA GAG AGA-AGA AAA AAG AGA*GGA TTA TTT	RE-RRKKR*GLF	HP	AM183672
10	AGA GAG AGA-AGA AGA AAG AGA*GGA TTA TTT	RE-RRRKR*GLF	HP	AM183673
11	AGA GAG AGA-AGA AAA AAG AGA*GGA TTA TTT	RE-RRKKR*GLF	HP	AM183674
12	AGA GAA AGA AGA AAA AAA AAG AGA*GGA CTA TTT	RERRKKKR*GLF	HP	AM183671
13	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	AF028709
14	AGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	RERRRKKR*GLF	HP	AM183669
15	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
16	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
17	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
18	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
19	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
20	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
21	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study
22	GGA GAG AGA AGA AGA AAA AAG AGA*GGA CTA TTT	GERRRKKR*GLF	HP	This study

[a] *, actual site of cleavage of the HA_0 precursor.
[b] LP, low pathogenicity; HP, highly pathogenic.
[c] Accession numbers are from the GenBank database.

used as an internal control assay which ensures the successful amplification of H5-specific viral RNA. In addition, isolates of most of the other HA subtypes (H1 to H4, H6 to H13, and H16) were investigated with both FliH5 assays, and no cross-reactivity with non-H5 subtypes was observed (data not shown). Therefore, simultaneously positive FliH5-CS-FAM and FliH5-HEX assays clearly confirm the presence of HPAIV of an H5 Qinghai-like virus in the sample. A sample yielding a positive signal only in the EuH5-FAM rRT-PCR and/or the FliH5-HEX rRT-PCR requires conventional sequencing of the cleavage site for characterization and exact pathotyping.

The reported assay was further validated with field samples of the recent H5N1 outbreaks in Germany. Analysis of 100 AIV H5-negative samples of cloacal and tracheal swabs could confirm the results obtained using the newly developed cleavage site rRT-PCR. In addition, more than 70 samples of wild-bird carcasses which tested positive for HPAIV H5N1 by rRT-PCR and conventional sequencing were investigated using the novel cleavage site rRT-PCR FliH5-CS-FAM. In all cases, the sequencing results could be rapidly confirmed.

Based on the data described here, we propose a cascade style of molecular diagnostic measures for the monitoring of wild birds for HPAIV H5 of Qinghai parentage currently circulating in large parts of Asia and Europe. In the first step, the presence of influenza A viral sequences is ascertained by a generic, e.g., M-gene-specific, rRT-PCR enhanced by an internal control. If positive, rRT-PCR assays targeting H5- and H7-specific sequences should be performed. If also positive, the cleavage site should be amplified by conventional PCR for nucleotide sequencing. In the case of H5-specific sequences, the FliH5 rRT-PCR assays are a versatile, rapid, and highly sensitive alternative for the detection of Qinghai-like viruses confirming the presence of an HPAIV. Even samples yielding weakly positive signals in the EuH5 assay can often be pathotyped provided they harbor viruses of the Qinghai lineage.

In conclusion, the presented cleavage site-specific rRT-PCR using TaqMan probes is particularly useful for rapid pathotyping of HPAIV H5N1 strains of the Qinghai lineage and is also more suitable than a recently reported universal rRT-PCR system for discriminating between highly pathogenic H5 influenza viruses and those of low pathogenicity by use of SYBR green binding and melting point analysis (6). Finally, the system presented here was successfully operated during the 2006 German H5N1 outbreak in wild birds, and it is part of routine diagnostics of the German OIE and National Reference Laboratory for Avian Influenza.

We thank Ulrike Polenz, Karin Lissek, and the lab team of the OIE and German National Reference Laboratory for Avian Influenza for excellent technical assistance.

REFERENCES

1. **Anonymous.** Terrestrial Animal Health Code OIE. Chapter 2.7.12. Avian influenza. http://www.oie.int/eng/normes/mcode/en_chapitre_2.7.12.htm.
2. **Capua, I., and F. Mutinelli.** 2001. Low pathogenicity (LPAI) and highly pathogenic (HPAI) avian influenza in turkeys and chicken, p. 13–20. *In* I. Capua and F. Mutinelli (ed.), A colour atlas and text on avian influenza. Papi Editore, Bologna, Italy.
3. **Lee, C. W., and D. L. Suarez.** 2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J. Virol. Methods **119:**151–158.
4. **Ng, E. K., P. K. Cheng, A. Y. Ng, T. L. Hoang, and W. W. Lim.** 2005. Influenza A H5N1 detection. Emerg. Infect. Dis. **11:**1303–1305.
5. **Payungporn, S., S. Chutinimitkul, A. Chaisingh, S. Damrongwantanapokin, C. Buranathai, A. Amonsin, A. Theamboonlers, and Y. Poovorawan.** 2006. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J. Virol. Methods **131:**143–147.
6. **Payungporn, S., S. Chutinimitkul, A. Chaisingh, S. Damrongwantanapokin,**

B. Nuansrichay, A. Amonsin, R. O. Donis, A. Theamboonlers, and Y. Poovorawan. 2006. Discrimination between highly pathogenic and low pathogenic H5 avian influenza A viruses. Emerg. Infect. Dis. **12:**700–701.
7. **Perdue, M. L., and D. L. Suarez.** 2000. Structural features of the avian influenza virus hemagglutinin that influence virulence. Vet. Microbiol. **74:** 77–86.
8. **Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. **40:**3256–3260.
9. **Steinhauer, D. A.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1–20.
10. **Wood, G. W., J. W. McCauley, J. B. Bashiruddin, and D. J. Alexander.** 1993. Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Arch. Virol. **130:**209–217.