

Detection of avian influenza virus and Newcastle Disease virus by duplex One Step RT PCR

Research Article

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Abstract: Newcastle disease Virus (NDV), a member of the *Paramyxoviridae* family, and Influenza virus, from the *Orthomyxoviridae* family, are two main avian pathogens that cause serious economic problems in poultry farming. NDV strains are classified into three major pathotypes: velogenic, mesogenic, and lentogenic. Avian influenza viruses (AIV) are also divided into: low pathogenic (LPAI) and highly pathogenic (HPAI) strains. Both viruses are enveloped, single stranded, negative-sense RNA viruses which give similar symptoms ranging from sub-clinical infections to severe disease, including loss in egg production, acute respiratory syndrome, and high mortality, depending on their level of pathogenicity. This similarity hinders diagnosis when based solely on clinical and post mortem examination. Most of the currently available molecular detection methods are also pathogen-specific, so that more than one RT-PCR is then required to confirm or exclude the presence of both pathogens. To overcome this disadvantage, we have applied a One Step Duplex RT-PCR method to distinguish between those two pathogens. The main objective of the project was to develop a universal, fast, and inexpensive method which could be used in any veterinary laboratory.

Keywords: Newcastle disease virus • Influenza virus • Differentiation • One Step RT-PCR • Duplex

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1. Introduction

Avian influenza virus (AIV) is a member of the *Orthomyxoviridae* family. It has a spherical, enveloped capsid composed of a matrix protein. The virus contains linear, segmented, single-stranded RNA of negative polarity, covered by nucleoprotein. Eight segments encode twelve proteins. On the surface of the virion, there are ion channels and two main glycoproteins, hemagglutinin and neuraminidase, which are responsible for the diversity of this virus. Highly pathogenic avian influenza (HPAI) strain cause severe symptoms in the respiratory tract, as well as in the digestive and nervous systems which lead to a high mortality rate of up to 100%. This group includes, in particular, some H5 and H7 influenza strains. Other strains cause milder symptoms: apathy, decrease in laying capacity, and mild respiratory tract disorders, and are known as LPAI (low pathogenic avian influenza)

[1,2]. The differences in virulence level have a molecular basis, namely changes in the cleavage site of HA0. In HPAI strains, in the cleavage site, there is a region rich with basic amino acids. This motif can be cleaved by the majority of enzymes present in various cells of the host organism [3].

Newcastle disease virus (NDV) belongs to *Mononegavirales* order, *Paramyxoviridae* family, and *Avulavirus* genus. The viral particle is composed of a matrix protein, surface glycoproteins (hemagglutinin-neuraminidase and a fusion protein), and has a lipid double layer on its surface. The genome of NDV is linear, non-segmented, single-stranded RNA encoding 8 proteins [4]. NDV strains are classified into one of the three major pathotypes: velogenic, mesogenic, and lentogenic [5], according to pathogenicity *in vivo*. Similarly to influenza virus, NDV pathogenicity has a molecular background. The product of F gene of highly pathogenic strains

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(velogenic) is recognized by a common, subtilisin-like protease, while the products of genes of low pathogenic strains (mesogenic, lentogenic) are recognized only by trypsin-like proteases present mainly in the respiratory tract [6,7].

Newcastle disease virus and avian influenza virus cause similar symptoms in infected birds, which makes quick diagnosis difficult. The symptoms are observed in the digestive tract, respiratory tract, and nervous system. The incubation period normally lasts 2 to 6 days, and this timeframe depends on the level of virulence of the strain and the pre-infection condition of the bird. The most frequent clinical symptoms are apathy, loss of appetite, diarrhoea, secretion from nostrils and beak, breathing difficulties, strong convulsions, neck torsion, drooping wings, paralysis. In internal organs, hemorrhagic lesions occur. Also, a significant decrease in laying capacity, and laying eggs without shells, can be observed [1,8,9].

Traditional methods of isolation and identification of viruses, such as propagation in chicken embryos or cell culture followed by identification in hemagglutination-inhibition test (HI) are time-consuming and laborious. Rapid-developing molecular biology tools are an alternative to traditional diagnostic methods and allow fast and definite identification of a particular pathogen in a sample.

The previously developed tests are focus mostly on identification of only a few types of influenza serotypes (H3, H5, H7, and H9) and NDV. The primers used in those tests are based on fragments of hemagglutinin gene (for detection of influenza virus) and fusion (F) protein (for detection of NDV) [10-13]. These genes are the most variable fragments of AIV and NDV genome respectively, therefore applying such methods may lead to some false negative results.

Naturally, there are a few more publications related to detection of NDV and AIV [14-18] but the main disadvantage of the tests presented there is the need to use two-step methods, which are more complicated, expensive and time-consuming.

In this study, we present a new duplex One step RT-PCR assay for the simultaneous detection and differentiation of avian influenza virus and Newcastle disease virus. Using primers designed to conserve fragments of the genes of both viruses assures the universality of this method. One step RT-PCR method reduces the time and cost of analysis.

2. Experimental Procedures

2.1 Virus isolates and RNA extraction

Eighteen NDV strains, fourteen influenza and twelve other (negative control strains) viruses listed in

Table 1 were isolated from allantoic fluids of SPF embryonated eggs using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). All strains with the exception of A/ostrich/Denmark-Q/72420/96 and A/African starling/England/983/79 (source: AHVLA Weybridge, UK), were from the collection of the National Veterinary Research Institute (Pulawy, Poland). APMV strains were purchased from x-OvO Limited (Dunfermline, United Kingdom). Reovirus (Reo S1133) and infectious bronchitis virus (IB K+ 4/91) were components of Nobilis REO 1133 and Nobilis IB 4-91 vaccines (Intervet/Schering-Plough Animal Health, Holland).

2.2 Primers design and Multiplex One Step RT-PCR reaction

In silico analyses were performed with Geneious software (<http://www.geneious.com/>) using sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Two sets of primers based on the conserved fragments of the M genes of NDV and IV were used in this study: Primers: IV-F-VII/25 (5'-AGATGAGTCTTCTAACCGAGGTCG-3') and IV-R-VII/100 (5'-TGCAAAAACATCTTCAAGTCTCTG-3') amplifies a 100 bp long fragment of the matrix protein gene of AIV [19], while primers: NDV-F-4011 (5'-GTCCCAAATACCGGAGACCT-3') and NDV-R-4142 (5'-TTGTTTGCCACAACCCTACAG-3') are specific to the conserved fragment of matrix protein gene of NDV, and the amplification product is 152 bp long. BLAST sequence analyses were also performed to confirm the specificity of the designed primers.

The reaction was carried out according to the Transcriptor One-Step RT-PCR Kit protocol (Roche Diagnostics, Mannheim, Germany). The reaction mixture (50 µl) contained: viral RNA (5 µl), both sets of primers (0.3 µM each), buffer (1x), Transcriptor Enzyme Mix, and water. The conditions of the reaction were established through optimization and are presented in Table 2. The results were visualized in 2.5% agarose gel.

2.3 RT-PCR sensitivity and specificity

The detection limit was determined using a known number of copies of M genes fragments of both viruses. *In vitro* transcription of RNA was performed using TranscriptAid T7 High Yield Transcription Kit (Fermentas, Thermo Fisher Scientific, Vilnius, Lithuania), according to protocol instructions. The concentration of plasmid RNA was determined by spectrophotometry. Copy number was calculated and the RNA samples were serially, 10-fold diluted to serve as standards for determination of the sensitivity of One Step RT-PCR assay.

No	Strain	Pathotype / Lineage/ Serotypes	Results of test	
			AIV	NDV
1	Ulster 2C – reference	L (1)	-	+
2	LaSota – reference AF077761	L (2)	-	+
3	Clone 30 – commercial vaccine	L (2)	-	+
4	APMV-1/chicken/Poland/111/90	L (2)	-	+
5	APMV-1/chicken/Poland/89/90	L (2)	-	+
6	APMV-1/chicken/Poland/299/92	L (2)	-	+
7	APMV-1/chicken/Poland/117/90	L (2)	-	+
8	APMV-1/chicken/Poland/548/04	L (2)	-	+
9	APMV-1/turkey/Poland/549/04	L (2)	-	+
10	APMV-1/chicken/Poland/18/91	L (2)	-	+
11	APMV-1/chicken/Poland/Radom/70	V (3b)	-	+
12	Italy/2736/00 – reference AY562989	V (4)	-	+
13	APMV-1/pigeon/Poland/AR1/95	V (4)	-	+
14	APMV-1/pigeon/Poland/AR4/95	V (4)	-	+
15	APMV-1/pigeon/Poland/AR5/92	V (4)	-	+
16	APMV-1/pigeon/Poland/M470/05	V (4)	-	+
17	APMV-1/pigeon/Poland/AR2/95	M (4)	-	+
18	APMV-1/pigeon/Poland/AR7/98	M (4)	-	+
19	A/mallard/Poland/275/09/H2N?	H2N?	+	-
20	A/mallard/Poland/227/08/H3N3	H3N3	+	-
21	A/mallard/Poland/214/09/H3N8	H3N8	+	-
22	A/mallard/Poland/96/10/H3N9	H3N9	+	-
23	A/buzzard/Poland/266B/07/H5N1	H5N1	+	-
24	A/greylag goose/Poland/74/10/H5N2	H5N2	+	-
25	A/ostrich/Denmark-Q/72420/96 /H5N2	H5N2	+	-
26	A/swan/Poland/467/06/H5N1	H5N1	+	-
27	A/swan/Poland/937/06/H5N1	H5N1	+	-
28	A/African starling/England/983/79/H7N1	H7N1	+	-
29	A/mallard/Poland/16/06/H7N7	H7N7	+	-
30	A/mallard/Poland/01/08/H7N7	H7N7	+	-
31	A/mallard/Poland/41/09/H7N7	H7N7	+	-
32	A/common gull/Poland/241/11/H13N?	H13N?	+	-
33	APMV – 2	NA	-	-
34	APMV – 3	NA	-	-
35	APMV - 4	NA	-	-
36	APMV - 6	NA	-	-
37	APMV - 7	NA	-	-
38	APMV - 8	NA	-	-
39	APMV - 9	NA	-	-
40	Coronavirus 165/08 5-8	NA	-	-
41	Rotavirus G036/10 1-5	NA	-	-
42	Gumboro virus 131/92	NA	-	-
43	Reovirus Reo S1133 (Nobilis REO 1133, Intervet)	NA	-	-
44	IB K+ 4/91 (Nobilis IB 4-91, Internet)	NA	-	-

Table 1. List of NDV, AIV strains and heterologous pathogens investigated by duplex One Step RT-PCR. Results of analysis.

L, lentogenic; M, mesogenic; V, velogenic; NA, not applicable

	Step	Temp.	Time	Cycles	
Reverse transcription		50°C	30 min	1	
Initial denaturation		94°C	7 min	1	
PCR profile	Denaturation	94°C	10 s	10	
	Annealing	53°C	30 s		
	Elongation	68°C	10 s		
	Denaturation	94°C	10 s		
	Annealing	60°C	30 s		25
	Elongation	68°C	15 s		
Final Elongation		68°C	7 min	1	

Table 2. Conditions of One Step RT-PCR reaction.

The specificity of the newly developed assay was investigated using the RNA extracted from NDV and AIV strains, as well as from 12 other avian viruses presented in Table 1.

2.4 Detection of NDV and AIV from experimentally co-infected birds

In order to check the new method and to compare it to the previously described tests, an experiment with co-infected chickens was performed. Four-week-old SPF chickens were infected with both viruses: NDV – LaSota and AIV – H7N1 using a dose of $10^{6.0}$ EID₅₀ and $10^{7.5}$ EID₅₀, respectively, intraocularly and intranasally. Swabs from the cloaca and trachea were collected on the fourth and fifth day of the experiment and examined by the new method and by two previously described tests [12,19]. Animal experiments in this study were approved by the Local Animal Welfare Committee (22/2006).

3. Results

3.1 Detection and differentiation of NDV and IV strains by duplex One Step RT-PCR assay; specificity of the assay

Eighteen strains of Newcastle Disease Virus (including ten lentogenic, two mesogenic, and six velogenic isolates) and twelve avian influenza virus strains were tested. The expected PCR products of 152 bp and 100 bp of length, respectively, were amplified in all NDV/AIV samples. The new One Step RT-PCR assay was shown to detect and differentiate both viruses even in co-infection (Figure 1, Table 1).

The specificity of the new assay was investigated by analysing 12 heterologous avian pathogens (Table 1). No amplification products were detected

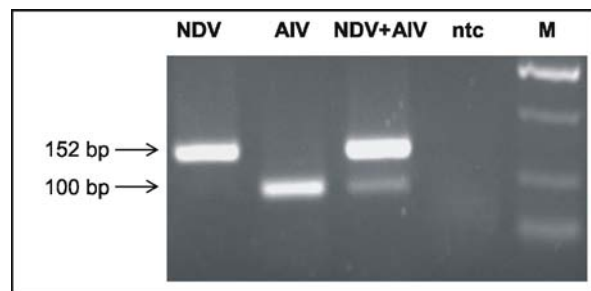


Figure 1. Analysis of duplex One Step RT-PCR reaction. 2.5% Agarose gel with EtBr. ntc – no template control, M – Mass marker (Hyper Ladder II, BioLine). NDV and AIV – single infection by Newcastle Disease Virus or avian influenza virus; NDV+AIV – co-infection.

when templates originating from those twelve other, unrelated viruses were used, while all the templates from NDV and AIV strains gave distinctive products.

3.2 Analysis of infected animals and detection limit

The detection limit of this assay was assessed by analysing serial dilutions of *in vitro* transcribed RNA of M genes of NDVs and AIVs. The detection limits were 10^3 copies of RNA per reaction, even in co-infection. Additional analysis in the presence of contamination with other viruses' RNA did not change the detection limits.

Moreover, samples from chickens infected with two viruses: NDV (LaSota) and AIV (H7N1), were also analysed by the new method as well as two previously described methods. The results of the analysis show the possibility to use this new assay as an alternative method for detection and differentiation of both viruses (Table 3). The new test also detects viruses in samples with low concentrations of pathogens (Ct value higher than 30)

		Chicken no 1				Chicken no 2			
		AIV		NDV		AIV		NDV	
		A	OS	B	OS	A	OS	B	OS
4 dpi	O	20.48	+	n.d.	n.d.	20.71	+	29.82	+
4 dpi	C	22.76	+	29.74	+	24.80	+	n.d.	n.d.
5 dpi	O	20.68	+	27.34	+	21.69	+	33.69	+
5 dpi	C	22.07	+	n.d.	n.d.	23.40	+	n.d.	n.d.
		Chicken no 3				Chicken no 4			
		AIV		NDV		AIV		NDV	
		A	OS	A	OS	A	OS	B	OS
4 dpi	O	21.68	+	n.d.	n.d.	21.98	+	35.00	+
4 dpi	C	23.94	+	n.d.	n.d.	19.55	+	33.39	+
5 dpi	O	21.86	+	34.55	+	23.34	+	n.d.	n.d.
5 dpi	C	25.01	+	34.97	+	20.72	+	n.d.	n.d.

Table 3. Comparison of different methods for detection of influenza virus (H7N1) and NDV (LaSota) in infected chickens. Oral (O) and cloacal (C) swabs were collected 4 and 5 days after infection (dpi).

AIV – Avian Influenza Virus; NDV – Newcastle Disease Virus; dpi – day post infection; O – Oral swab; C – Cloacal swab; “+” – positive; n.d. – not detected; A – Ct values obtained from real-time PCR method of detection based on Spackman et al. [19]; B – Ct values obtained from real-time PCR method of detection based on Kim et al. [12]; OS – One Step RT-PCR Method.

4. Discussion

In this study, we present a new duplex One Step RT-PCR assay for the detection and differentiation of avian influenza and Newcastle disease viruses. The test is relatively quick and very easy to perform. It is based on the analysis of conserved fragments of genes coding M proteins of both viruses. Both genes in both viruses show the highest level of conservation, and thus can serve as a universal tool for diagnostics and differentiation of NDVs and AIVs. Owing to the substantial difference in product lengths, the risk of a false assessment of the sample is minimized. At the same time, it allows detection of both viruses in co-infection.

In previously described tests for differentiation of these two viruses, the authors selected hemagglutinin gene for influenza virus and fusion protein gene for NDV. Other tests are more universal but need a two-step procedure. However, these genes are very variable, and the tests based on their detection may fail in some cases. Moreover, those tests focus on the detection of particular subtypes of AIV (H3, H5, H7, and/or H9). Though they are very useful, they do not allow detection of other subtypes of influenza virus that pose a danger to wild and domestic birds [11,13,16,18,20,21].

The advantage of the newly developed test is the ability to detect a broad spectrum of isolates, as the primers bind to the conserved fragment of M genes. This method is naturally less sensitive when compared to real-time PCR assays,

however it may be a good alternative for laboratories without RTqPCR equipment. Moreover, the one step approach used in this method reduces the time and cost of analysis.

This assay may be the first step of the complete analysis of infected birds. The international regulations require a more precise characterization of the detected virus based on the virulence markers located in HA (AIV) or F (NDV) genes [22,23]. Therefore, the method can serve as a useful preliminary tool, but all positive samples should be submitted to a more specific molecular test, e.g. HA- and F-specific, for determination of the pathogenicity of NDV and AIV and its subtypes [13,22-24].

5. Conclusions

Birds infected by Newcastle disease or influenza virus show very similar symptoms which are hard to distinguish by veterinarians even during necropsy. For this reason, the new One Step RT-PCR assay for direct detection and differentiation of NDV and IV was developed. The conserved fragments of M genes used to design primers and probes make this method widely applicable. The assay can be applied by veterinary diagnosticians for the preliminary differentiation of NDV and influenza virus.

The ability to detect both these major, avian pathogens, even in co-infections, through a very easy procedure, makes this method very attractive for application in veterinary laboratories.

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References

- [1] Capua I., Alexander D.J., Avian influenza and human health, *Acta Trop.*, 2002, 83, 1–6
- [2] Alexander D.J., A review of avian influenza in different bird species, *Vet. Microbiol.*, 2000, 74, 3-13
- [3] Suarez D.L., Schultz-Cherry S., Immunology of avian influenza virus: a review, *Dev. Comp. Immunol.*, 2000, 24, 269-283
- [4] Lamb R.A., Collins P.L., Kolakofsky D., Melero J.A., Nagai Y., Oldstone M.B.A., et al., Family paramyxoviridae, In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (Eds.), *Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses*, Elsevier Academic Press, 2005
- [5] Beard C., Hanson R., Newcastle Disease, In: Hofstad M., Barnes H., Calnek B., Reid W., Yoder H., (Eds.), *Disease of Poultry*, 8th ed., Iowa State University Press, 1984
- [6] Collins M.S., Strong I., Alexander D.J., Pathogenicity and phylogenetic evaluation of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses" based on the nucleotide sequence of the fusion protein gene, *Arch. Virol.*, 1996, 141, 635-647
- [7] Nagai Y., Klenk H.D., Rott R., Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus, *Virology*, 1976, 72, 494-508
- [8] Panda A., Huang Z., Elankumaran S., Rockemann D.D., Samal S.K., Role of fusion protein cleavage site in the virulence of Newcastle disease virus, *Microb. Pathog.*, 2004, 36, 1-10.
- [9] Seal B.S., King D.J., Bennet J.D., Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis, *J. Clin. Microbiol.*, 1995, 33, 2624-2630
- [10] Creelan J.L., Graham D.A., McCullough S.J., Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction, *Avian Pathol.*, 2002, 31, 493-499
- [11] Chen H., Zhang J., Sun D., Zhang D., Cai X., Liu X., et al., Rapid discrimination of H5 and H9 subtypes of avian influenza viruses and Newcastle disease virus by multiplex RT-PCR, *Vet. Res. Commun.*, 2008, 32, 491–498
- [12] Kim L.M., Suarez D.L., Afonso C.L., Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real time reverse transcription polymerase chain reaction assay, *J. Vet. Diagn. Invest.*, 2008, 20, 414-425
- [13] Tang Q., Wang J., Bao J., Sun H., Sun Y., Liu J., et al., A multiplex RT-PCR assay for detection and differentiation of avian H3, H5, and H9 subtype influenza viruses and Newcastle disease viruses, *J. Virol. Methods*, 2012, 181, 164-169
- [14] Pang Y., Wang H., Girshick T., Xie Z., Khan M.I., Development and Application of a Multiplex Polymerase Chain Reaction for Avian Respiratory Agents, *Avian Dis.*, 2002, 46, 691-699
- [15] Soaresa P.B.M., Dem'etrioa C., Sanfilippoa L., Kawanotob A.H.N., Brentanoc L., Durigona E.L., Standardization of a duplex RT-PCR for the detection of Influenza A and Newcastle disease viruses in migratory birds, *J. Virol. Methods*, 2005, 123, 125–130
- [16] Farkas T., Antal M., Sami L., German P., Kecskemeti S., Kardos G., et al., Rapid and Simultaneous Detection of Avian Influenza and Newcastle Disease Viruses by Duplex Polymerase Chain Reaction Assay, *Zoonoses Public Health.*, 2007, 54, 38–43
- [17] Slomka M.J., Coward V.J., Banks J., Löndt B.Z., Brown I.H., Voermans J., et al., Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union, *Avian Dis.*, 2007, 51, 227-234
- [18] Rashid S., Naeem K., Ahmed Z., Saddique N., Abbas M.A., Malik S.A., Multiplex polymerase chain reaction for the detection and differentiation

- of avian influenza viruses and other poultry respiratory pathogens, *Poult. Sci.*, 2009, 88, 2526-2531
- [19] Spackman E., Senne D.A., Myers T.J., Bulaga L.L., Garber L.P., Perdue M.L., et al., 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.*, 2002, 40, 3256-3260
- [20] Deng M.J., Long L., Xiao X.Z., Wu Z.X., Zhang F.J., Zhang Y.M., et al., Immuno-PCR for one step detection of H5N1 avian influenza virus and Newcastle disease virus using magnetic gold particles as carriers, *Vet. Immunol. Immunopathol.*, 2011, 141, 183-189
- [21] Malik Y.S., Patnayak D.P., Goyal S.M., Detection of three avian respiratory viruses by single-tube multiplex reverse transcription–polymerase chain reaction assay, *J. Vet. Diagn. Invest.*, 2004, 16, 244–248
- [22] Anon., 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) (Text with EEA relevance) (2006/437/EC), 2006
- [23] Anon., Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012. Chapter 2.3.14: Newcastle disease (version adopted in 2012). Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf, 2012
- [24] Nidzworski D., Rabalski L., Gromadzka B., Detection and differentiation of virulent and avirulent strains of Newcastle Disease Virus by real-time PCR, *J. Virol. Methods*, 2011, 173, 144-149