Typing of Poultry Influenza Virus (H5 and H7) by Reverse Transcription-Polymerase Chain Reaction

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ABSTRACT

The ability of the influenza *Orthomixovirus* to undergo to continually antigenically changes that can affect its pathogenicity and its diffusion, explains the growing seriousness of this disease and the recent epizoozies in various parts of the world. There have been 15 HA and 9 NA type A sub-types of the influenza virus identified all of which are present in birds. Until now the very virulent avian influenza viruses identified were all included to the H5 and H7 sub-types.
We here show that is possible to identify the H5 and H7 sub-types with reverse transcription-polymerase chain reaction (RT-PCR) by using a set of specific primers for each HA sub-type. The RT-PCR is a quick and sensitive method of identifying the HA sub-types of the influenza virus directly from homogenised organs.

Key Words: Poultry, Avian influenza virus, Hemagglutinine, Reverse transcription-polymerase chain reaction (RT-PCR).

RIASSUNTO

TIPIZZAZIONE DEI VIRUS INFLUENZALI AVIARI (H5 E H7) TRAMITE RT-PCR

La capacità dell'Orthomixovirus dell'influenza di andare incontro a continue variazioni antigeniche, determinanti la patogenicità e la diffusibilità, spiega l'accresciuta importanza di questa malattia e le recenti epizoozie segnalate in varie parti del mondo.
Nel virus influenzale di tipo A sono stati identificati 15 sottotipi di HA e 9 NA, tutti presenti negli uccelli. Finora i virus influenzali aviari che hanno presentato caratteri d'elevata virulenza sono tutti appartenenti ai sottotipi H5 e H7.
E' possibile identificare i due sottotipi H5 e H7 tramite una reverse transcription-polymerase chain reaction (RT-PCR) *utilizzando un set di primers specifici per ogni sottotipo HA.*
La RT-PCR si presenta come un rapido e sensibile strumento per l'identificazione dei sottotipi HA del virus influenzale direttamente da omogenato d'organi.

Parole chiave: *Pollame, Virus influenzale aviare, Emoagglutinina,* RT-PCR.

Introduction

Influenza was first described by Hippocrates in 412 B.C., and the miniscule virus has continually mutated throughout the centuries, spreading the disease right up to the latest H5N1 epidemics which hit Asia in 2003 and 2004. The influenza virus is part of the Orthomyxoviridae family (genus) and is divided into three genus: A, B and C.

The A type viruses have essentially avian origins and only occasionally do they jump the species barrier to affect other animals or man. Recently, knowledge of the virus, its pathogenesis and prevention, have greatly increased but unfortunately this has not allowed us to avoid great (economic) losses due to recurrent epizoozies from highly viru-

lent strains in different parts of the world.

The virus contains 8 separate segments of RNA, which codify genes of at least 10 different proteins. This unusual genetic structure explains why rearrangement of the segments occurs so frequently. Exchange of genetic segments can easily occur which can cause up to 256 different descendents.

The A type influenza strains are characterised by the structural changes of 2 glycoproteines; hemagglutinine (HA) and neuroaminidase (NA) which project from the surface of the viral particles. Sub-type sub-division is based on a combination of the 15 HA and 9 NA which have so far been identified, all of which are present in birds (Alexander, 1993; Easterday *et al.*, 1997).

Epidemics occur when one or the other of these proteins undergoes a mutation. The unforeseeable nature of the influenza virus comes from their ability to change the surface HA and NA proteins, thus escaping the immune system's vigilance.

If we consider the epidemiological polyedric aspects, the speed of the influenza diagnosis and therefore quick confirmation of the infection becomes particularly important. For these reasons, we can say that reverse transcription-polymerase chain reaction (RT-PCR) has given particularly encouraging results.

To date, all highly pathogenic stains that have been isolate are A influenza viruses of the H5 and H7 sub-types.

The molecular base for distinguishing the antigenic differences of the HA sub-type are based on the amino-acid sequence differences between 20% and 74% and so this percentage is reflected in the nucleotide sequence of the HA sub-type (Ming-Shiuh *et al.*, 2001).

These characteristics are fundamental for the typing of HA sub-types by RT-PCR as the PCR is determined by the difference in the nucleotide sequence. We have here evaluated a quick identification procedure to identity H5 and H7 subtypes of the influenza virus using RT-PCR.

Material and methods

Samples

For the study we used organs (tracheal exudates, lungs, cecal tonsils) in that previously the following avian influenza virus strains were isolated and identified: H7N1/99 chicken and H7N3/02 turkey. Moreover, starting from allantoic fluid, the following strains were also used: H5N2/73 England (turkey), H6N2/90 (turkey) and H9N2/86 (chicken).

Identification of the Type A influenza virus with RT-PCR

The viral RNA was extracted using the Rneasy® Minikit (Qiagen, Valencia, CA, USA) protocol. Once we had the RNA sample we proceeded to the retro-transcription using ProstarTM Kit (Stratagene®, USA). The retro-transcription product was then used directly in the PCR with the addition of a reagent and the tampon which are part of the Accuprime TaqPCRx DNA Polymerase (Invitrogen® USA). The research of the viral genome also includes the use of M52C and M253R primers (Fouchier *et al.*, 2000), which amplifies a region coding for the matrix protein of the type A influenza virus.

Typing H5 and H7 with RT-PCR

The cDNA obtained by retro-transcription was used for a further PCR according to above protocol. To differentiate the H5 and H7 subtypes, sets of specific primers for each sub-type in respect to

Table 1. Primer used for the HA-sub-typing of avian influenza viruses by RT-PCR.

Primer	Primer sequences	PCR product (bp)
H5-155F	5′ ACACATGCYCARGACATACT 3′	545
H5-699R	5′ CTYTGRTTYAGTGTTGATGT 3′	
H7-12F	5′ GGGATACAAAATGAAYACTC 3′	634
H7-645R	5′ CCATABARYYTRGTCTGYTC 3′	

Table 2. Results of identification and subtyping.

Sample	AIV strain	Type	Subtype
Allantoic fluid	H5N2/73 Turkey England	A	H5 (545bp)
Allantoic fluid	H9N2/86 Chicken Italy	A	"
Allantoic fluid	H6N2/90 Turkey Italy	A	"
Organs	H7N1/99 Chicken Italy	A	H7 (634bp)
Organs	H7N3/02 Turkey Italy	A	"

their sequence and characteristics were used as triggers (Ming-Shiuh *et al.*, 2001) (Table 1). The reactions occurred in the following amplification profile: 95°C for 3 minutes, (hot start) followed by 35 cycles of three steps: 95°C for 30 seconds (denaturation), 55°C for 40 seconds (annealing), 72°C for 40 seconds (extension), at the end of cycle there was an additional 10 minutes at 72°C for any possible extensions. The amplified product then underwent an electrophoresis course in agarose gel at 1% with ethidium bromide and was visualised using a UV trans-illuminator.

Results and discussion

Results about avian influenza virus (AIV) identification and subtyping are shown in table 2. Given the variable nature of the influenza virus, it is almost certain that it can escape immune surveillance, thus making it difficult to keep this disease under control.

The particular characteristics of AIV make quick identification and characterisation of the antigenic variants indispensable to be able to intervene as quickly as possible in the more convenient way.

Furthermore, early identification if the new A type influenza virus subtypes, which could be possible causes of pandemics, must be one of the main objectives to insure that vaccines include the immunological characteristics of the virus prevalently in circulation. RT-PCR response perfectly to the requirements of identifying the H5 and H7 subtypes both for its precision and its speed (48 hours).

Conclusions

RT-PCR technique could be a valid test in support of classical diagnosis at the same time being an efficient control technique of poultry breeding. It has been shown to be a sensitive and specific method both using homogenised organs and liquid allantoid.

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