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Rapid and Highly Sensitive Neuraminidase Subtyping of Avian Influenza Viruses by Use of a Diagnostic DNA Microarray▽†

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Rapid neuraminidase subtyping of avian influenza viruses from diagnostic samples is crucial considering the existence of permanently emerging and evolving strains. Here we report an easy-to-use, low-cost microarray for neuraminidase subtyping following fragment amplification by a generic, neuraminidase-specific reverse transcription-PCR (RT-PCR). This method enables highly specific characterization with a sensitivity equal to that of matrix gene-specific real-time RT-PCR.

Avian influenza viruses (AIV) are classified into 16 hemagglutinin (HA; H1 to H16) and 9 neuraminidase (NA; N1 to N9) subtypes on the basis of antigenic and/or genetic differences of these surface glycoproteins (2, 3, 5). Acknowledging the potential threat of the AIV pool for carrying prepandemic human-pathogenic viruses, exemplified by transspecies transmissions of highly pathogenic AIV (HPAIV) directly from infected poultry to humans, surveillance studies of influenza A virus in wild birds and poultry have been intensified recently (12, 13). Real-time reverse transcription-PCR (rRT-PCR) and other molecular diagnostic techniques are used with preference in such programs because of their superior sensitivity, suitability for rapid and high-throughput analysis, and reduced risk of the operator handling infectious material. However, molecular subtyping of AIV from an rRT-PCR-positive sample, which is required to further understand the epidemiology of these viruses, can be challenging if commonly used conventional RT-PCRs followed by sequencing do not achieve the same sensitivity as the screening method (1, 4, 10, 14).

We recently described a highly sensitive microarray assay for HA sub- and pathotyping (7, 8) which is based on an RT-PCR amplifying an HA gene fragment encoding the HA_0 cleavage site of all influenza A viruses (6). The ArrayTube system (Clondiag, Jena, Germany) utilized is a small and inexpensive platform with spot-on low-density microarrays integrated in reaction tubes and with signal amplification by enzyme-catalyzed local TMB precipitation staining. Hybridization and analysis are fast and are easily conducted with standard laboratory equipment complemented by a simple transmission reader. For higher throughput, the system can be operated in a completely automated 96-well microplate format (ArrayStrip and ArrayMate reading device).

Here we present a new, ready-to-use ArrayTube assay for NA subtyping which is based on an RT-PCR amplifying a fragment of the NA genes of all influenza A viruses. In an extensive validation study, the assay was proven to exhibit high specificity and sensitivity.

ArrayTubes with 106 oligonucleotide probes specific for the NA gene were fabricated at Clondiag (Jena, Germany) as described previously (11). Sequence characteristics and print patterns can be found in Table S1 in the supplemental material. NA subtype-specific probes and hybridization controls were spotted in triplicate. The array also contained positive

TABLE 1. Sensitivity of the new ArrayTube assay for AIV of subtypes N1, N2, N3, and N7

Virus and dilution	M gene-specific rRT-PCR result[a]	RT-PCR result[b]	ArrayTube assay result[c]
A/whooper swan/Germany/R65/06 (H5N1) HPAIV			
10^{-5}	30.57	+	ND
10^{-6}	33.99	+/−	+
10^{-7}	36.63	−	+
10^{-8}	No C_T	−	−
10^{-9}	No C_T	−	−
A/turkey/Germany/22/96 (H9N2)			
10^{-5}	31.69	+	ND
10^{-6}	35.18	+/−	+
10^{-7}	38.40	−	+
10^{-8}	40.98	−	+
10^{-9}	No C_T	−	−
A/mallard/Germany/Wv677/04 (H2N3)			
10^{-5}	33.21	+	ND
10^{-6}	36.38	+/−	+
10^{-7}	39.20	−	+
10^{-8}	40.73	−	−
10^{-9}	No C_T	−	−
A/chicken/Germany/R28/03 (H7N7) HPAIV			
10^{-5}	31.00	+ +	ND
10^{-6}	34.24	+	+
10^{-7}	38.08	−	+
10^{-8}	40.80	−	+
10^{-9}	No C_T	−	−

[a] C_T (dR) value for M gene-specific rRT-PCR (15).
[b] ++ and +, different intensities of the bands by agarose gel electrophoresis; +/−, weak band; −, no band visible.
[c] ND, not determined.

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TABLE 2. Characterization of influenza A viruses from diagnostic samples

Sample no.	Virus[a]	M gene-specific rRT-PCR result[b]	RT-PCR result[c]	ArrayTube assay result
1	A/duck/Germany/R4-Lippe/08 (H1N1)	26.64	++	N1
2	A/Anas platyrhynchos/Germany/R91/08 (H3N8)	31.32	+	N8
3	A/Branta canadanensis/Germany/R107/08 (H11N9)	34.06	+	N9
4	A/Cygnus olor/Germany/R108/08 (H4Nx)	36.95	+	N6
5	A/Anser albifrons/Germany/R165/08	32.38	+	N2
6	A/Anser albifrons/Germany/R167/08	29.08	++	N8
7	A/Anser albifrons/Germany/R168/08	31.58	+	N2
8	A/Anser albifrons/Germany/R169/08	32.74	+	N2
9	A/Anas platyrhynchos/R234/08 (H1N1)	29.91	++	N1
10	A/Anser anser/Germany/R239/08 (H9N2)	33.96	+	N2
11	A/Anser anser/Germany/R240/08 (H9N2)	32.34	+	N2
12	A/Anser anser/Germany/R248/08 (H9N2)	22.33	++	N2
13	A/Struthio camelus/South Africa/R296/2/08 (H5N2) HPAIV	26.35	+/−	N2
14	A/Struthio camelus/South Africa/R296/4/08 (H5N2) HPAIV	18.98	++	N2
15	A/Struthio camelus/South Africa/R296/5/08 (H5N2) HPAIV	25.86	++	N2
16	A/Struthio camelus/South Africa/R296/7/08 (H5N2) HPAIV	30.50	−	N2
17	A/Callonetta leucophrys/Germany/R641/2/08 (H6N2)	23.40	++	N2
18	A/Anas platyrhynchos/Germany/R665/2/08 (H5Nx)	36.73	−	N3
19	A/Anas platyrhynchos/Germany/R695/08 (H5Nx)	28.47	+	N2
20	A/Anas platyrhynchos/Germany/R701/08 (H5Nx)	34.42	−	N2
21	A/Anas platyrhynchos/Germany/R726/08 (H5Nx)	29.37	+	N3
22	A/Anas platyrhynchos/Germany/R734/1/08 (H5N3)	24.23	++	N3
23	A/domestic duck/Germany/R786/08 (H5N1) HPAIV	24.84	++	N1
24	A/domestic duck/Germany/R821/08 (H5N1) HPAIV	36.91	−	N1
25	A/domestic duck/Germany/R836/08 (H5N1) HPAIV	36.68	−	N1
26	A/domestic goose/Germany/R1110/08 (H6N2)	30.95	++	N2
27	A/domestic goose/Germany/R1125/08 (H6N2)	30.49	++	N2
28	A/mallard/Germany/R1140/2008 (H6N2)	16.01	+++	N2
29	A/mallard/Germany/R1142/2008 (H6N2)	14.54	+++	N2
30	A/Anser anser/Germany/R1416/08 (H2N9)	24.31	+++	N9
31	A/Anas platyrhynchos/Germany/R1419/08	30.67	++	N9
32	A/Anas platyrhynchos/Germany/R1423/08 (H5Nx)	33.74	+	N3
33	A/Anas platyrhynchos/Germany/R1430/08 (H10Nx)	29.02	+++	N9
34	A/Anas platyrhynchos/Germany/R1433/08	33.08	+	N9
35	A/Anser anser/Germany/R1467/08 (H2N9)	25.24	+++	N9
36	A/Anas platyrhynchos/Germany/R1516/08 (H9N2)	36.55	−	N2
37	A/Anas platyrhynchos/Germany/R1521/08 (H9N2)	33.19	+	N2
38	A/Anas platyrhynchos/Germany/R1522/08 (H9N2)	30.11	+	N2
39	A/Anas platyrhynchos/Germany/R1523/08 (H11N9)	28.57	++	N9
40	A/Anser aegyptiacus/Germany/R1524/08	32.84	++	N9
41	A/Anas platyrhynchos/Germany/R1526/08 (H2N3)	33.97	+	N3
42	A/wild bird/Germany/R1527/08 (H6N2)	32.47	+	N2
43	A/Cygnus olor/Germany/R1541/08 (H7N3)	34.55	+/−	N3
44	A/Anas platyrhynchos/Germany/R1594/08 (H11N1)	28.22	++	N1
45	A/Anas platyrhynchos/Germany/R1597/08 (H11N1)	28.01	++	N1
46	A/Anas platyrhynchos/Germany/R1599/08 (H9N2)	28.65	+++	N8, N2
47	A/Anas platyrhynchos/Germany/R1601/08 (H5N2)	32.91	+	N2
48	A/Anas platyrhynchos/Germany/R1603/08	30.89	++	N9
49	A/Anas platyrhynchos/Germany/R1604/08	27.67	++	N9
50	A/Anas platyrhynchos/Germany/R1609/08	29.11	++	N2
51	A/Anser anser/Germany/R1625/08 (H6N2)	29.73	++	N2
52	A/Anser anser/Germany/R1628/08 (H6N2)	30.35	++	N2
53	A/Meleagris gallopavo/Germany/R1649-53/08 (H5N3)	30.09	+++	N3
54	A/domestic duck/Germany/R1769/08 (H6N2)	33.60	−	N2
55	A/domestic duck/Germany/R1774/08 (H6N2)	29.06	+/−	N2
56	A/domestic duck/Germany/R1779/08 (H6N2)	32.92	+/−	N2
57	A/Meleagris gallopavo/Germany/R1914-6/08 (H5N3)	25.84	+++	N3
58	A/Meleagris gallopavo/Germany/R2040/08	38.02	−	—[d]
59	A/Meleagris gallopavo/Germany/R2045/08 (H5N3)	32.56	+	N3
60	A/Anas platyrhynchos/Germany/R2316/08	32.20	+	N1
61	A/Anas platyrhynchos/Germany/R2319/08	31.70	+	N1
62	A/Cygnus cygnus/Germany/R2324/08 (H6N5)	34.22	+	N5
63	A/Cygnus cygnus/Germany/R2325/08 (H6N5)	33.74	+	N5
64	A/duck/Germany/R2331/0 8 (H5N3)	27.86	++	N3
65	A/duck/Germany/R2342/0 8 (H5N3)	31.15	++	N3
66	A/Meleagris gallopavo/Germany/R2379/08 (H5N3)	26.88	++	N3

Continued on following page

TABLE 2—*Continued*

Sample no.	Virus[a]	M gene-specific rRT-PCR result[b]	RT-PCR result[c]	ArrayTube assay result
67	A/Anser albifrons/R2/09 (H6N8)	27.10	++	N8
68	A/Anser albifrons/R3/09 (H6N8)	31.99	+	N8
69	A/Meleagris gallopavo/Germany/R23/09 (H3N2)	34.14	−	N2
70	A/Meleagris gallopavo/Germany/R24/09 (H3N2)	35.48	−	N2
71	A/Larus canus/Germany/R127/09	27.08	−	N6
72	A/Larus argentatus/Germany/R128/09	29.24	−	N6
73	A/Larus argentatus/Germany/R129/09	29.42	+/−	N6
74	A/Anas platyrhynchos/Germany/R131/09	21.21	++	N2
75	A/Anas platyrhynchos/Germany/R132/09	36.67	−	N2
76	A/Anas platyrhynchos/Germany/R133/09	32.28	+/−	N2

[a] Subtyping was not successful for all samples. NA subtypes were determined by subtype-specific RT-PCRs followed by sequencing (4) and/or by sequencing of the entire NA gene (10).
[b] C_T (dR) value for M gene-specific rRT-PCR (15).
[c] +++ to +, different intensities of bands by agarose gel electrophoresis; +/−, weak band; −, no band visible.
[d] For sample no. 58, no ArrayTube assay result could be achieved.

controls for the staining procedure (3′-C-7-amino- and 5′-biotin-modified oligonucleotides) and negative controls (spotting buffer). Influenza A virus isolates (allantoic fluids) as well as field samples (cloacal swabs from wild and domestic birds) were obtained from the repository of the Office International des Epizooties (OIE) and German National Reference Laboratory for Avian Influenza. Viral RNAs were purified with a QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Specificity tests were conducted with RNAs or DNAs from Newcastle disease virus strain La Sota, reovirus strain 1133 (Intervet), infectious bursitis disease virus D78, infectious laryngotracheitis virus A489 (Intervet), *Mycoplasma gallinarum*, and *Mycoplasma gallinaceum*. A universal primer set for amplification of an ~620-bp fragment of the NA genes of all influenza A viruses was designed. It consisted of 5′-biotinylated (Bio) primers NA886.1Fbio (5′-Bio-ATC GAR GAR TGY TCN TGY TA-3′), NA886.3Fbio (5′-Bio-GTC GAR GAR TGY TCH TGB TA-3′; positions 846 to 865 [GenBank accession no. NC_007361]), and AIV(647)Rbio (5′-Bio-*GCA GTA TAT CGC TTG ACA* AGT AGA AAC AAG G-3′; positions 1458 to 1446 [GenBank accession no. NC_007361] [nonviral universal tail is shown in italics]). Target RNA was reverse transcribed, amplified, and biotin labeled by a one-step "pan"-NA RT-PCR with the SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The reaction was carried out analogously to a previously published "pan"-HA RT-PCR (6), but using 2 μl NA-specific primer mix panNA-Mix 4.1-bio [10 pmol/μl (each) of primers NA886.1Fbio, NA886.3Fbio, and AIV(647)Rbio]. Amplicons were verified by agarose gel electrophoresis and submitted to microarray analysis without further purification. Microarray hybridization and data analysis were performed as described by Gall et al. (8). An rRT-PCR specific for the matrix (M) gene (15) was performed for comparison purposes. NA subtypes of diagnostic samples were determined by subtype-specific RT-PCRs followed by partial sequencing of the NA gene (4) and/or sequence analysis of the entire NA gene (10).

For validation of the ArrayTube assay, we utilized a panel of 94 influenza A virus reference strains of all NA subtypes (see Table S2 in the supplemental material)—mainly from poultry and wild birds—originating from Eurasia, North America, and Australia. A recent swine-origin influenza virus (H1N1) from a patient in Germany was also investigated as a representative of the ongoing pandemic. NA subtypes were accurately determined by the microarray assay for all strains. In contrast, Newcastle disease virus strain La Sota, reovirus strain 1133, infectious bursitis disease virus D78, infectious laryngotracheitis virus A489, *Mycoplasma gallinarum*, and *Mycoplasma gallinaceum* were not detected (not shown).

The sensitivity of the new assay was compared to that of a standard rRT-PCR specific for the M gene (15) by using 10-fold serial dilutions of RNAs from reference strains of the four most relevant NA subtypes in RNA-safe buffer (9) {0.05% Tween 20, 0.05% sodium azide, 50 ng/μl of carrier RNA [poly(A) homopolymer; Amersham Biosciences, Piscataway, NJ]} as templates for both assays. Comparable sensitivities were observed for the selected strains of the N1, N2, and N7 subtypes (Table 1), while for A/Mallard/Germany/Wv677/04 (H2N3) the ArrayTube assay was found to be 10-fold less sensitive than the rRT-PCR.

The reported microarray assay was further validated with 76 diagnostic samples, comprising cloacal swabs from poultry and wild birds (threshold cycle [C_T] values for the M gene ranged from 14.54 to 38.02 for these samples), including material from an outbreak of HPAIV H5N1 clade 2.2 in Germany in 2008 (Table 2). A total of 74 samples (97.4%) was successfully analyzed, and NA subtypes determined by the ArrayTube assay perfectly corresponded to results obtained by subtype-specific RT-PCRs and/or sequencing (4, 10). Among the nine known NA subtypes, only N4 and N7 were not represented in the panel of samples. In one sample (no. 46), the N8 and N2 subtypes were detected simultaneously, and direct PCR sequencing was unsuccessful due to two superimposed sequences; an H9N2 virus was isolated from this sample. The microarray analysis of the sample with the lowest viral RNA load (no. 58) remained negative.

The validation data presented here demonstrate the feasibility of a novel ArrayTube assay for highly specific NA subtyping of AIV with a sensitivity comparable to that of rRT-PCR specific for the M gene (15). Within 24 h, the NA subtypes could be ascertained for all virus isolates and the majority of field samples included in the study. The assay clearly surpasses the potential of conventional molecular tools

for NA subtyping (1, 4, 10) with regard to sensitivity. Nevertheless, sequencing of NA genes remains an important tool for studies of the phylogeny of AIV.

In conclusion, the established microarray assay addresses the requirements of diagnostic laboratories for a rapid, easy-to-use, and affordable system enabling NA subtyping of influenza A virus-positive samples within surveillance programs of both wild bird and poultry populations. The ArrayTube assay described here is now integrated into the routine diagnostics of the OIE and German National Reference Laboratory for Avian Influenza. It was merged with a previously described assay for HA subtyping and pathotyping (8) to form a single microarray integrated in eight-well strips (ArrayStrip), which is currently under validation and allows processing of large sample numbers for complete HA and NA subtyping.

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REFERENCES

1. **Alvarez, A. C., M. E. G. Brunck, V. Boyd, R. Lai, E. Virtue, W. Chen, C. Bletchly, H. G. Heine, and R. Barnard.** 2008. A broad spectrum, one-step reverse-transcription PCR amplification of the neuraminidase gene from multiple subtypes of influenza A virus. Virol. J. **5:**77. doi:10.1186/1743-422X-5-77.
2. **Anonymous.** 2005. Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.7.12. Avian influenza. OIE, Paris, France. http://www.oie.int/eng/normes/mmanual/A_00037.htm.
3. **Anonymous.** 2007. Terrestrial animal health code. Chapter 2.7.12. Avian influenza. OIE, Paris, France. http://www.oie.int/eng/normes/mcode/en_chapitre_2.7.12.htm.
4. **Fereidouni, S. R., E. Starick, C. Grund, A. Globig, T. C. Mettenleiter, M. Beer, and T. Harder.** 2009. Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. Vet. Microbiol. **135:**253–260.
5. **Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus.** 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. **79:**2814–2822.
6. **Gall, A., B. Hoffmann, T. Harder, C. Grund, and M. Beer.** 2008. Universal primer set for amplification and sequencing of HA_0 cleavage sites of all influenza A viruses. J. Clin. Microbiol. **46:**2561–2567.
7. **Gall, A., B. Hoffmann, T. Harder, C. Grund, D. Höper, and M. Beer.** 2009. Design and validation of a microarray for detection, hemagglutinin subtyping, and pathotyping of avian influenza viruses. J. Clin. Microbiol. **47:**327–334.
8. **Gall, A., B. Hoffmann, T. Harder, C. Grund, R. Ehricht, and M. Beer.** 2009. Rapid haemagglutinin subtyping and pathotyping of avian influenza viruses by a DNA microarray. J. Virol. Methods **160:**200–205.
9. **Hoffmann, B., K. Depner, H. Schirrmeier, and M. Beer.** 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J. Virol. Methods **136:**200–209.
10. **Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. **146:**2275–2289.
11. **Monecke, S., and R. Ehricht.** 2005. Rapid genotyping of methicillin-resistant Staphylococcus aureus (MRSA) isolates using miniaturised oligonucleotide arrays. Clin. Microbiol. Infect. **11:**825–833.
12. **Munster, V. J., C. Baas, P. Lexmond, T. M. Bestebroer, J. Guldemeester, W. E. P. Beyer, E. de Wit, M. Schutten, G. F. Rimmelzwaan, A. D. M. E. Osterhaus, and R. A. Fouchier.** 2009. Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. J. Clin. Microbiol. **47:**666–673.
13. **Parmley, E. J., N. Bastien, T. F. Booth, V. Bowes, P. A. Buck, A. Breault, D. Caswell, P.-Y. Daoust, J. C. Davies, S. M. Elahi, M. Fortin, F. Kibenge, R. King, Y. Li, N. North, D. Ojkic, J. Pasick, S. P. Pryor, J. Robinson, J. Rodrigue, H. Whitney, P. Zimmer, and F. A. Leighton.** 2008. Wild bird influenza survey, Canada, 2005. Emerg. Infect. Dis. **14:**84–87.
14. **Phipps, L. P., S. C. Essen, and I. H. Brown.** 2004. Genetic subtyping of influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. J. Virol. Methods **122:**119–122.
15. **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.