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### ► To cite this version:

S.R. Fereidouni, E. Starick, C. Grund, A. Globig, T.C. Mettenleiter, et al.. Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. *Veterinary Microbiology*, 2009, 135 (3-4), pp.253. 10.1016/j.vetmic.2008.09.077 . hal-00532513

**HAL Id: hal-00532513**

**<https://hal.science/hal-00532513>**

Submitted on 4 Nov 2010

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## Accepted Manuscript

Title: Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses

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PII: S0378-1135(08)00467-7  
DOI: doi:10.1016/j.vetmic.2008.09.077  
Reference: VETMIC 4220

To appear in: *VETMIC*

Received date: 7-5-2008  
Revised date: 22-9-2008  
Accepted date: 29-9-2008

Please cite this article as: Fereidouni, S.R., Starick, E., Grund, C., Globig, A., Mettenleiter, T.C., Beer, M., Harder, T., Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.077

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2 **Rapid molecular subtyping by reverse transcription polymerase chain**  
3 **reaction of the neuraminidase gene of avian influenza A viruses**

4

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7

8 **Running title:**

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10 RT-PCR subtyping of avian influenza A virus neuraminidase

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35

36 **Abstract**

37

38 Accurate identification of hemagglutinin (HA) and neuraminidase (NA) subtypes of influenza  
39 A viruses is an integral part of monitoring programs targeting avian influenza viruses (AIV).  
40 Use of highly sensitive molecular screening methods such as pan influenza-specific real-time  
41 RT-PCR (rRT-PCR) yields an increasing number of samples which are positive for AIV RNA  
42 but negative by virus isolation and, therefore, require molecular, instead of serological,  
43 subtyping. We developed specific RT-PCR assays for all known nine AIV NA subtypes.  
44 Validation using 43 reference isolates from different animal species revealed good  
45 performance characteristics regarding sensitivity and specificity. On basis of serial tenfold  
46 dilution series of reference isolates a benchmark value of  $C_t$  32 in an M gene-specific rRT-  
47 PCR became evident below which all nine NA subtypes were readily detectable by the  
48 subtype-specific RT-PCRs. For subtypes N1, N2, N4 and N6 detection was extended to  
49 dilutions with  $C_t$  values of up to 35. Diagnostic applicability of the whole set of conventional  
50 NA-specific RT-PCRs was evaluated by analysis of 119 different diagnostic samples from  
51 wild birds which proved to be positive for AIV by M gene-specific rRT-PCR. Diagnostic  
52 sensitivity and specificity was confirmed by sequencing NA amplicons from 41 field isolates  
53 generated from this set and by NA inhibition assays. A universal molecular HA/NA subtyping  
54 algorithm for rRT-PCR positive avian influenza virus monitoring samples is proposed which  
55 may complement classical serological subtyping of influenza A virus isolates.

56

57 **Key words**

58 Influenza A virus, Neuraminidase, Subtyping, RT-PCR

## 59 Introduction

60

61 Influenza A viruses are classified as subtypes on the basis of antigenic and/or genetic diffe-  
62 rences of their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To  
63 date, 16 subtypes of HA (H1–H16) and 9 subtypes of NA (N1–N9) have been identified  
64 (Fouchier et al., 2005).

65

66 The recent geographic expansion of highly pathogenic avian influenza viruses (HPAIV) of  
67 subtype H5N1 prompted intensified virological monitoring both in poultry and wild bird  
68 populations all around the world (Hlinak et al., 2006; Munster et al., 2006, Gaidet et al, 2007;  
69 Parmely et al, 2008). The majority of these surveys is based on molecular methods such as  
70 real-time reverse transcription PCR (rRT-PCR), which have been proven superior regarding  
71 sensitivity and suitability for high throughput analyses when compared to classical diagnostic  
72 measures such as virus isolation in embryonated specific pathogen free (SPF) chicken eggs  
73 and serological virus characterization (Duan et al., 2007; Munster et al., 2007; Spackman et  
74 al., 2007). While the above mentioned monitoring programs aim in particular at the detection  
75 of AIVs of subtypes H5 (particularly H5N1) and H7, a plethora of non-H5/H7 AIV positive  
76 samples is encountered, especially in wild bird monitoring programs. Since these samples are  
77 of importance with respect to further understanding the epidemiology of AIVs, a  
78 characterization of HA and NA subtypes is required. While virus isolation and subsequent  
79 serological characterization of isolates by hemagglutination inhibition (HI) and neuraminidase  
80 inhibition (NI) tests would be sufficient, the quality of samples and/or the amount of virus  
81 present may hamper virus isolation. In addition, such tests are time consuming and demand an  
82 array of specific antisera and references antigens of high quality. Therefore, rapid and reliable  
83 molecular tools for HA/NA subtyping are needed.

84

85 Several studies focussed on the identification of different HA subtypes by specific RT-PCR  
86 assays (Stockton et al., 1998; Starick et al., 2000; Herrmann et al., 2001; Lee et al., 2001;  
87 Munch et al., 2001; Takao et al., 2002; Spackman et al., 2003), or by a pan-HA RT-PCR  
88 targeting the more conserved HA2 section of the HA gene and subsequent sequencing of the  
89 amplicons or microarray detection (Phipps et al., 2004; Gall et al., 2008). However, only few  
90 published studies have focussed on the subtype identification of influenza virus  
91 neuraminidases. These concentrate mainly on subtypes N1 and N2 associated with human

92 influenza viruses (Wright et al., 1995; Li et al., 2001; Takao et al., 2002; Barr et al., 2006;  
93 Mak et al., 2006; Payungporn et al., 2006; Zou et al., 2007). Recently, a pair highly  
94 degenerated primers has been published which might be suitable for amplification of an NA  
95 gene fragment from all nine subtypes (Alvarez et al., 2008).

96

97 In this study, the development and evaluation of single subtype-specific RT-PCR assays  
98 suitable for the molecular typing of all nine AIV-NA subtypes known to date is reported. The  
99 results enabled the proposal of an improved algorithm for molecular HA/NA subtyping of  
100 influenza A viruses.

101

102

## 103 **Materials & Methods**

104

105 **Origin of influenza virus reference strains, field samples and sera.** Reference isolates and  
106 sera are maintained in the virus repository of the OIE and National Reference Laboratory for  
107 Avian Influenza at the Friedrich-Loeffler-Institut (NRL-AI). Several of these viruses were  
108 donated by reference laboratories in Canada, Dubai, Italy, U.K. and Vietnam. A total of 119  
109 of AIV RNA positive field samples was obtained in the framework of nationwide monitoring  
110 programs for AIV in wild birds in Germany during 2001-2007 (unpublished), in other  
111 countries (Fereidouni et al., 2005). The majority of samples originating from wild birds in  
112 Germany had been pre-tested by rRT-PCR for influenza virus M gene by the regional  
113 laboratories of the Federal States of Germany and were subsequently sent to the NRL-AI for  
114 confirmation and further characterization. Virus isolation from these samples was attempted  
115 as previously described (Fereidouni et al., 2005). Other avian pathogenic viruses included  
116 infectious bronchitis virus (IBV, M41 strain), infectious laryngotracheitis virus (ILTV, A-489  
117 strain), infectious bursal disease virus (IBDV, D78 strain) and avian paramyxoviruses  
118 (APMV) types 1-9 (APMV-1: La Sota strain; APMV-2: chicken/California/Yucaipa/56;  
119 APMV-3: parakeet/Netherlands/449/75; APMV-4: duck/Hungary/D3/75; APMV-6:  
120 duck/HongKong/189/77; APMV-7: dove/Tn/u/75; APMV-8: goose/Delaware/1057/76;  
121 APMV-9: duck/New York/22/78) were provided by the virus collection at the Friedrich-  
122 Loeffler-Institut.

123

124 **Primer design.** Initially, a comprehensive selection of mammalian and avian viral NA full  
125 length gene sequences available from the Influenza Sequence Database  
126 (<http://www.flu.lanl.gov/index.html>) and Genbank was aligned (clustal X, version 1.83). The  
127 alignment finally comprised 2104 N1, 794 N2, 163 N3, 23 N4, 51 N5, 152 N6, 49 N7, 138  
128 N8 and 71 N9 sequences. Primers specific for each single NA subtype and hybridizing to all  
129 published sequences within that subtype were selected whereby tolerating up to four  
130 degenerated sites per primer (Table 1).

131

132 **Reverse transcription-PCR (RT-PCR).** RNA isolation was performed manually from  
133 allantoic fluids (virus isolates) or swab fluids (diagnostic samples) using either the High Pure  
134 Viral RNA Kit (Roche) or the QIAamp viral RNA kit (Qiagen). The RT-PCR assays were  
135 performed separately for each NA-subtype on the basis of an one-step protocol using the  
136 Superscript III-based one-step RT-PCR kit (Invitrogen) according to the manufacturer's  
137 instructions. All tests were accompanied by one negative RNA preparation control and one  
138 positive RT-PCR control. The cycling conditions consisted of 30 min at 50°C during the  
139 reverse transcription phase and then an initial denaturation at 95°C for 2 min followed by 5  
140 touch-down PCR cycles starting with 94°C for 15 s, 60°C (decrement of 1°C per cycle) for 30  
141 s, 68°C for 1 min and followed by 30 cycles of 94°C for 15 s, 54°C for 15 s, 68°C for 1 min  
142 and a final extension at 68°C for 5 min.

143

144 **Real-time RT-PCR for primary detection of influenza A viruses.** A real-time RT-PCR  
145 assay targeting a highly conserved region of the M gene was performed as described  
146 (Spackman et al., 2002).

147

148 **Neuraminidase inhibition (NI) assay.** The protocol described by Van Deusen et al. (1983)  
149 using fetuin as substrate was followed with some modifications. Briefly, allantoic fluid  
150 containing an AIV isolate was diluted 1:4 using one volume of CaCl<sub>2</sub>/NaCl-solution (pH 5.6)  
151 and two volumes of phosphate buffer (pH 5.9). Virus dilutions were incubated in 48-well  
152 microtiter plates (Costar) with 1:10 and 1:20 diluted antisera followed by incubation with  
153 fetuin (F3004, SIGMA). Sodium periodate (NaIO<sub>4</sub>, S1147, SIGMA) dissolved in ortho-  
154 phosphoric acid served as an oxidant, and the reaction was completed by sodium meta-arsenit  
155 (1287, FLUKA) dissolved in H<sub>2</sub>SO<sub>4</sub> and thio-barbituric acid (T-5500, SIGMA) (pH 9.0). All

156 reagents with the exception of thio-barbituric acid (200  $\mu$ l) were used in amounts of 50  $\mu$ l.  
157 Positive controls for each subtype (reference isolates) were included in each assay.

158

159 **Sequencing.** PCR amplicons were purified from agarose gels using the QIAquick Gel  
160 Extraction kit (Qiagen). Purified DNA fragments were cycle-sequenced in both directions  
161 using the same primers as employed for the amplification. The Prism Big Dye Terminator  
162 v1.1 cycle sequencing kit (Applied Biosystems) was used and products were analysed on an  
163 automatic sequencer (ABI-377, Applied Biosystems). Assembled nucleotide sequences were  
164 then used in BlastN2 database searches for subtype specification (Altschul et al., 1990).

165

166

## 167 **Results**

168

169 **NA subtype-specific RT-PCR design.** The alignment of a comprehensive set of NA full  
170 length sequences from all 9 subtypes revealed an extensive sequence diversity within a  
171 subtype, in particular for subtypes N1 and N2 (data not shown).

172

173 Therefore, primer pairs specific for each single NA subtype but containing up to four  
174 degenerate positions were designed and evaluated using RNA templates isolated from  
175 reference isolates representing all nine NA subtypes. Based on their high specificity for a  
176 single subtype and broad reactivity within that subtype, nine primer pairs were finally chosen  
177 (Table 1) from a panel of 34 primer pairs initially evaluated.

178

179 In silico analysis (BLASTN2 search, corrected for short sequences, against the GB Virus  
180 database) of finally chosen primer sequences still revealed up to two mismatches with target  
181 sequences of some strains of the homologous subtype; yet, these mismatches did not affect  
182 the five most 3' nucleotides of the primers (data not shown). On the other hand no relevant  
183 homologies (exceeding two matches out of the five most 3' nucleotides) of primers with  
184 sequences of strains of heterologous NA subtypes were detected.

185

186 **Analytical sensitivity and specificity.** The analytical sensitivity of each NA subtype-specific  
187 RT-PCR was assessed using  $\log_{10}$  dilutions of RNA extracted from AIV reference isolates  
188 carrying the matching NA. The number of strains available ranged from ten (N1) to one (N4)



189 per subtype (Table 2). Results were compared with an rRT-PCR targeting the M gene  
190 fragment (Spackman et al., 2002) and carried out with the same  $\log_{10}$  dilution series of AIV  
191 RNA. Fragments of predicted size were amplified for all NA subtypes from RNA dilutions of  
192 reference isolates for which  $C_t$  values of  $\leq 32$  were obtained in the M-specific rRT-PCR. For  
193 NA subtypes 1, 2, 4 and 6, amplicons were also obtained from RNA dilutions of  $C_t$  values of  
194  $\leq 35$  (data not shown).

195

196 The analytical specificity of each of the NA subtype-specific primer pairs was tested by RT-  
197 PCR in separate reactions in a checker board fashion with RNA templates extracted from a  
198 total of 43 influenza A virus reference isolates originating from different host species such as  
199 humans, swine, horses or birds which represented all nine NA subtypes of avian influenza  
200 viruses (Table 2). For all nine primer pairs, amplicons were obtained exclusively with RNA of  
201 the homologous NA subtype. The amplicon size of these PCR products matched the fragment  
202 length as expected on basis of the primers' locations.

203

204 Genomic RNA and DNA extracted from other important avian viruses such as infectious  
205 bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian paramyxoviruses  
206 (APMV) serotypes 1 to 9 and infectious bursal disease virus (IBDV) tested negative in all NA  
207 subtype-specific RT-PCRs (data not shown).

208

209 **Diagnostic sensitivity and specificity.** A total of 119 samples from a variety of avian hosts  
210 had tested positive by rRT-PCR for the influenza A virus M gene fragment, and had been  
211 characterized by H5/H7 subtype-specific rRT-PCR assays or by sequence analysis of an HA2  
212 fragment (Phipps et al., 2004). Out of these 119 AIV RNA positive samples 41 yielded an  
213 AIV isolate. All 119 samples were analysed with the nine NA subtype-specific RT-PCR  
214 assays and could be subtyped successfully (Table 3: AIV RNA positive samples with negative  
215 results by virus isolation,  $n=78$ ; table 4: AIV RNA positive samples yielding a virus isolate,  
216  $n=41$ ). Results obtained with RNA extracted from the field sample and from the  
217 corresponding virus isolate, respectively, matched completely.

218

219 For the further assessment of performance characteristics of the NA subtype specific RT  
220 PCRs with diagnostic material, amplicates from all 41 AIV field isolates were sequenced  
221 directly to confirm the specificity as assessed by amplicon size and predicted by primer

222 identity. In all cases, the NA subtype predicted by amplicon size and specific primer pair used  
223 for amplification, and the sequence obtained proved to match (Table 4). For the same set of  
224 AIV field isolates, the NI assay was used as an additional specificity control. Again, a  
225 complete match of results was achieved (Table 4). Analysis of available full length sequences  
226 revealed one or two primer mismatches for some isolates which, however, did not influence  
227 the successful amplification of all viruses tested.

228

229

## 230 **Discussion**

231

232 We developed a full set of subtype-specific RT-PCR assays for the identification of all nine  
233 influenza A virus neuraminidase subtypes known to date. These conventional RT-PCR assays  
234 proved to be highly specific and revealed, in comparison to a pan-influenza virus real-time  
235 RT-PCR assay targeting the M gene, a satisfactory analytical sensitivity. Analyses of 119  
236 AIV field samples demonstrated that the new NA-specific RT-PCR assays can be used for the  
237 characterization of RNA obtained directly from diagnostic specimens. All 119 investigated  
238 field samples, irrespective of their  $C_t$  value in rRT-PCR, could be correctly characterized with  
239 regard to the NA subtype demonstrating a high diagnostic sensitivity. Alvarez et al. (2008)  
240 employing a single highly degenerated universal NA specific primers only showed results for  
241 cultured, high-titred virus suspension and not for RNA directly extracted from clinical  
242 samples. The high specificity of the nine RT-PCR assays described here could be also shown  
243 by the investigation of well-characterized AIV reference viruses and non-AIV avian viruses.  
244 Clearly discernable unique amplicates were produced for the different NA subtypes. The  
245 method described by Alvarez et al. (2008), in contrast, produced complex patterns of  
246 amplicates of different sizes even from cultured virus material. In our study, NA subtyping  
247 results of recent field virus isolates by subtype-specific RT-PCR was fully confirmed by NI  
248 assays and sequence analyses. In silico analyses of NA sequences of various mammalian  
249 hosts suggested furthermore that these nine RT-PCR assays are also suitable for NA  
250 subtyping of influenza A viruses of mammalian species.

251

252 Advantages of influenza A virus molecular subtyping directly from diagnostic samples are  
253 obvious: (i) independence from labour- and time-consuming virus isolation (requirement of  
254 embryonated SPF chicken eggs) and hemagglutination-inhibition (HI) and NI assays

255 (requirement of specific antisera, handling of hazardous substances), (ii) acquisition of NA-  
256 specific sequences possible, (iii) reliable results within a single working day, (iv) increased  
257 biosecurity as no replication-competent viruses need to be handled. Microarray hybridization  
258 assays such as described by Kessler et al. (2004) or Sengupta et al. (2003) and RT-PCR using  
259 one uniform primer pair with subsequent sequencing (Alvarez et al., 2008) are also suitable  
260 for NA subtyping of avian influenza viruses. In contrast, however, to conventional NA  
261 subtype-specific RT-PCRs these assays require expensive equipment and consumables and  
262 are usually not available in less well equipped laboratories.

263

264 Positive results with more than one NA subtype-specific assay would signal a double  
265 infection rather than false-positive amplification by promiscuous primer binding which has  
266 not been observed in our study so far. Corroborating the *in silico* analysis showing only  
267 limited homology of primers to heterologous NA subtype sequences in the first five  
268 nucleotide positions at the 3' end, the checker board amplification experiments likewise gave  
269 no evidence for misbinding of primers that would eventually lead to false-positive  
270 amplicates.

271

272 Simultaneous infections with more than one influenza A virus subtype have repeatedly been  
273 reported in wild birds. In fact, such co-infections are the necessary basis of genetic  
274 reassortment leading to the formation of new reassortant strains. Given the fact that two HA  
275 and two NA subtypes were detected by molecular means within the same sample, there are no  
276 further tools, apart from virus isolation, to retrieve the original HA/NA combinations  
277 molecularly. Even with successful virus isolation one may argue that reassortment cycles  
278 might have occurred during replication in the egg and that the resulting viruses do not  
279 necessarily represent the original subtype combinations.

280

281 In summary, our conventional RT-PCRs are suitable for NA subtyping of AIV directly from  
282 diagnostic specimens, provided the quality and quantity of RNA is adequate. In our hands,  
283 samples with  $C_t$  values equal to or below 32 in M gene-specific rRT-PCRs allowed an  
284 unambiguous NA subtyping; for some NA subtypes (N1, N2, N4, N6) even samples with  
285 lower viral loads were suitable for typing. Attempts to establish multiplex PCR assays for the  
286 NA subtyping are under way.

287

288 For a full molecular subtyping of AIVs from diagnostic specimens with positive results by  
289 rRT-PCR (M gene), we suggest the following procedure:

290

291 (i) Perform an HA2 RT-PCR according to Phipps et al. (2004) *or* the Pan HA RT-PCR  
292 as recommended by Gall et al. (2008).

293 (ii) Sequence the respective HA amplicon and identify the HA subtype by using a  
294 BlastN2 search.

295 (iii) Short-list putative NA subtypes matching the identified HA subtype as a possible  
296 partner according to the BlastN2 results.

297 (iv) Perform subtype-specific NA RT-PCR assays according to this list.

298 (v) If no specific amplicon is identified, perform subtype-specific NA RT-PCR assays  
299 for the remaining subtypes.

300

301 By using this system, we identified a number of rare HA subtypes (H8, H16) and unusual  
302 HA/NA combinations with currently less than four hits in the Genbank database (H13N8 - 1  
303 hit, H10N4 - 2 hits, H1N6 - 3 hits, H11N1 - 3 hits, and H10N8 - 3 hits. Among the known  
304 HA/NA subtypes only representatives of H12, H14, H15, and N5 were not found in our  
305 sample set. While a full molecular HA/NA subtyping is possible, virus isolation should  
306 nevertheless attempted with AIV RNA positive samples to allow future biological  
307 investigations of these viruses.

308

### 309 **Acknowledgment**

310 We like to thank Bianca Kinnemann, Kathrin Steffen and Bianka Hillmann for excellent  
311 technical assistance. The study has been financially supported by grants from the Federal  
312 Government of Germany (FSI project 1-3.6) and by the European network of excellence  
313 'EPIZONE'. We are indebted to our colleagues from regional diagnostic laboratories in  
314 Germany for continuously supplying AIV-positive samples and isolates. We are also grateful  
315 to our colleagues from the AIV Community Reference Laboratory at Weybridge, U.K. (Dres.  
316 D. Alexander, I. Brown, R. Manvell), and from the O.I.E. Reference Laboratories at Padova,  
317 Italy (Dr I. Capua), and Winnipeg, Canada (Dr J. Pasick), as well as Dres. Wernery, Dubai,  
318 United Arab Emirates, who made available AIV reference strains and field isolates,  
319 respectively.

320

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**Table 1.** Primers used in this study for RT-PCR and sequencing of influenza A virus neuraminidase (N) gene fragments.

Name	Orientation	Sequence (5' → 3')	Length (bp)	Position in reference sequence	Expected product size (bp)	Accession no. Of reference sequence
N1-F	Forward	AGR <sup>1</sup> CCTTGYYTCTGGGTTGA	20	1209-1228	126	AJ416625
N1-R	Reverse	ACCGTCTGGCCAAGACCA	18	1334-1317		
N2-F	Forward	GCATGGTCCAGYTCAAGTTG	20	548-567	362	AJ574906
N2-R	Reverse	CCYTTCCAGTTGTCTCTGCA	20	909-890		
N3-F	Forward	AGATCRGGCTTTGAARTCATCAAAGT	26	1126-1151	247	CY005741
N3-R	Reverse	CATTGTCTARTCCACAGAAAGTAACTATAC	30	1372-1343		
N4-F	Forward	TGGATAAGATTCAACAGTGA	20	925-944	456	CY004989
N4-R	Reverse	GGTATCAGAATTAACACCACA	21	1380-1360		
N5-F	Forward	GTTATTGGGTAATGACRGAYGGTC	24	724-747	193	CY004374
N5-R	Reverse	GGTCTATTCATTCCATTCCAA	21	916-896		
N6-F	Forward	GCIACAGGAATGACACTATC	20	52-71	265	CY004311
N6-R	Reverse	GRATGTGCCATGARTTTAC	19	316-298		
N7-F	Forward	GTYGACAAYAACAATTGGTCAGG	23	1211-1233	183	AY340077
N7-R	Reverse	CCCAACTGRGAITGGGCT	18	1393-1376		
N8-F	Forward	GGTCAGGATAYAGYGGTTCYTTTAC	25	1216-1240	145	AX225077
N8-R	Reverse	CCACACATCACAATGGAGCT	20	1360-1341		
N9-F	Forward	AACACIGACTGGAGTGGYTAC	21	1216-1236	166	CY014689
N9-R	Reverse	GGAATTCTGTRCTGGAACAC	20	1381-1362		

<sup>1</sup> Codes for ambiguous bases position and NTP analogues: R=A/G, Y=C/T, I=Inosine

**Table 2.** Reference strains used in this study for RT-PCR validation.

NA-subtype	Strain designation	Subtype	GenBank accession no NA gene
1	A/swine/Bakum/5/95	H1 N1	N.A.
	A/swine/Wisconsin/67	H1 N1	CY026293
	A/swine/Iowa/15/30	H1 N1	EU139833
	A/FortMonmouth/1/47	H1 N1	CY009614
	A/chicken/Scotland/59	H5 N1	CY015083
	A/chicken/Indonesien/R134/03	H5 N1	AM183681
	A/chicken/China/1204/04	H5 N1	AM183680
	A/duck/Vietnam/TG24-01/05	H5 N1	AM183678
	A/chicken/Italy/473/99	H7 N1	N.A.
	A/chicken/FPV/Rostock/45/34	H7 N1	<a href="#">CY015048</a>
	A/Hong kong/1/68	H3 N2	AF348184
	A/mallard/Postdam/178-4 /83	H2 N2	DQ017487
	A/duck/Potsdam/1402-6/86	H5 N2	CY005778
	2	A/swine/Bakum/909/93	H3 N2
A/Johannesburg/33/94		H3 N2	AJ457946
A/chicken/Italy/8/98		H5 N2	EF597309
A/turkey/Mass/3740/65		H6 N2	AJ574906
A/turkey/Wisconsin/1/66		H9 N2	CY014665
A/turkey/Germany/90/95		H9 N2	N.A.
3		A/tern/South Africa/61	H5 N3
	A/duck/Alberta/48/76	H7 N3	N.A.
	A/turkey/Italy/2043/03	H7 N3	CY022615
	A/black-headed gull/Sweden/5/99	H16 N3	<a href="#">AY684905</a>
4	A/turkey/Ontario/6118/68	H8 N4	<a href="#">AB289344</a>
5	A/mallard duck/Alberta/60/76	H12 N5	CY004374
	A/turkey/Germany/R41/98	H6 N5	N.A.
	A/mallard/Astrakhan/263/1982	H14 N5	<a href="#">AB289336</a>
6	A/duck/Czech/56	H4 N6	AB295612
	A/duck/England/1/56	H11 N6	AB288846
	A/gull/Maryland/704/77	H13 N6	AY207553
	A/duck/Potsdam/2216-4/84	H5 N6	<a href="#">CY005771</a>
7	A/equi/Prague/1/56	H7 N7	CY005802
	A/swan/Germany/64/81	H7 N7	N.A.
	A/turkey/Ireland/PV8/98	H7 N7	N.A.
	A/chicken/Germany/N/49	H10 N7	<a href="#">CY014673</a>
8	A/equine/Miami/1/63	H3 N8	L06580
	A/duck/Ukraine/1/63	H3 N8	L06576
	A/turkey/Germany/R83/99	H6 N8	N.A.
	A/quail/Italy/1117/65	H10 N8	CY005796
	A/guinea fowl/Hungary/1/69	H10 N8	N.A.
9	A/chicken/Italy/22A/98	H5 N9	CY022623
	A/turkey/Ontario/7732/66	H5 N9	N.A.
	A/wedge-tailed shearwater/Australia/2576/79	H15 N9	<a href="#">CY005407</a>

N.A. – not available

**Table 3.** Results of NA subtyping of AIV RNA from 78 out of 119 diagnostic swab samples; results of remaining 41 field samples with successful virus isolation are listed in table 4.

NA-subtype	Strain designation	HA Subtype (HA2 sequencing)	NA Subtype (determined by RT-PCR)
1	A/mallard/Germany/R355/07	H1	N1
	A/Vietnam/1194/04	H5	N1
	A/mallard/Germany/R1649/07	H1	N1
	A/wild duck/Germany/R3036/07	H1	N1
2	A/mallard/Germany/Wv1226-30/03	H3	N2
	A/mallard/Germany/Wv436/04	H3	N2
	A/mallard/Germany/Wv1310-04K/03	H5	N2
	A/teal/Germany/Wv1378-79K/03	H5	N2
	A/mallard/Germany/Wv474-77K/04	H5	N2
	A/duck/British Columbia/2606/05	H5	N2
	A/quail/UAE/R1187/07	H9	N2
	A/falcon/UAE/R1217/07	H9	N2
	A/golden pheasant/Germany/R1081/07	H5	N2
	A/teal/Germany/Wv180-2/05	H7	N2
	A/turkey/Germany/R617/07	H6	N2
	A/mallard/Germany/1313/03	H5	N2
	A/black-headed gull/Germany/R2622/06	H13	N2
	A/mallard/Germany/SU62/06	H6	N2
	A/herring gull/Germany/R2792/06	H16	N2
	A/mallard/Germany/Wv476k/04	H5	N2
	A/guinea fowl/Germany/D73/85	H2	N2
	A/mallard/Germany/R2769/07	H5	N2
	A/mallard/Germany/R2784/07	H5	N2
	A/mallard/Germany/R2486/07	H9	N2
A/mallard/Germany/R2886/07	H11	N2	
A/mallard/Germany/R2889/07	H9	N2	
A/mallard/Germany/R2798/07	H6	N2	
3	A/mallard/Germany/Wv1317-21/03	H2	N3
	A/mallard/Germany/Wv481/04	H2	N3
	A/mallard/Germany/Wv672/04	H2	N3
	A/mallard/Germany/Wv1349-51K/03	H5	N3
	A/mute swan/Germany/R1042/07	H5	N3
	A/herring gull/Germany/R2788/06	H16	N3
	A/duck/Italy/636/03	H7	N3
	A/mallard/Germany/sko-212-219/07	H7	N3
4	A/mallard/Germany/R2127/07	H2	N3
	A/mallard/Germany/1682-85/03	H10	N4
	A/moorhen/Germany/Wv1703-04/03	H10	N4
6	A/mallard/Germany/Wv1677-81/03	H10	N4
	A/mallard/Germany/Wv1806-09/03	H4	N6
	A/mallard/Germany/Wv1754-57/03	H4	N6
	A/herring gull/Germany/Wv1136-40/03	H13	N6
	A/jackdaw/Germany/Wv1141/03	H13	N6
	A/mallard/Germany/SK50/06	H4	N6
	A/mallard/Germany/R2619/07	H4	N6
	A/mallard/Germany/R1648/07	H4	N6
	A/mallard/Germany/R1740/07	H4	N6
	A/mallard/Germany/R2897/07	H1	N6
7	A/mallard/Germany/R2924/07	H4	N6
	A/mallard/Germany/R2796/07	H9	N6
	A/mallard/Germany/Wv1776-80/03	H7	N7
	A/mallard/Germany/Wv190/05	H7	N7
	A/chicken/Germany/R28/03	H7	N7
	A/turkey/Germany/R11/01	H7	N7
	A/mallard/Germany/Wv1722/03	H10	N7
	A/mallard/Germany/Wv1015/04	H10	N7
	A/mallard/Germany/Wv907/04	H10	N7
	A/mallard/Germany/R2074/07	H10	N7
8	A/mallard/Germany/Wv1303-04/03	H3	N8
	A/mallard/Germany/Wv50/05	H3	N8
	A/mallard/Germany/Wv548/04	H3	N8
	A/mallard/Germany/Wv64-67/05	H3	N8
	A/ostrich/Germany/R1312/06	H6	N8
	A/mallard/Germany/R2188/29/06	H6	N8
	A/mallard/Germany/R2188/57/06	H6	N8
	A/mallard/Germany/R2187/2/06	H6	N8
	A/black-headed gull/Germany/R2064/06	H13	N8
	A/mallard/Germany/R2217/2/06	H3	N8
	A/mallard/Germany/R2218/06	H3	N8
	A/mallard/Germany/R2760/06	H3	N8

	A/black-headed gull/Germany/R2603/06	H13	N8
	A/mallard/Germany/R2322/07	H3	N8
	A/mallard/Germany/R1767/07	H6	N8
	A/mallard/Germany/R2484/07	H3	N8
9	A/mute swan/Germany/R1162/07	H11	N9
	A/mallard/Germany/R2706/07	H2	N9
	A/mallard/Germany/R2925/07	H11	N9
	A/duck/Germany/R2994/07	H11	N9

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**Table 4.** AIV field isolates used for validation of NA subtype RT-PCR assays by NI assay and NA sequencing (diagnostic sensitivity and specificity).

NA subtype	Identification of the AIV strain	HA Subtype (HA2 sequencing)	NA Subtype (determined by RT-PCR)	NA Subtype (determined by sequencing)	NI assay
1	A/mallard/Germany/Wv41/04	H7	N1	N1	N1
	A/mallard/Germany/R2843/06	H1	N1	N1	N1
	A/teal/Germany/Wv632/05	H5	N1	N1	N1
	A/turkey/Ontario/182/2000	H7	N1	N1	N1
2	A/mallard/Quebec/2323-19/06	H5	N2	N2	N2
	A/goose/Manitoba/428/06	H5	N2	N2	N2
	A/duck/Germany/R113/95	H9	N2	N2	N2
	A/mallard/Germany/R2551/06	H3	N2	N2	N2
3	A/chicken/British Columbia/04	H7	N3	N3	N3
	A/mallard/Germany/R2557/06	H5	N3	N3	N3
	A/teal/Germany/R14/06	H2	N3	N3	N3
	A/mallard/Iran/V31/04	H7	N3	N3	N3
4	A/quail/Germany/R44/06	H8	N3	N.A.	N3
	A/common buzzard/Germany/R1184/06	H10	N4	N4	N4
	A/mute swan/Germany/R2201/06	H10	N4	N4	N.A.
	A/mute swan/Germany/R736/06	H7	N4	N4	N4
6	A/mallard/Iran/V16/04	H8	N4	N4	N4
	A/mallard/Germany/Wv1806-09k/03	H4	N6	N6	N6
	A/mallard/Germany/R31/06	H4	N6	N6	N6
	A/black-headed gull/Germany/R2622/06	H13	N6	N6	N6
	A/mallard/Germany/R33/06	H4	N6	N6	N6
	A/black-headed gull/Germany/R2220/06	H13	N6	N6	N.A.
7	A/mallard/Germany/R1995/06	H4	N6	N6	N.A.
	A/mallard/Alberta/8734/07	H7	N7	N7	N7
	A/mute swan/Germany/R57/06	H7	N7	N7	N7
	A/greylag goose/Germany/R752/06	H7	N7	N7	N7
	A/pochard/Germany/R916/06	H7	N7	N7	N7
	A/teal/Germany/Wv1773/05	H7	N7	N7	N7
	A/mallard/Iran/V15/04	H10	N7	N7	N7
8	A/mallard/Iran/V40/04	H10	N7	N7	N7
	A/black-headed gull/Germany/R2613/06	H13	N8	N8	N8
	A/mallard/Germany/R2185/2/06	H6	N8	N8	N8
	A/egyptian goose/Germany/R2187/2/06	H6	N8	N8	N8
	A/coot/Germany/R61/1/06	H10	N8	N8	N8
	A/black-headed gull/Germany/R2609/06	H13	N8	N8	N8
9	A/mallard/Iran/V10/04	H3	N8	N8	N8
	A/mallard/Alberta/329/06	H5	N9	N9	N9
	A/mallard/British Columbia /544/05	H5	N9	N9	N9
	A/mallard/Germany/R2219/06	H11	N9	N9	N9
	A/wigeon/Germany/R636/07	H11	N9	N9	N9

N.A. = No results were obtained