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MOLECULAR METHODS FOR THE DETECTION OF AVIAN INFLUENZA TYPE A VIRUSES

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Abstract

Two RT-PCR methods with primers aimed at NP and M genes for the detection of avian influenza virus (AIV) in allantoic fluids of SPF embryonated eggs as well as in tissues of SPF chickens infected experimentally are described. Both methods proved to be equally specific but RT-PCR aimed at M gene seemed to be more sensitive (10 EID_{50}) than the second one (100 EID_{50}). To detect AIV in chicken tissues, SPF chickens were inoculated intraocularly with 10^6 EID_{50} of A/African starling/England/983/79 reference strains (H7N1) and 5 d after inoculation. various tissue samples were aseptically collected, examined with PCR-based methods and subjected to virus isolation on SPF embryos. The results showed high concordance between the used methods, but some discrepancies were also noted.

Key words: avian influenza virus, isolation, RT-PCR, detection, diagnosis.

Avian influenza viruses (AIV) belong to the *Influenzavirus A* genus of the *Orthomyxoviridae* family and their genome is single negative-stranded, segmented RNA. Avian influenza viruses can be divided into subtypes on the basis of two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Currently, 16 HA and 9 NA subtypes are known (6, 15). All 16 HA NA subtypes are found in aquatic birds, which are considered as the reservoir of all influenza A viruses responsible for their spreading in the nature (1, 2, 6, 17). AIVs differ in their virulence and have been grouped into two pathotypes: low pathogenic (LPAI) and highly pathogenic avian influenza (HPAI). In chickens, only the H5 and H7 subtypes have so far been characterized as HPAI, but not all H5 and H7 isolates belong to this pathotype (2). High pathogenicity is primarily determined by the presence of multiple basic amino acids at the cleavage site of the HA protein (12, 18). In the diagnosis of avian influenza, the methods recommended by OIE Manual and EU Council Directive comprise virus isolation on SPF or SAN embryonated eggs and identification in the haemagglutination inhibition (HI) test (4, 11). Recently, OIE regulations have admitted to replace, under some circumstances, the isolation in embryos by RT-PCR. However, the molecular methods proposed to detect influenza type A viruses contained no specific sets of primers and pointed only at nucleoprotein (NP) or matrix (M) genes, which are known to posses highly conserved regions (8, 9, 13).

The aim of the present study was to evaluate two RT-PCR methods based on NP and M genes for rapid detection of influenza type A virus in experimentally infected chicken embryos and chickens.

Material and Methods

Virus strains and genome isolation. All 16 influenza A virus strains, of avian and non-avian origin, used in this study are listed in Table 1. The full name (indicating source and geographic origin, date of isolation) of these strains, HA and NA subtypes as well as HA titer are mentioned.

Prior to testing in RT-PCR, all the strains were propagated on SPF embryonated eggs (Valo-Lohmann, Germany) and allantoic fluid was used for viral genome extraction. Viral RNAs were extracted with the RNeasy Mini Kit (Qiagen, CA, USA) as recommended by the supplier.

Virus isolation assay. Virus isolation was performed on 9-11 SPF embryonated eggs (Valo-Lohmann, Germany) according to the Annex III of the Council Directive 92/40/EEC (4).

cDNA synthesis and polymerase chain reaction (PCR). The one-tube reverse transcription (RT)-PCR was carried out according to the manufacturer instruction (OneStep RT-PCR Kit, Qiagen). The 50 µl reaction mixture contained 5 µl of extracted RNA, 0.4 mM each dATP, dGTP, dCTP, dTTP, 10 mM primers, 1xRT-PCR buffer, 1xQ buffer and 2 µl of Qiagen OneStep Enzyme Mix.

Table 1
Virus isolates of avian and non-avian origins used in the study

Influenza A virus strain	HA subtypes	NA subtypes	HA titer	Lanes on agarose gels in Fig. 1
A/Ostrich/Denmark/725/96	5	2	128	1
A/African starling/England/983/79	7	1	128	2
A/Turkey/Poland/85/95	7	2	-	3
A/Turkey/Poland/95/95	7	4	1024	4
A/Seagull/Poland/236-245/02	5	2	32	5
A/Robin/Poland/119-128/02	5	-	128	6
A/Equine/Praque/1/56	7	7	256	7
A/Equine/Kentucky/1/81	3	8	256	8
A/Swine/Belgium/1/98	1	1	512	9
A/Swine/England/17394/96	1	2	128	10
A/Swine/Flanders/1/98	3	2	512	11
A/Swine/Poland/KPP1/04	1	1	nd	12
A/Swine/Poland/ JPW1/04	1	1	nd	13
A/Swine/Poland/GWW1/04	1	1	512	14
A/Swine/Poland/SPT1/05	1	1	64	15
A/Swine/Poland/EWW1/05	1	1	nd	16

nd - not detected

Table 2
Primers used for the detection of influenza A viruses by RT-PCR

Primer	Target/localization*	Sequence	Reference
IVA-M1	M/249-265	5'AGCGTAGACGCTTTGTC'3	(13)
IVA-M2	M/833-849	5'GACGATCAAGAATCCAC'3	
NP-1200F	NP/1094-1115	5'CAG(A/G)TACTGGGC(A/T/C)ATAAG(A/G)AC'3	(8)
NP-1529R	NP/1502-1524	5'GCATTGTCTCCGAAGAAATAAG'3	

* primer positions refer to the sequences for A/chicken/FPV/Weybridge (M, accession nr M23929), A/chicken/British Columbia/04 (NP, accession nr AY650273).

Table 3
Programme parameters for reverse transcription, PCR and the size of obtained products

Primer	Reverse transcription	PCR programme	PCR product (bp)
IVA-M1 IVA-M2	50^0C, 30 min	A	601
NP-1200F NP-1529R		B	330

A: 95^0C, 15 min; 40x (94^0C, 1 min; 50^0C, 1 min; 72^0C, 1 min); 72^0C, 10 min
B: 95^0C, 15 min; 40x (94^0C, 1 min; 55^0C, 1 min; 72^0C, 1 min); 72^0C, 10 min

The primers used in the study and the programme parameters are shown in Tables 2 and 3, respectively. The PCR products were separated on a 2% agarose gel stained with ethidium bromide and visualized by ultraviolet (UV) transillumination.

Sensitivity of RT-PCR. The sensitivity of the RT-PCR was established by ten-fold diluting of allantoic fluid containing A/Ostrich/Denmark/725/96 ($10^{7,5}$ EID_{50}/0.1 ml). Subsequently, RNA was isolated and RT-PCR was performed according to the procedure described above. The highest dilution with positive RT-PCR signal was determined by two virus detecting RT-PCR methods.

Specificity of RT-PCR. To evaluate specificity of the method, cDNA of the following RNA viruses was used: paramyxovirus serotype 3, avian influenza virus (H5N2 and H7N1), infectious bursal disease virus (vaccinal 228E strain and very virulent 99/150 Polish field strain), and avian infectious bronchitis virus (strains M-41 and 4/91).

Experimental design. Four 4 week-old SPF chickens (Valo-Lohmann, Germany) kept in isolation were inoculated intraocularly and intranasally with 10^6 EID_{50} of the A/African starling/England/983/79 virus. Five days post inoculation (*p.i.*) tracheal and cloacal swabs as well as tissue samples from the trachea, lung, liver, spleen, heart, brain, kidneys, bursa of Fabricius, duodenum, caecal tonsils, and rectum were aseptically collected.

Supernatants of the organs (used for virus isolation as well as for RT-PCR) were prepared according to the Annex III of the Council Directive 92/40/EEC (4). Tracheal and cloacal swabs were suspended in PBS with antibiotics (1 ml/swab) and after

1 h of incubation at room temperature and centrifugation, supernatants were harvested. All the supernatants were pooled in batches of four. Additionally, pooled supernatants of the trachea, lungs, liver, spleen, kidneys, heart and brain (pooled sample N°1) and duodenum, caecal tonsils and rectum (pooled sample N°2) were also used as separate samples.

Results

Using both sets of primers, NP1200F and NP1529R as well as IVA-M1 and IVA-M2 in RT-PCRs, all 16 tested samples of avian and non-avian origin produced the characteristic amplicons, 330 bp and 601 bp, respectively (Figs. 1a and 1b). Three samples (A/Swine/Poland/KPP1/04, A/Swine/Poland/JPW1/04, and A/Swine/Poland/EWW1/05) were positive in RT-PCR tests despite the fact they were negative in HA tests. This can indicate that PCR-based methods are more sensitive than the HA test. No cross reaction was found with other RNA viruses used in the study (data not shown). Sensitivity of the RT-PCR based on NP-gene primers was established at 100 EID_{50}/0.1 ml (Fig. 2a) and the second RT-PCR based on M-gene primers was at 10 EID_{50}/0.1 ml (Fig. 2b).

Table 4 shows the results of two RT-PCR methods and virus isolation performed on tissue samples collected from SPF chickens 5 days *p.i.* By RT-PCR method based on NP-gene the positive results were obtained in 6 samples per 13 tested (6/13). On the other hand, the M-based RT-PCR method gave 5 positive results per 13 tested (5/13). Positive result of virological examination was noted in 6 samples but there were some discrepancies between the identities of these samples.

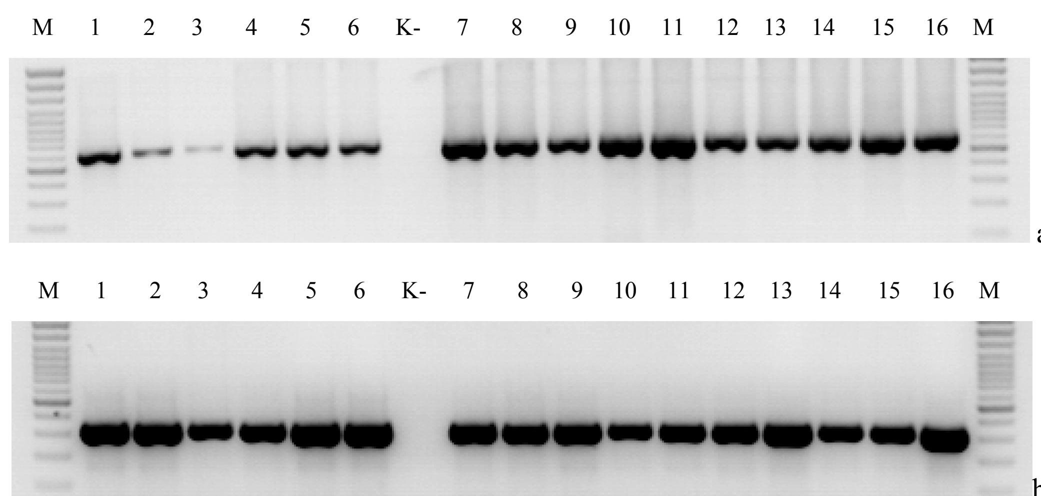

Fig. 1. Electrophoresis of the RT-PCRs based on M- (a) and on NP-gene primers (b). Lanes 1 to 16 influenza strains as listed in Table 1. M-marker GeneRuler 100 bp DNA Ladder (Fermentas, Lithuania), K- negative control.

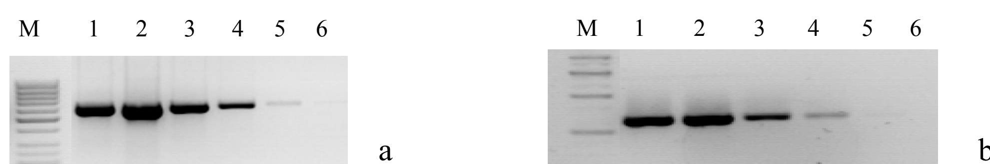

Fig. 2. Results of the sensitivity studies of the RT-PCRs based on M- (a) and on NP-gene primers (b). M-marker GeneRuler 100 bp DNA Ladder (Fermentas, Lithuania), lane 1-positive control, lane 2-$10^{3,5}$ EID_{50}, lane 3-1000 EID_{50}, lane 4-100 EID_{50}, lane 5-10 EID_{50}, lane 6-1 EID_{50},

Table 4

Comparison of two RT-PCR methods and virus isolation for the detection of AIV in tissues of infected chickens

	Cloacal swabs	Tracheal swabs	Trachea	Lungs	Liver	Spleen	Heart	Brain	Kidneys	Bursa of Fabricius	Duodenum	Caecal tonsils	Rectum	Pooled sample No. 1	Pooled sample No. 2
VI	+	-	+	-	-	-	-	-	-	+	-	+	-	+	+
RT-PCR (NP)	+	-	+	-	-	-	-	-	-	+	-	+	+	-	+
RT-PCR (M)	+	-	-	-	-	-	-	-	-	+	-	+	+	-	+

Gray areas – positive test results.

Discussion

There are many different RT-PCR methods applied for AIV detection (3, 5, 8, 9, 13). The sensitivity and specificity of PCR-based methods are dependent on the sequences of chosen primers. The most frequently used primers to detect influenza virus are based on highly conserved regions of NP and M gene. Since both M and NP genes are type-specific, it was concluded that M-based and NP-based PCR should be capable to detect influenza A viruses not only of avian origin but also from other hosts. The NP (nucleoprotein) gene is coded by segment 5 of influenza A virus and functions as viral RNA carrier and takes part in transcription. The M (matrix) gene is located in segment 7 of AIV. In the present study, two primers sets were applied and evaluated for diagnostic purpose: one based on NP-gene according to Lee *et al*. (8) and the second one based on M-gene according to Starick *et al*. (13). Our experimental results confirmed that these two primers sets do not cross-react with other RNA viruses and are suitable for the detection of influenza A virus strains independently of the subtype and the host species as well as various organs and tissues of experimentally infected chickens. However, different sensitivity of these two methods indicates the M-based RT-PCR method as more suitable for the detection of influenza A virus strains. These two methods used for AIV detection differed only by primers, the rest of reagents was similar. But, it should be pointed out that not only primers influence on final effects of AIV detection by RT-PCR. Different laboratories developped their own procedures with different reagents to perform the RT-PCR method. As described Starick and Werner (14) the other factors having effect on results are methods of RNA isolation, applied conditions of RT and PCR (e.g. extension time or cycles number). There is the need for full standardization including not only primers but also all reagents and significant parameters to perform RT-PCR.

Due to a high number of different influenza virus subtypes, preliminary diagnosis should be targeted at the detection of possibly the broadest spectrum of influenza virus strains. This can be accomplished by amplifying genome fragments common for all influenza type A viruses, like M or NP gene. In our study, selected methods proved to be useful since the detection of influenza viruses, regardless of H or N subtype and host species, was successful. It should be noted that positive findings must be followed by haemagglutinin subtype determination, primarily H5 and H7 (responsible for high pathogenic avian influenza). Although false negative reactions have not been found in our study, results interpretation should be undertaken carefully especially in relation to NP-based PCR. Jestin *et al.* (7) failed to detect all influenza strains included in their study when using NP-based PCR.

Both applied PCR-based methods proved to be useful for the AIV detection directly in chicken tissues and a high degree of correlation with virus isolation test was observed. Some discrepancies, however, were also noted. Collection of samples was carried out 5 d post infection because the high amount of the virus was expected to be found at that very time. The H7N1 strain as a LPAI replicates mainly in the respiratory and intestinal tracts (10). In some field outbreaks, LPAI viruses have produced pancreatitis and nephritis, but the mechanism for such infections is unknown. (16). In our study, differences concerned pooled sample No. 1 (VI positive, both RT-PCR negative), rectum (VI negative, both RT-PCR positive), and trachea (VI and RT-PCR (NP) positive, RT-PCR (M) negative). There are several reasons for this phenomenon. The production of influenza virus infection requires multiple replication cycles of the virus in the host animal and necessitates a prerequisite for intra- and extracellular cleavage of the surface H protein (HA0) into two subunits, HA1 and HA2 strain, to produce these replication cycles (16). This could influence the difference in results of AIV detection in the pooled sample No 1. On the other hand, RT-PCR is very sensitive to the presence of some inhibitors, which could explain differences in the results in the case of the rectum and trachea.

The RT-PCR methods applied for the diagnosis of avian influenza virus infection offer a significant reduction of time required to obtain the final result. It could be achieved within a single working day (8-12 h) in comparison with the classical methods. By virus isolation, positive results may be obtained in 24 h or more after inoculation but a negative diagnosis may require up to 2 weeks. The PCR methods offer additional benefits, as differentiation between H5 and H7 subtypes of AIVs. Moreover, the combination of the RT-PCR method with primers encompassing cleavage site of HA gene and further direct sequencing enables to distinguish an isolate with low or high virulence and can replace the *in vivo* method (1-day-old chick inoculation) of the virulence assessment.

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