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MOLECULAR APPROACHES FOR THE IDENTIFICATION OF AVIAN INFLUENZA VIRUSES

MOLEKULARNI PRISTUP IDENTIFIKACIJI VIRUSA INFLUENCE PTICA

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Summary

The recently raised awareness of the threat of a new influenza pandemic has stimulated the interest in the detection of influenza A viruses in the secretions of a wide variety of birds and mammals. Influenza A viruses are subtyped conventionally according to the characteristics of the external glycoproteins, haemagglutinin (HA) and neuraminidase (NA). All 16 HA and 9 NA subtypes have been isolated from aquatic birds that are believed to be a natural host and reservoir for influenza viruses. Poultry, specifically chickens and turkeys, are not considered to be a normal host for the virus, although transmission from wild birds to poultry occurs routinely. Although most HA subtypes have been found in poultry, particular emphasis is placed on the H5 and H7 HA subtypes of avian influenza virus because only these subtypes are known to cause highly pathogenic avian influenza (HPAI) in poultry. Recently developed molecular methods enable fast, accurate and reliable approach to identify influenza virus and its subtypes.

Key words: Avian influenza virus; H5, H7; Typing; Polymerase chain reaction

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INTRODUCTION

Influenza viruses can infect people, birds, pigs, horses, seals, whales and other animals, but wild birds are the natural hosts for these viruses [1]. Certain birds, particularly water birds, act as hosts for influenza viruses by carrying the virus in their intestines. Infected birds shed the virus in saliva, nasal secretions and feces. Fecal-to-oral transmission is the most common mode of virus spread between birds. Domesticated birds may became infected with avian influenza virus through direct contact with infected waterfowl or other infected poultry, or through contact with surfaces or materials that have been contaminated with virus. When this happens, avian influenza outbreaks can occur among poultry.

Influenza type A viruses are divided into subtypes based on two proteins on the surface of the virus; hemagglutinin (HA) and neuraminidase (NA). There are 16 different HA and 9 different NA subtypes [2, 3, 4]. Many different combinations of HA and NA proteins are possible. Usually, influenza viruses do not cause lethal disease in wild birds, indicating that they have achieved an optimal level of adaptation in this natural reservoir. However, in domestic poultry, such as chickens and turkeys, as well as in ratites, the virus can cause severe disease and very high mortality.

Influenza type A viruses infecting poultry can be further classified as either highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI). Only H5 and H7 subtypes have been responsible for a severe systemic disease reaching up to 100% mortality in poultry and designated as HPAI [5]. Because LPAI viruses can evolve into HPAI viruses, outbreaks of H5 and H7 LPAI are closely monitored by animal health officials.

Until recent times HPAI was considered a rare disease in domestic poultry with only 17 episodes being reported worldwide in the 40-year period 1959 to 1998 [6]. However, further outbreaks have occured since 1999, resulting in eight episodes involving 12 countries in 7 years covering 1997 to March 2004. Recently, there also appears to have been a marked increase in the numbers of LPAI outbreaks caused by H5 and H7 viruses. Detailed characterization of the H5 and H7 subtypes is therefore particularly important. A reverse transcriptase polymerase chain reaction (RT-PCR) assays followed by sequencing of the HA gene enable rapid detection and characterization of these viruses.

BIOLOGICAL AND ANTIGENIC PROPERTIES OF INFLUENZA VIRUSES

Influenza viruses are enveloped, segmented, single-standed negative sense RNA members of the *Orthomyxoviridae* family. They are clasified as type A, B and C. The typing is based on antigenic differences on the nucleoprotein (NP) and matrix (M1) protein of the virus. All avian influenza viruses are classified as type A.

By electron microscopy, the influenza A viruses are pleomorphic, including virions that are roughly spherical (approx. 120 nm in diameter) and filamentous. Two distinct types of spikes (approx. 16 nm in length), corresponding to the HA and NA molecules, reside on the surface of the virions. These two glycoproteins are anchored to the lipid envelope derived from the plasma membrane of host cells by short sequences of hydrophobic amino acids. HA enables the virion to attach to the cell surface [7] and is responsible for its hemagglutinating activity [8]. HA elicits virus-neutralizing antibodies that are important in protection against infection. NA cleaves terminal sialic acid from glycoproteins or glycolipids, thus allowing virus particles to become free from host cell receptors and new virions to be released from the cell [9]. Antibodies to NA are also important in host protection [10].

The antigenicity of influenza viruses changes gradually by point mutation (antigenic drift) or drastically by genetic reasortment (antigenic shift) [2]. Immunological pressure on HA and NA is thought to drive antigenic drift which is more often detected in human influenza viruses. Among avian influenza viruses, antigenic drift has also been detected but to a lesser extent [11, 12], possibly because of limited immunological pressure in short-lived birds.

Antigenic shift is caused by the reassortment of genes from two different influenza viruses that have infected a single cell [13]. Theoretically, 256 different combinations of RNA can be produced from the shuffling of the eight different genomic segments of the virus. Mixed infections occur relatively frequently in nature and can lead to genetic reasortment [14, 15].

MOLECULAR DETERMINANTS OF VIRUS PATHOGENICITY

Depending on their pathogenicity in chickens and turkeys, avian influenza A viruses are classified as virulent or avirulent. Most of them isolated in the field are avirulent; virulent viruses have been isolated from apparently healthy waterfowl only near a chicken influenza outbreak [3, 16].

Although the virulence of avian influenza virus is polygenic, the HA glycoprotein plays a pivotal role [17, 18]. It initiates infection by mediating virus binding to cell receptors and by promoting release of the viral RNP through membrane fusion [19]. Post-translational proteolytic activation of the precursor HA molecule (HA0) into HA1 and HA2 subunits by host proteases generates a fusogenic domain at the amino terminus of HA2, which mediates fusion between the viral envelope and the endosomal membrane. Hence, proteolytic activation of the HA molecule is essential for infectivity [20, 21] and for spread of the virus through the host's body [17]. The HAs of avirulent avian influenza viruses are usually cleaved only in a limited number of cell types, so that the

viruses cause only localized infections in the respiratory or intestinal tract, or both, resulting in mild or asymptomatic infections. By contrast, the HAs of virulent avian viruses are cleaved in a broad range of different host cells and therefore are capable of causing lethal systemic infection in poultry.

The HA cleavability is determined by the amino acid sequence at the cleavage site of HA0. Amino acid sequence comparisons between naturally occuring avirulent and virulent avian influenza viruses have showh that HAs with restricted cleavability (avirulent type) usually have a single arginine (R) whereas those with high cleavability (virulent type) have multiple basic residues arginine or lysine (R or K) situated immediately upstream of the cleavage site [22] (Table 1).

Table 1. HA cleavage site sequence of avian influenza virus isolates

Virus subtype	Cleavage site amino acid sequence													
									–	–	–	HA1	||	HA2 –
Avirulent	H5	P	Q	–	–	–	–	R	E	T	R		G	L
	H5	P	Q	–	–	–	–	R	K	T	R		G	L
	H5	P	Q	–	–	–	–	K	K	K	R		G	L
Virulent	H5	P	Q	R	E	T	R	R	Q	K	R		G	L
	H5	P	Q	–	–	R	R	R	K	K	R		G	L
	H5	P	Q	R	K	R	K	R	K	T	R		G	L
Avirulent	H7	P	E	X	P	–	–	–	–	K	X	R	G	L
Virulent	H7	P	E	P	S	–	K	K	R	R	K	R	G	L
	H7	P	E	P	P	–	K	K	R	R	K	R	G	L
	H7	P	E	I	P	K	K	K	K	K	K	R	G	L

X – nonbasic amino acid

DETECTION AND SUBTYPING OF INFLUENZA VIRUSES

Since the circulating influenza virus has the potential to change by genetic drift and shift, methods used for surveillance should have specificity allowing detection of antigenically and genetically diverse influenza strains. Due to the potential of avian influenza viruses to spread between avian and non-avian hosts the identification of an isolate is important.

In the last two decades the development of molecular methods based on the amplification and sequencing of specific genome segments provides new alternatives for rapid detection and characterisation of these viruses. Polymerase chain reaction (PCR) is a powerful technique that enables the identification of virus genomes even when they are present at very low levels in animal samples. Since the genome of an influenza virus is single-stranded RNA, a DNA copy (cDNA) which is complementary to viral RNA

must be synthesized prior to the PCR reaction. Reverse transcriptase (RT) is a polymerase used to synthesize such cDNA. The obtained cDNA is used for PCR that consists of 30 – 40 cycles of denaturation, annealing and extension. These repeating cycles enable exponential amplification of a target DNA sequence.

By cycling through various temperatures, PCR results in the extension of a sequence–specific primer by a DNA polymerase such as DNA polymerase isolated from *Thermus aquaticus* (*Taq*). The DNA polymerase reads the template and incorporates a complimentary nucleotide yielding a newly assembled complimentary strand. For the sensitivities and specificities of PCR-based methods the choice of primer sequences is the most crucial parameter. Primer pairs used in PCR reactions are designed based on known sequences. For a rapid detection of all influenza A viruses primers fitting to highly conserved regions of matrix (M) or nucleoprotein (NP) genes were mostly selected [23, 24, 25]. A general influenza A diagnostic PCR is important both for the detection of virus from an actual outbreak but also for the screening of potential carriers of influenza A. In our laboratory, reference virus strains of different subtypes were detected succesfully by the RT-PCR assay amplifying a part of the nucleoprotein or the matrix protein gene (Fig. 1).

For further subtyping of influenza A viruses, specific primer sets were developed to differentiate the HA subtypes. It is important to identify H5 and H7 hemagglutinin subtypes while they are known subtypes with the risk of becoming highly pathogenic

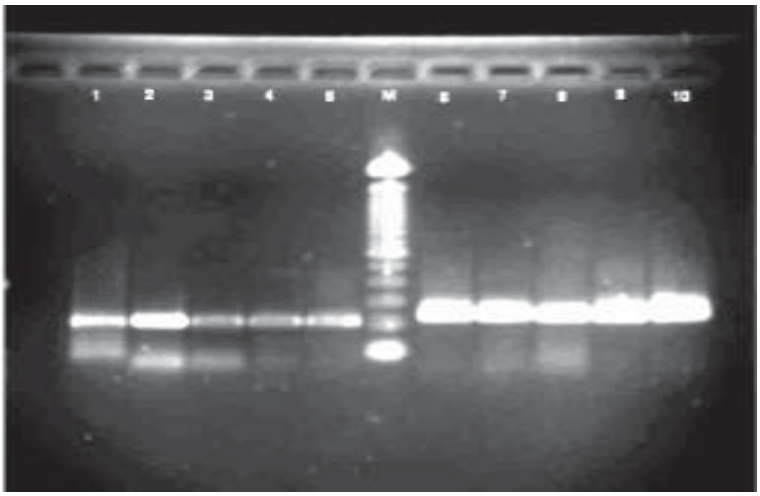

Fig. 1. Amplification of nucleoprotein (1-5) and matrix protein gene (6-10) sequences of avian influenza type A strains. **1, 6**: H9N2; **2, 7**: H7N3; **3, 8**: H5N1; **4, 9**: H6N2; **5, 10**: H9N2; M: 100 bp DNA step ladder (Promega). Oligonucleotide primers used for amplification were described by Munch et al., 2001 [25] (NP) and Fouchier et al., 2000 [24] (M), respectively.

in chickens and turkeys. Detailed characterization of these two subtypes includes amplification of the H5 or H7 genome region and sequencing of their HA cleavage sites. Because of the high variability of the HA genes, several primer pairs have been used in these investigations [23, 25, 26, 27, 28, 29, 30]. The subtype-specific RT-PCR assays for H5 and H7 avian influenza viruses were also introduced in our laboratory using primers described by Starick and co-workers [30]. In the starting experiments the inactivated samples of reference strains (H5N2, H7N1) obtained from VLA, Weybridge UK, were used for RT-PCR optimization (not shown). Further investigations including animal specimens are still in progress. They will elucidate a practical use of this method in avian influenza virus subtyping.

Recently, a real-time RT-PCR assay was developed using hydrolysis probes for the detection of avian influenza virus and the H5 and H7 subtypes. The specific primers and probes were directed to regions of the avian influenza virus matrix gene that are conserved among most type A influenza viruses. The H5 and H7 primers and probes are directed to H5 and H7 HA gene regions that are conserved among North American avian influenza viruses [31]. Comparing to classical RT-PCR assay, this method enabled even faster detection of type A influenza virus and further characterization of positive samples for the H5 and H7 subtypes.

CONCLUSIONS

To date, influenza A viruses representing 16 HA and 9 NA subtypes have been detected in wild birds and poultry throughout the world. They reside naturally in wild bird species where they are generally nonpathogenic but sometimes cause significant morbidity and mortality upon transmission to other species, including domestic birds and mammals. In the last 10 years, the incidence of highly pathogenic avian influenza in domestic poultry has increased substantially. Highly pathogenic avian influenza (HPAI) viruses, which are restricted to H5 and H7 subtypes, are capable of causing severe respiratory and mortality as high as 100% in infected poultry flocks. Although HPAI viruses are primarily of major concern to the poultry industry, because they cause severe economic losses, they have also become a human health concern because of their ability to transmit directly to humans. New diagnostic approaches including molecular methods for the amplification and direct sequencing of specific genome segments enables rapid detection and characterization of many different influenza virus strains circulating in the nature. These methods became also a necessary tool for assesing the virulence potential of avian influenza viruses in poultry.

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Sažetak

Svijest o prijetnji nove pandemije influence potaknula je zanimanje za dokaz virusa influence A u sekretima različitih vrsta ptica i sisavaca. Virusi influence A uobičajeno se tipiziraju na osnovi osobina njihovih površinskih glikoproteina, hemaglutinina (HA) i neuraminidaze (NA). Svih 16 HA i devet NA podtipova izdvojeno je iz vodenih ptica koje su prirodni domaćin i rezervoar virusa influence. Perad, posebice kokoši i pure, ne smatra se uobičajenim domaćinom virusa iako se virus redovito s divljih ptica prenosi na perad. Premda su mnogi HA podtipovi izdvojeni iz peradi, posebna pozornost pridaje se podtipovima H5 i H7 virusa influence ptica, jer samo oni pripadaju visoko patogenim sojevima za perad. Nedavno razvijene molekularne metode omogućuju brzu, točnu i pouzdanu identifikaciju virusa influence i njegovih podtipova.

Ključne riječi: Virus influence ptica; H5, H7; Tipizacija; Lančana reakcija polimerazom