



Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet



Short communication

Rapid haemagglutinin subtyping and pathotyping of avian influenza viruses by a DNA microarray

Astrid Gall^a, Bernd Hoffmann^a, Timm Harder^a, Christian Grund^a, Ralf Ehrlich^b, Martin Beer^{a,*}

^a Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald - Insel Riems, Germany

^b CLONDIAG GmbH, Löbstedter Straße 103 - 105, 07749 Jena, Germany

A B S T R A C T

Article history:

Received 16 February 2009
Received in revised form 3 May 2009
Accepted 7 May 2009
Available online xxx

Keywords:

Avian influenza virus
Microarray
Haemagglutinin subtype
Pathotype
HA₀ cleavage site

Rapid and reliable methods are fundamental for the comprehensive characterization of emerging and evolving avian influenza viruses. Although microarrays provide new possibilities with their parallel approach, their use in diagnostic laboratories is still limited due to economical and practical factors. An easy-to-use, low-cost microarray-based assay for haemagglutinin subtyping and pathotyping of avian influenza viruses and specific detection of highly pathogenic H5N1/Asia clade 2.2 is described as a novel diagnostic tool. The ArrayTube™ platform is user-friendly, inexpensive and allows processing of many samples. The sensitivity of the assay developed was comparable to real-time RT-PCR, and the simultaneous detection of different subtypes was possible. Validation with 90 influenza A virus isolates representing all 16 haemagglutinin subtypes and 44 field samples (cloacal swabs from wild and domestic birds) demonstrated the feasibility of the system for sensitive and specific characterization of AIV. Facilitating haemagglutinin subtyping and pathotyping for the majority of influenza A-positive cloacal swabs within 24 h, the new assay enables detailed AIV diagnosis even in less well-equipped laboratories.

© 2009 Elsevier B.V. All rights reserved.

Avian influenza viruses (AIV) are classified into 16 haemagglutinin (HA; H1–H16) and 9 neuraminidase (NA; N1–N9) subtypes based on antigenic and/or genetic differences of these surface glycoproteins (Anon., 2005, 2007; Fouchier et al., 2005). Furthermore, highly pathogenic avian influenza viruses (HPAIV) and AIV of low pathogenicity (LPAIV) are differentiated biologically by the determination of intravenous pathogenicity indices, or on the molecular basis by sequence analysis of the cleavage site within the haemagglutinin precursor protein HA₀ (Anon., 2005, 2006, 2007; Wood et al., 1993).

Therefore, the need for rapid and reliable diagnostic assays with a high information content is growing constantly. New molecular methods have the advantage of accelerating diagnosis and reducing the risk of handling infectious material (Fereidouni et al., 2008, 2009; Höper et al., 2009; Hoffmann et al., 2007; Spackman et al., 2002). Nevertheless, especially within surveillance programmes or during outbreaks of highly pathogenic avian influenza, inherent limitations of conventional molecular tools become evident (Globig et al., 2009). Microarrays are promising alternatives which surpass the potential of (real-time) RT-PCR and sequencing as they can detect hundreds or thousands of genes in parallel. Recently, various microarrays have been described for detection, differential diagnosis and subtyping of influenza A viruses (Dawson et al., 2006; Gall

et al., 2008b; Han et al., 2008; Kessler et al., 2004; Li et al., 2007; Lin et al., 2007; Liu et al., 2006; Lodes et al., 2006; Quan et al., 2007; Sengupta et al., 2003; Townsend et al., 2006; Wang et al., 2008). To date, high costs and insufficient sensitivity of the microarray platforms and technically demanding and time consuming protocols have been limiting factors for widespread use for routine diagnostic investigations.

The ArrayTube™ (AT) system (Clondia, Jena, Germany) represents a small and comparatively cost-effective platform using spot-on low density microarrays integrated in reaction tubes and signal amplification by enzyme-catalyzed local precipitation staining. Hybridization and analysis are conducted fast and easily using standard laboratory equipment complemented by a simple transmission reader. No extensive special expertise or training is required. The possibility of operating the system in a completely automated 96 well microplate-format (ArrayStrip™ system) is available for high-throughput laboratories.

This report describes a ready-to-use ArrayTube™ assay targeting the HA₀ cleavage region of avian influenza viruses. It permits, within a single approach, haemagglutinin subtyping, a general pathotyping and the specific detection of HPAIV H5N1/Asia clade 2.2, which occurs outside of Southeast Asia in Europe and Africa since 2006 (Starick et al., 2008; Vijaykrishna et al., 2008).

ArrayTubes™ with 99 Influenza A-specific oligonucleotide probes which have been validated previously (Gall et al., 2008b) were fabricated at Clondia (Jena, Germany) as described in Monecke and Ehrlich (2005). Sequence characteristics and print

* Corresponding author. Tel.: +49 38351 7200; fax: +49 38351 7151.
E-mail address: martin.beer@fli.bund.de (M. Beer).

pattern can be found in [supplementary file 1](#). Influenza A-specific probes and hybridization controls were spotted in triplicate. The array also contains positive controls for the staining procedure (3'-C7 Amino- and 5'-Biotin-modified oligonucleotides) and negative controls (spotting buffer).

Influenza A virus isolates (allantoic fluids) and field samples (cloacal swabs from wild and domestic birds) were obtained from the repository of the German National Reference Laboratory for Avian Influenza. Viral RNA was purified with the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. Target RNA was reverse transcribed, amplified and biotin-labelled by a one-step pan haemagglutinin RT-PCR as described in [Gall et al. \(2008a\)](#) modified by use of 5'-biotinylated primers. *In vitro*-transcribed RNA from a synthetic gene (GeneArt, Regensburg, Germany) was applied as a labelled positive control (LPC) resulting in an amplicon of 255 bp. RT-PCR products were verified by agarose gel electrophoresis and submitted to microarray analysis without further purification. The ArrayTube™ vessel was conditioned by washing twice with 500 µl hybridization buffer (0.25 M Na₂HPO₄, 1 mM EDTA, 4.5% SDS in 1× SSC, pH 7.25) at 30 °C for 5 min. All incubations were carried out on a horizontal tube thermoshaker at 550 rpm (Thermomixer comfort; Eppendorf, Ham-

burg, Germany). For denaturation, 0.1–5 µl biotinylated product (depending on the DNA concentration) were adjusted to a final volume of 100 µl with hybridization buffer in a separate tube, heated at 95 °C for 5 min and chilled on ice subsequently for 1 min. After transfer into the ArrayTube™, hybridization was allowed to proceed at 50 °C for 60 min. The supernatant was discarded, and the ArrayTube™ was washed consecutively with 500 µl 2× SSC/0.01% Triton X-100 (30 °C, 5 min), 500 µl 2× SSC (30 °C, 5 min) and 500 µl 0.2× SSC (30 °C, 5 min). Vacant binding sites were blocked by incubation with 100 µl of a 2% solution of skim milk powder in 6× SSPE/0.005% Triton X-100 at 30 °C for 15 min. Subsequently, the ArrayTube™ was incubated with 100 µl of a 1:5000 dilution of Poly-HRP Streptavidin (Pierce/Thermo Fisher Scientific, Rockford, IL, USA) in 6× SSPE/0.005% Triton X-100 at 30 °C for 15 min, followed by three washing steps as described above. Finally, 100 µl of the peroxidase substrate SeramunGrün® prec (Seramun Diagnostica, Heidesee, Germany) were added. Hybridization signals were measured after incubation at 25 °C for 10 min (without shaking) using an ATR03 transmission reader (Clondiag) and processed with the IconoClust® software version 3.0r0 (Clondiag). Normalized spot intensities were calculated according to the equation: $NI = 1 - (M/BG)$, with NI being the normalized intensity, *M* the aver-

Table 1
Influenza A viruses used in this study.

No.	Isolate	Acc. no. ^a	No.	Isolate	Acc. no. ^a
1	A/USSR/90/77 (H1N1)	CY010372	46	A/Mallard/NVP/41/04 (H7N1)	
2	A/Fort Monmoth/1/47 (H1N1)	CY009612	47	A/turkey/Italy/472/99 (H7N1)	AJ704811
3	A/Denver/1/57 (H1N1)	CY008988	48	A/TY/18-2/2000 (H7N1)	AF497552
4	A/duck/Potsdam/177/83 (H2N2)	CY005765	49	A/swan/Potsdam/64/81 (H7N3)	AM922155
5	A/bantam/Germany/DZ4/85 (H2N2)	AM922141	50	A/duck/Alberta/48/76 (H7N3)	AF497554
6	A/Mallard/Germany/Wv1317-21/03 (H2N3)	AM922142	51	A/turkey/Italy/2043/03 (H7N3)	CY022613
7	A/Mallard/Germany/Wv677/04 (H2N3)	AM922143	52	A/Ck/BC/04 (H7N3)	AY611524
8	A/Mallard/Germany/Wv943-45/04 (H2N3)	AM922144	53	A/Mall/AB/8734/2007 (H7N7)	
9	A/HongKong/1/68 (H3N2)	EF409245	54	A/chicken/Germany/R28/03 (H7N7)	AJ620350
10	A/Mallard/439/04 (H3N2)		55	A/duck/Potsdam/15/80 (H7N7)	AJ704797
11	A/Mallard/Germany/Wv1303-04/03 (H3N8)	AM087224	56	A/turkey/Germany/R11/01 (H7N7)	AJ704812
12	A/Mallard/Germany/Wv64-67/05 (H3N8)		57	A/turkey/Ireland/PV8/98 (H7N7)	AJ704799
13	A/duck/Ukraine/1/63 (H3N8)	AB292668	58	A/turkey/Ontario/6118/68 (H8N4)	CY014659
14	A/Mallard/Germany/Wv1806-09/03 (H4N6)	AM922147	59	A/Anser spec./Germany/R44/2006 (H8N3)	AM922158
15	A/Mallard/Wv1754-57/03 (H4N6)	AM922148	60	A/turkey/Wisconsin/66 (H9N2)	CY014663
16	A/Mallard/Wv1732-34/03 (H4N6)	AM922149	61	A/turkey/Germany/176/95 (H9N2)	AF218101
17	A/Mallard/Germany/Wv1027/04 (H4N6)		62	A/chicken/Emirates/R66/02 (H9N2)	AM922159
18	A/Anas platyrhynchos/Germany/R622-Friesland/08 (H4N6)		63	A/iran/541 (H9N2)	AJ781825
19	A/HongKong/156/97 (H5N1)	AF046088	64	A/chicken/Germany/90/95 (H9N2)	AF218099
20	A/whooper swan/Germany/R65/06 (H5N1)	DQ464354	65	A/turkey/Germany/22/96 (H9N2)	AJ781820
21	A/ck/CXLA/1204/2004 (H5N1)	AM183671	66	A/Moorhen/LKStendal/Wv1703-04 (H10N4)	
22	A/ck/Indonesien/R60/05 (H5N1)	AM183670	67	A/Mallard/NVP/1682-85 (H10N4)	
23	A/ck/Vietnam/P41/05 (H5N1)	AM183672	68	A/Mallard/NVP/Wv1677-81/03 (H10N4)	AM922160
24	A/dk/Vietnam/TG24-01/05 (H5N1)	AM183677	69	A/Mallard/Föhr/Wv1781-82/03 (H10N7)	
25	A/Teal/Wv632/05 (H5N1)	AM408214	70	A/chicken/Germany/N/49 (H10N7)	CY014671
26	A/chicken/Italy/8/98 (H5N2)	AJ305306	71	A/guinea fowl/Hungary/1/69 (H10N8)	
27	A/Mall/QC/2323-19/2006 (H5N2)		72	A/quail/Italy/1117/65 (H10N8)	CY014644
28	A/Goose/MB/428/06 (H5N2)		73	A/duck/England/56 (H11N6)	CY014679
29	A/DK/BC/26-6/05 (H5N2)	DQ309440	74	A/Mallard/Föhr/Wv1499-1503/03 (H11N9)	
30	A/Mallard/Föhr/Wv1349-51/03 (H5N3)	AM087222	75	A/Fulica atra/Germany/R1128/06 (H11N1)	
31	A/chicken/Italy/22/98 (H5N9)	CY022621	76	A/Anas platyrhynchos/Germany/R2219/2006 (H11N9)	AM922161
32	A/Mal/AB/392/2006 (H5N9)		77	A/duck/Alberta/60/76 (H12N5)	AB288334
33	A/Mal/BC/544-2005 (H5N9)		78	A/pilot whale/Maine/328/84 (H13N2)	M26091
34	A/Wigeon/Föhr/Wv579/05 (H6N5)		79	A/gull/Maryland/704/77 (H13N6)	CY014694
35	A/turkey/Hessen/R04/99 (H6N2)	AJ507207	80	A/gull/Stralsund/Wv1136-40/03 (H13N6)	AM922163
36	A/turkey/Hartzfehn/R26/99 (H6N2)	AJ507208	81	A/Larus ridibundus/R2603/06 (H13N8)	
37	A/turkey/LTZ/R63/02 (H6N2)		82	A/Larus ridibundus/Germany/R2609/06 (H13N8)	
38	A/Anas crecca/Germany/R633-NRW/08 (H6N2)		83	A/Larus ridibundus/Germany/R2613/06 (H13N8)	
39	A/turkey/Grub/R41/98 (H6N5)		84	A/Larus ridibundus/Germany/R2622/06 (H13N8)	
40	A/turkey/Grub/R42/98 (H6N5)		85	A/Larus ridibundus/Germany/R2064/2006 (H13N8)	AM922164
41	A/turkey/Grub/R43/98 (H6N5)	AJ507206	86	A/Ma/Gur/263/82 (H14N5)	M35997
42	A/turkey/Heidemark/R83/99 (H6N7)		87	A/She/WA/2576/79 (H15N9)	CY006010
43	A/broiler/Italy/445/99 (H7N1)	AJ580353	88	A/BHG/Sweden/5/99 (H16N3)	AY684891
44	A/chicken/Brescia/19/02 (H7N7)	AM922154	89	A/Larus argentatus/Germany/R2788/06 (H16N3)	
45	A/hen/Italy/444/99 (H7N1)	AJ704810	90	A/Larus argentatus/Germany/R2792/06 (H16N3)	

^a Acc. nos. for the haemagglutinin gene from EMBL/GenBank®/DDBJ.

Table 2
 Sensitivity of the new ArrayTube assay for avian influenza viruses of subtypes H5 and H7.

	rRT-PCR Ct (dR) ^a				RT-PCR ^b	ArrayTube
	M	H5	H7	H5N1 Qinghai		
A/whooper swan/Germany/R65/06 (H5N1) HP						
10 ⁻⁵	31.04	29.82	nd	33.05	+	+
10 ⁻⁶	34.52	32.88	nd	36.26	+ –	+
10 ⁻⁷	37.96	36.13	nd	No Ct	+ –	+
10 ⁻⁸	41.71	42.73	nd	No Ct	–	–
A/chicken/Italy/8/98 (H5N2) HP						
10 ⁻⁵	32.49	30.45	nd	No Ct	+	+
10 ⁻⁶	35.83	33.48	nd	No Ct	+	+
10 ⁻⁷	38.03	37.13	nd	No Ct	–	–
10 ⁻⁸	No Ct	40.35	nd	No Ct	–	–
A/swan/Potsdam/64/81 (H7N3)						
10 ⁻⁵	33.91	nd	33.45	nd	+++	+
10 ⁻⁶	37.67	nd	37.08	nd	++	+
10 ⁻⁷	41.77	nd	40.85	nd	+	+
10 ⁻⁸	No Ct	nd	No Ct	nd	–	–

^a Ct values of rRT-PCR specific for M- and H5-gene (Spackman et al., 2002), H7-gene (unpublished) and HPAIV H5N1 of the Qinghai lineage (Hoffmann et al., 2007).
^b Pan haemagglutinin RT-PCR as described in Gall et al. (2008a). +++ to +, different intensities of bands in agarose gel electrophoresis; + –, weak band; –, no band visible.

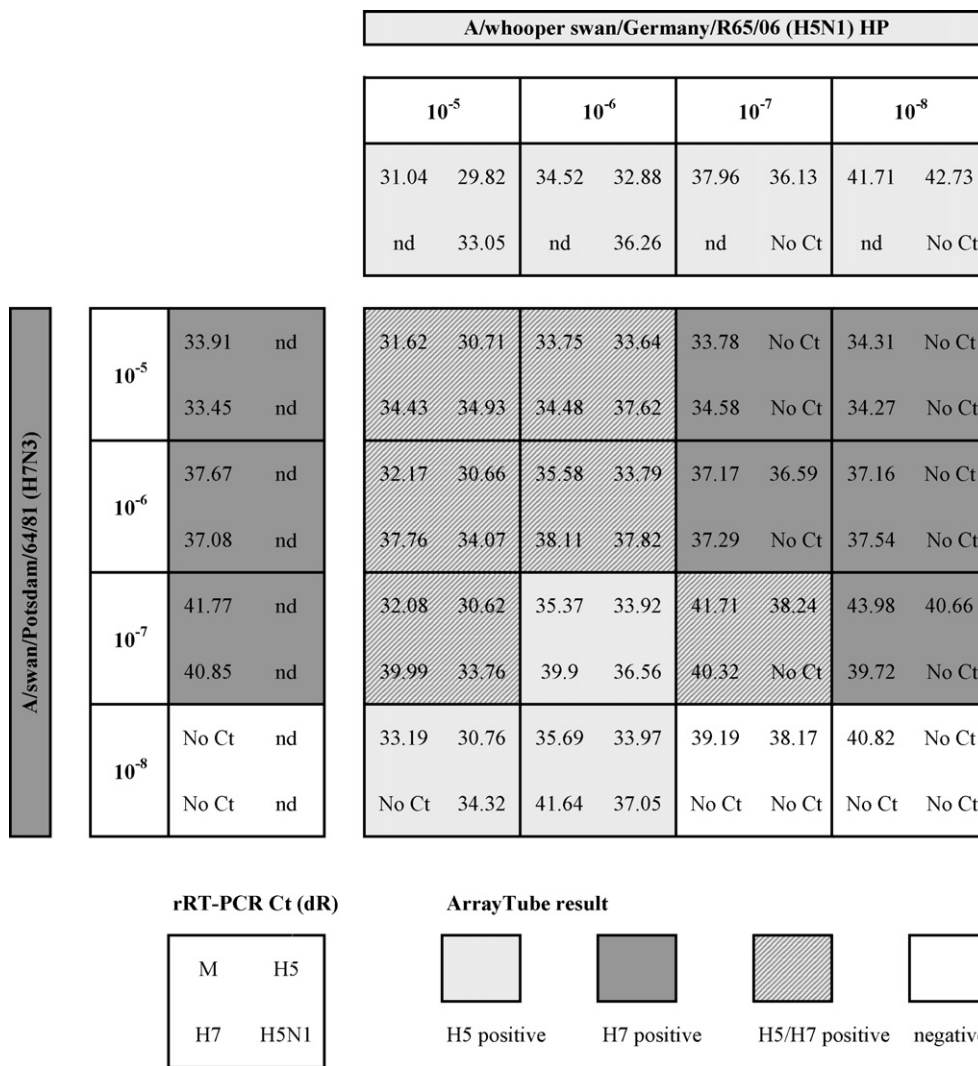


Fig. 1. Sensitivity of simultaneous detection of influenza A viruses of the notifiable subtypes H5 and H7. Detection by the ArrayTube™ is indicated as shaded fields: light grey, H5 positive; dark grey, H7 positive; striped, simultaneous detection of H5 and H7; white, negative for H5 and H7. For each sample, Ct values of the rRT-PCRs specific for the M- (top left), H5- (top right) and H7-gene (bottom left) and for HPAIV H5N1 of the Qinghai lineage (bottom right) are shown.

age (mean) intensity of triplicate spots, and BG the local background intensity. Values for the normalized spot intensity NI ranged from 0 (no signal, bright spots, negative result) to 1 (strong signal, dark spots, positive result). A sample was considered negative when all signal intensities except the internal staining control and the hybridization controls were below 0.07.

Real-time RT-PCR (rRT-PCR) specific for the matrix (M)- and H5-gene (Spackman et al., 2002), H7-gene (unpublished) and for HPAIV H5N1 of the Qinghai lineage (Hoffmann et al., 2007) were performed for comparison purposes. HA subtype and pathotype of diagnostic samples were determined by sequence analysis of an HA₂ fragment (Phipps et al., 2004) and/or the HA₀ cleavage site (Anon., 2006; Gall et al., 2008a; Wood et al., 1993).

For validation of the ArrayTube™ assay, a panel of influenza A virus reference strains – mainly from poultry and wild birds (subtypes H1–16, both pathotypes) – originating from Eurasia, North America and Australia was utilized (Table 1). For 87 (96.7%) of the 90 analyzed strains, HA subtypes and pathotypes were accurately determined, and highly pathogenic H5N1/Asia clade 2.2 was detected specifically. The strains A/duck/England/56 (H11N6), A/Mallard/Föhr/Wv1499-1503/03 (H11N9) and A/Larus

ridibundus/Germany/R2622/06 (H13N8), however, could not be characterized definitely due to signals from probes for more than one subtype.

Furthermore, the sensitivity of the new assay was compared to rRT-PCR assays used within the German National Reference Laboratory for Avian Influenza. In a first step, 10-fold dilution series of RNA from three reference strains in RNA-safe buffer (Hoffmann et al., 2006; 0.05% Tween 20, 0.05% sodium azide, 50 ng/μl of carrier RNA [poly(A) homopolymer; Amersham Biosciences, Piscataway, NJ, USA]) were tested (Table 2). In a second step, performance and sensitivity with regard to simultaneous detection and characterization of two subtypes were investigated. To this end, 16 RNA mixtures were prepared in a chequerboard manner from 10-fold dilution series of RNA from reference strains of the H5 and H7 subtype and tested subsequently (Fig. 1). For the selected strains, the M-gene-specific and H5-subtype-specific rRT-PCRs (Spackman et al., 2002) were more sensitive, the H7-subtype-specific rRT-PCR (unpublished) had a comparable sensitivity, and the rRT-PCR specific for HPAIV H5N1 of the Qinghai lineage (Hoffmann et al., 2007) was less sensitive than the ArrayTube™ assay. The H5 and H7 subtype could be detected simultaneously in six of the mixed sam-

Table 3
Characterization of influenza A viruses from diagnostic samples.

No.	Sample ^a	M specific rRT-PCR ^b	RT-PCR ^c	ArrayTube
1	A/Anas platyrhynchos/Germany/R1649/07 (H1N1)	27.97	++	H1
2	A/Dendrocygna viduata/Germany/R2484/07 (H3N8)	28.86	+++	H3
3	A/Anas platyrhynchos/Germany/R2710/07 (H2N9)	34.34	+ –	H2
4	A/Anas platyrhynchos/Germany/R2723/07 (H5Nx)	33.56	+	LP H5
5	A/Anas platyrhynchos/Germany/R2769/07 (H5N2)	37.57	+	LP H5, H12
6	A/Anas platyrhynchos/Germany/R2776/07	34.39	+	LP H5
7	A/Anas platyrhynchos/Germany/R3240/07 (H1N1)	29.45	+	H1
8	A/Wild goose/Germany/R3250/07 (H6N1)	28.29	+	H6
9	A/Duck/Germany/R4-Lippe/08 (H1N1)	26.64	++	H1
10	A/Anas platyrhynchos/Germany/R91/08 (H3N8)	31.32	++	H3
11	A/Branta canadensis/Germany/R105/08 (H11N9)	23.88	++	H11
12	A/Branta canadensis/Germany/R107/08	34.06	–	–
13	A/Cygnus olor/Germany/R108/08 (H4Nx)	36.95	+ –	H4
14	A/Anser albifrons/Germany/R165/08	32.38	+ –	H9
15	A/Anser albifrons/Germany/R167/08	29.08	+	H6
16	A/Anser albifrons/Germany/R168/08	31.58	+	H9
17	A/Anser albifrons/Germany/R169/08	32.74	++	H9
18	A/Anas platyrhynchos/R234/08 (H1N1)	29.91	+	H1
19	A/Anser anser/Germany/R239/08	33.96	+ –	H9
20	A/Anser anser/Germany/R240/08 (H9N2)	32.34	–	H9
21	A/Anser anser/Germany/R248/08 (H9N2)	22.33	++	H9
22	A/Anas platyrhynchos/Germany/R292/1/08 (H1N1)	26.19	+	H1
23	A/Struthio camelus/South Africa/R296/2/08 (H5N2) HP	26.35	+	HP H5
24	A/Struthio camelus/South Africa/R296/4/08 (H5N2) HP	18.98	++	HP H5
25	A/Struthio camelus/South Africa/R296/5/08 (H5N2) HP	25.86	+	HP H5
26	A/Struthio camelus/South Africa/R296/7/08 (H5N2) HP	30.50	–	HP H5
27	A/Chlamydotis undulata/Dubai/R316/08 (H9N2)	23.80	+ –	H9
28	A/Callonetta leucophrys/Germany/R641/2/08 (H6N2)	23.40	++	H6
29	A/Anas platyrhynchos/Germany/R665/2/08 (H5Nx)	36.73	+ –	LP H5
30	A/Anas platyrhynchos/Germany/R695/08 (H5Nx)	28.47	+	LP H5
31	A/Anas platyrhynchos/Germany/R701/08 (H5Nx)	34.42	+ –	LP H5
32	A/Anas platyrhynchos/Germany/R726/08 (H5Nx)	29.37	+	LP H5
33	A/Anas platyrhynchos/Germany/R734/1/08 (H5N3)	24.23	++	LP H5
34	A/Domestic duck/Germany/R786/08 (H5N1) HP	24.84	+++	HP H5N1/Asia
35	A/Domestic duck/Germany/R821/08 (H5N1) HP	36.91	–	HP H5N1/Asia
36	A/Domestic duck/Germany/R836/08 (H5N1) HP	36.68	+	HP H5N1/Asia
37	A/Domestic goose/Germany/R990/08	34.73	+ –	H6
38	A/Tadorna tadorna/Germany/R1018/1/08	31.37	–	H6
39	A/Anas platyrhynchos/Germany/R1019/1/08	33.30	–	H6
40	A/Anas platyrhynchos/Germany/R1020/1/08	35.86	++	–
41	A/Anas platyrhynchos/Germany/R1021/1/08	37.14	+	–
42	A/Anas platyrhynchos/Germany/R1022/1/08	29.13	++	H11
43	A/Anas platyrhynchos/Germany/R1023/2/08	29.57	+	H11
44	A/Anas platyrhynchos/Germany/R1048/2/08	36.73	–	–

^a Subtyping was not successful for all samples. HA subtypes determined by sequencing of HA₂ gene (Phipps et al., 2004) and/or HA₀ cleavage site (Gall et al., 2008a).

^b Ct (dR) (Spackman et al., 2002).

^c Pan haemagglutinin RT-PCR as described in Gall et al. (2008a). +++ to +, different intensities of bands in agarose gel electrophoresis; + –, weak band; –, no band visible.

ples by the microarray assay. Simultaneous detection of multiple subtypes is important as co-infections of aquatic wild birds with two viruses are substantial part of avian influenza epidemiology. In addition, an analytical sensitivity of 10 copies/reaction was determined using a 10-fold dilutions series of the labelled positive control (data not shown).

The microarray assay was validated further with 44 samples, comprising cloacal swabs from poultry and wild birds (Ct values for the M gene ranged from 18.98 to 37.57 for these samples), including material of the recent outbreak of HPAIV H5N1/Asia clade 2.2 in Germany in 2008 (Table 3). A total of 40 cloacal swabs (90.9%) could be examined successfully. HA subtypes and pathotypes determined by the ArrayTube™ assay corresponded perfectly to results obtained by sequencing of the HA₂ fragment (Phipps et al., 2004) and/or the HA₀ cleavage site (Anon., 2006; Gall et al., 2008a; Wood et al., 1993). For the four samples which remained negative by the ArrayTube™ assay, sequencing and virus isolation were also not successful, and very low viral genome loads (Ct values for the M gene ≥ 34.06) were detected. Therefore, these samples may be considered as false-positive in rRT-PCR.

The limited validation data demonstrate the feasibility of the new ArrayTube™ assay for highly sensitive and specific characterization of avian influenza viruses. Within 24 h, both HA subtypes and pathotypes could be ascertained for the majority of virus isolates and field samples, provided RNA quantity/viral loads and RNA quality were adequate. In this study, all cloacal swabs with Ct values for the M gene-specific rRT-PCR of less than 34 and even a few with lower viral loads (Ct values up to 38) were suitable for direct microarray analysis.

In conclusion, this microarray assay addresses the requirements of diagnostic laboratories for a rapid, easy-to-use and affordable system enabling haemagglutinin subtyping and pathotyping of influenza A-positive samples. The ArrayTube™ assay is integrated into routine diagnosis of the OIE and German National Reference Laboratory for Avian Influenza. A comparable system for neuraminidase subtyping has been developed. Both assays have been combined into a single microarray integrated in eight well-strips (ArrayStrip™) which is under validation.

Acknowledgments

The authors would like to thank Anne Jüngling and the laboratory team of the Office International des Epizooties (OIE) and German National Reference Laboratory for Avian Influenza for excellent technical assistance, Konrad Sachse for the kind introduction to the use of the ArrayTube™ System and colleagues at the OIE Reference Laboratories for AI in Canada, Italy and the UK for supplying reference material. This work was supported by the Federal Ministry of Food, Agriculture and Consumer Protection, BMELV, Germany (FSI, project no. 1.1.) and the EU Network of Excellence, EPIZONE (Contract No FOOD-CT-2006-016236).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2009.05.004.

References

- Anon., 2005. Manual of Diagnostic Tests and Vaccines for Terrestrial Animal OIE. Chapter 2.7.12. Avian influenza. http://www.oie.int/eng/normes/mmanual/A_00037.htm.
- Anon., 2006. Commission decision of 4 August 2006 approving a Diagnostic Manual for Avian Influenza as provided for in Council Directive 2005/94/EC (notified under document number C(2006) 3477) (2006/437/EC). http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_237/l_23720060831en00010027.pdf.
- Anon., 2007. Terrestrial Animal Health Code OIE. Chapter 2.7.12. Avian influenza. http://www.oie.int/eng/normes/mcode/en_chapitre.2.7.12.htm.
- Dawson, E.D., Moore, C.L., Smagala, J.A., Dankbar, D.M., Mehlmann, M., Townsend, M.B., Smith, C.B., Cox, N.J., Kuchta, R.D., Rowlen, K.L., 2006. MChip: a tool for influenza surveillance. *Anal. Chem.* 78, 7610–7615.
- Fereidouni, S.R., Harder, T.C., Starick, E., 2008. Rapid pathotyping of recent H5N1 highly pathogenic avian influenza viruses and of H5 viruses with low pathogenicity by RT-PCR and restriction enzyme cleavage pattern (RECP). *J. Virol. Methods* 154, 14–19.
- Fereidouni, S.R., Starick, E., Grund, C., Globig, A., Mettenleiter, T.C., Beer, M., Harder, T., 2009. Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. *Vet. Microbiol.* 135, 253–260.
- Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D., 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* 79 (5), 2814–2822.
- Gall, A., Hoffmann, B., Harder, T., Grund, C., Beer, M., 2008a. Universal primer set for amplification and sequencing of HA₀ cleavage sites of all influenza A viruses. *J. Clin. Microbiol.* 46 (8), 2561–2567.
- Gall, A., Hoffmann, B., Harder, T., Grund, C., Höper, D., Beer, M., 2008b. Design and validation of a microarray for detection, hemagglutinin subtyping, and pathotyping of avian influenza viruses. *J. Clin. Microbiol.* 47 (2), 327–334.
- Globig, A., Staubach, C., Beer, M., Koeppen, U., Fiedler, W., Nieburg, M., Wilking, H., Starick, E., Teifke, J.P., Werner, O., Unger, F., Grund, C., Wolf, C., Roost, H., Feldhufen, F., Conraths, F.J., Mettenleiter, T.C., Harder, T.C., 2009. Epidemiological and ornithological aspects of outbreaks of highly pathogenic avian influenza virus H5N1 of Asian lineage in wild birds in Germany, 2006 and 2007. *Transboundary Emerg. Dis.* 56, 57–72.
- Han, X., Lin, X., Liu, B., Hou, Y., Huang, J., Wu, S., Liu, J., Mei, L., Jia, G., Zhu, Q., 2008. Simultaneously subtyping of all influenza A viruses using DNA microarrays. *J. Virol. Methods* 152, 117–121.
- Höper, D., Hoffmann, B., Beer, M., 2009. Simple, sensitive, and swift sequencing of complete avian influenza H5N1 genomes. *J. Clin. Microbiol.* 47 (3), 674–679.
- Hoffmann, B., Depner, K., Schirmer, H., Beer, M., 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.
- Hoffmann, B., Harder, T., Starick, E., Depner, K., Werner, O., Beer, M., 2007. Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. *J. Clin. Microbiol.* 45, 600–603.
- Kessler, N., Ferraris, O., Palmer, K., Marsh, W., Steel, A., 2004. Use of the DNA flow-through chip, a three-dimensional biochip, for typing and subtyping of influenza viruses. *J. Clin. Microbiol.* 42 (5), 2173–2185.
- Li, H., McCormac, M.A., Estes, R.W., Sefers, S.E., Dare, R.K., Chappell, J.D., Erdman, D.D., Wright, P.F., Tang, Y.-W., 2007. Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. *J. Clin. Microbiol.* 45 (7), 2105–2109.
- Lin, B., Blaney, K.M., Malanoski, A.P., Ligler, A.G., Schnur, J.M., Metzgar, D., Russell, K.L., Stenger, D.A., 2007. Using a resequencing microarray as a multiple respiratory pathogen detection assay. *J. Clin. Microbiol.* 45 (2), 443–452.
- Liu, R.H., Lodes, M.J., Nguyen, T., Siuda, T., Slota, M., Fujii, H.S., McShea, A., 2006. Validation of a fully integrated microfluidic array device for influenza A subtype identification and sequencing. *Anal. Chem.* 78, 4184–4193.
- Lodes, M.J., Suci, D., Elliott, M., Stover, A.G., Ross, M., Caraballo, M., Dix, K., Crye, J., Webby, R.J., Lyon, W.J., Danley, D.L., McShea, A., 2006. Use of semiconductor-based oligonucleotide microarrays for influenza A virus subtype identification and sequencing. *J. Clin. Microbiol.* 44 (4), 1209–1218.
- Monecke, S., Ehrlich, B., 2005. Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays. *Clin. Microbiol. Infect.* 11, 825–833.
- Phipps, L.P., Essen, S.C., Brown, I.H., 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.
- Quan, P.-L., Palacios, G., Jabado, O.J., Conlan, S., Hirschberg, D.L., Pozo, F., Jack, P.J.M., Cisterna, D., Renwick, N., Hui, J., Drysdale, A., Amos-Ritchie, R., Baumeister, E., Savy, V., Lager, K.M., Richt, J.A., Boyle, D.B., Garcia-Sastre, A., Casas, I., Perez-Brena, P., Briese, T., Lipkin, W.I., 2007. Detection of respiratory viruses and subtype identification of influenza A viruses by GreeneChipResp oligonucleotide microarray. *J. Clin. Microbiol.* 45 (8), 2359–2364.
- Sengupta, S., Onodera, K., Lai, A., Melcher, U., 2003. Molecular detection and identification of influenza viruses by oligonucleotide microarray hybridization. *J. Clin. Microbiol.* 41 (10), 4542–4550.
- Spackman, E., Senne, D.A., Myers, T.J., Buluga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., Suarez, D.L., 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 (9), 3256–3260.
- Starick, E., Beer, M., Hoffmann, B., Staubach, C., Werner, O., Globig, A., Strebelow, G., Grund, C., Durban, M., Conraths, F.J., Mettenleiter, T., Harder, T., 2008. Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus. *Vet. Microbiol.* 128, 243–252.
- Townsend, M.B., Dawson, E.D., Mehlmann, M., Smagala, J.A., Dankbar, D.M., Moore, C.L., Smith, C.B., Cox, N.J., Kuchta, R.D., Rowlen, K.L., 2006. Experimental evaluation of the FluChip diagnostic microarray for influenza virus surveillance. *J. Clin. Microbiol.* 44 (8), 2863–2871.

Vijaykrishna, D., Bahl, J., Riley, S., Duan, L., Zhang, J.X., Chen, H., Peiris, M., Smith, G., Guan, Y., 2008. Evolutionary dynamics and emergence of pan-zootic H5N1 influenza viruses. *PlosPathog* 4(9), e1000161, doi:10.1371/journal.ppat.1000161.

Wang, L.-C., Pan, C.-H., Severinghaus, L.L., Liu, L.-Y., Chen, C.-T., Pu, C.-E., Huang, D., Lir, J.-T., Chin, S.-C., Cheng, M.-C., Lee, S.-H., Wang, C.-H., 2008.

Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. *Vet. Microbiol.* 127, 217–226.

Wood, G.W., McCauley, J.W., Bashruddin, J.B., Alexander, D.J., 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.