Accepted Manuscript

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Authors: S.R. Fereidouni, E. Starick, C. Grund, A. Globig, T.C. Mettenleiter, M. Beer, T. Harder

PII: S0378-1135(08)00467-7
Reference: VETMIC 4220

To appear in: VETMIC

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


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

Fereidouni¹, S.R.; Starick¹, E.; Grund¹, C.; Globig¹, A.; Mettenleiter², T.C., Beer¹, M.; Harder¹,*, T.

Running title:

RT-PCR subtyping of avian influenza A virus neuraminidase

1 – Institute of Diagnostic Virology, 2- Institute of Molecular Biology, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, 17493 Greifswald – Insel Riems, Germany

Correspondent footnote:
Timm C. Harder, O.I.E. and National Reference Laboratory for Avian Influenza, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, D-17493 Greifswald – Insel Riems, Germany; phone: ++49 38351 7152, fax: ++49 38351 7275, e-mail: timm.harder@fli.bund.de
Abstract

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

Key words
Influenza A virus, Neuraminidase, Subtyping, RT-PCR
Introduction

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

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

Several studies focussed on the identification of different HA subtypes by specific RT-PCR assays (Stockton et al., 1998; Starick et al., 2000; Herrmann et al., 2001; Lee et al., 2001; Munch et al., 2001; Takao et al., 2002; Spackman et al., 2003), or by a pan-HA RT-PCR targeting the more conserved HA2 section of the HA gene and subsequent sequencing of the amplicons or microarray detection (Phipps et al., 2004; Gall et al., 2008). However, only few published studies have focussed on the subtype identification of influenza virus neuraminidases. These concentrate mainly on subtypes N1 and N2 associated with human
influenza viruses (Wright et al., 1995; Li et al., 2001; Takao et al., 2002; Barr et al., 2006; Mak et al., 2006; Payungporn et al., 2006; Zou et al., 2007). Recently, a pair highly degenerated primers has been published which might be suitable for amplification of an NA gene fragment from all nine subtypes (Alvarez et al., 2008).

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

Materials & Methods

Origin of influenza virus reference strains, field samples and sera. Reference isolates and sera are maintained in the virus repository of the OIE and National Reference Laboratory for Avian Influenza at the Friedrich-Loeffler-Institut (NRL-AI). Several of these viruses were donated by reference laboratories in Canada, Dubai, Italy, U.K. and Vietnam. A total of 119 of AIV RNA positive field samples was obtained in the framework of nationwide monitoring programs for AIV in wild birds in Germany during 2001-2007 (unpublished), in other countries (Fereidouni et al., 2005). The majority of samples originating from wild birds in Germany had been pre-tested by rRT-PCR for influenza virus M gene by the regional laboratories of the Federal States of Germany and were subsequently sent to the NRL-AI for confirmation and further characterization. Virus isolation from these samples was attempted as previously described (Fereidouni et al., 2005). Other avian pathogenic viruses included infectious bronchitis virus (IBV, M41 strain), infectious laryngotracheitis virus (ILTV, A-489 strain), infectious bursal disease virus (IBDV, D78 strain) and avian paramyxoviruses (APMV) types 1-9 (APMV-1: La Sota strain; APMV-2: chicken/California/Yucaipa/56; APMV-3: parakeet/Netherlands/449/75; APMV-4: duck/Hungary/D3/75; APMV-6: duck/HongKong/189/77; APMV-7: dove/Tn/u/75; APMV-8: goose/Delaware/1057/76; APMV-9: duck/New York/22/78) were provided by the virus collection at the Friedrich-Loeffler-Institut.
**Primer design.** Initially, a comprehensive selection of mammalian and avian viral NA full length gene sequences available from the Influenza Sequence Database (http://www.flu.lanl.gov/index.html) and Genbank was aligned (clustal X, version 1.83). The alignment finally comprised 2104 N1, 794 N2, 163 N3, 23 N4, 51 N5, 152 N6, 49 N7, 138 N8 and 71 N9 sequences. Primers specific for each single NA subtype and hybridizing to all published sequences within that subtype were selected whereby tolerating up to four degenerated sites per primer (Table 1).

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

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

**Neuraminidase inhibition (NI) assay.** The protocol described by Van Deusen et al. (1983) using fetuin as substrate was followed with some modifications. Briefly, allantoic fluid containing an AIV isolate was diluted 1:4 using one volume of CaCl$_2$/NaCl-solution (pH 5.6) and two volumes of phosphate buffer (pH 5.9). Virus dilutions were incubated in 48-well microtiter plates (Costar) with 1:10 and 1:20 diluted antisera followed by incubation with fetuin (F3004, SIGMA). Sodium periodate (NaIO$_4$, S1147, SIGMA) dissolved in orthophosphoric acid served as an oxidant, and the reaction was completed by sodium meta-arsenit (1287, FLUKA) dissolved in H$_2$SO$_4$ and thio-barbituric acid (T-5500, SIGMA) (pH 9.0). All
reagents with the exception of thio-barbituric acid (200 µl) were used in amounts of 50 µl. Positive controls for each subtype (reference isolates) were included in each assay.

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

Results

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

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

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

Analytical sensitivity and specificity. The analytical sensitivity of each NA subtype-specific RT-PCR was assessed using \( \log_{10} \) dilutions of RNA extracted from AIV reference isolates carrying the matching NA. The number of strains available ranged from ten (N1) to one (N4)
per subtype (Table 2). Results were compared with an rRT-PCR targeting the M gene
fragment (Spackman et al., 2002) and carried out with the same log_{10} dilution series of AIV
RNA. Fragments of predicted size were amplified for all NA subtypes from RNA dilutions of
reference isolates for which C_{t} values of ≤ 32 were obtained in the M-specific rRT-PCR. For
NA subtypes 1, 2, 4 and 6, amplicons were also obtained from RNA dilutions of C_{t} values of
≤ 35 (data not shown).

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

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

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

For the further assessment of performance characteristics of the NA subtype specific RT
PCRs with diagnostic material, amplificates from all 41 AIV field isolates were sequenced
directly to confirm the specificity as assessed by amplicon size and predicted by primer
identity. In all cases, the NA subtype predicted by amplicon size and specific primer pair used for amplification, and the sequence obtained proved to match (Table 4). For the same set of AIV field isolates, the NI assay was used as an additional specificity control. Again, a complete match of results was achieved (Table 4). Analysis of available full length sequences revealed one or two primer mismatches for some isolates which, however, did not influence the successful amplification of all viruses tested.

Discussion

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

Advantages of influenza A virus molecular subtyping directly from diagnostic samples are obvious: (i) independence from labour- and time-consuming virus isolation (requirement of embryonated SPF chicken eggs) and hemagglutination-inhibition (HI) and NI assays
(requirement of specific antisera, handling of hazardous substances), (ii) acquisition of NA-specific sequences possible, (iii) reliable results within a single working day, (iv) increased biosecurity as no replication-competent viruses need to be handled. Microarray hybridization assays such as described by Kessler et al. (2004) or Sengupta et al. (2003) and RT-PCR using one uniform primer pair with subsequent sequencing (Alvarez et al., 2008) are also suitable for NA subtyping of avian influenza viruses. In contrast, however, to conventional NA subtype-specific RT-PCRs these assays require expensive equipment and consumables and are usually not available in less well equipped laboratories.

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

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

In summary, our conventional RT-PCRs are suitable for NA subtyping of AIV directly from diagnostic specimens, provided the quality and quantity of RNA is adequate. In our hands, samples with C\textsubscript{T} values equal to or below 32 in M gene-specific rRT-PCRs allowed an unambiguous NA subtyping; for some NA subtypes (N1, N2, N4, N6) even samples with lower viral loads were suitable for typing. Attempts to establish multiplex PCR assays for the NA subtyping are under way.
For a full molecular subtyping of AIVs from diagnostic specimens with positive results by rRT-PCR (M gene), we suggest the following procedure:

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

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

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

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

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

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

Acknowledgment

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


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

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<th>Length (bp)</th>
<th>Position in reference sequence</th>
<th>Expected product size (bp)</th>
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1 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.

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N.A. = No results were obtained