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# DIFFERENTIATION OF HIGHLY PATHOGENIC STRAIN H5N1 OF AIV BY REAL TIME PCR IN THE FIRST OUTBREAK IN ROMANIA**

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**Orginal scientific paper

**Abstract**: Fast diagnosis of Avian Influenza is a prerequisite for confining outbreaks. Diagnosis implies the differentiation of virulent and non-virulent Avian Influenza virus. After starting with PCR screening for matrix protein followed by identifying the presence of H5 and N1 genes, the diagnosis methodology within Romanian Institute for Diagnosis and Animal Health has moved to rapid molecular tests for detecting the virulent and non-virulent strains.

During October-December 2005 over 3400 biological specimens from 16 affected poultry backyards from Eastern Romania were tested by Real-Time PCR in the first Romanian outbreak. Over 3000 specimens have been tested in 2006, in the second Romanian outbreak when commercial poultry farms from central and southern Romania were affected.

**Key words:** Avian Influenza, RT-PCR, Real–Time PCR, Romanian outbreak, LPAI, HPAI.

## Introduction and Literature Review

Avian influenza is a highly contagious viral disease affecting the respiratory, digestive and/or nervous system of many species of birds. It is caused by a Type A influenza virus. There are two types of avian influenza virus, low pathogenic (LPAI) and highly pathogenic (HPAI). Within the LPAI types there is evidence that certain H5 and H7 viruses may mutate and become highly pathogenic.

Typically HPAI presents suddenly with affected birds showing edema (swelling) of the head, cyanosis (blue discoloration) of the comb and wattles

(neck and throat area), dullness, a loss of appetite, respiratory distress, diarrhea and a drop in egg production. Birds can be infected with LPAI without showing any signs of disease. However, there can be considerable variation in the clinical picture and severity of the disease associated with the type of birds infected and whether birds are also suffering from other diseases.

Infected birds shed influenza virus in their saliva, nasal secretions, and feces. Susceptible birds become infected when they have contact with contaminated secretions or excretions or with surfaces that are contaminated with secretions or excretions from infected birds. Domesticated birds may become infected with avian influenza virus through direct contact with infected waterfowl or other infected poultry, or through contact with surfaces (such as dirt or cages) or materials (such as water or feed) that have been contaminated with the virus.

In Romania on 7 October, three domestic ducks were found positive for avian influenza in domestic birds in Ceamurlia de Jos Village, Danube Delta. Around 500 chickens were destroyed, and 2,500 turkeys as well as pigeons will be slaughtered. A three-kilometer radius has been quarantined.

Research suggests that currently circulating strains of H5N1 viruses are becoming more capable of causing disease (pathogenic) in animals than were earlier H5N1 viruses. One study found that ducks infected with H5N1 virus are now shedding more virus for longer periods without showing symptoms of illness.

## Material and Methods

RNA extraction was done with MagNA Pure Compact system (Roche, Germany) from tracheal and cloacae swabs, homogenate of brain, intestine and spleen, using specific primers and probes recommended by CRL Weybridge, United Kingdom for detecting Influenza virus A Matrix Protein and Influenza virus A H5.. The Real Time RT-PCR tests were performed simultaneously on three Light Cycler systems (Roche, Germany) that provided fast, easy and accurate results in identifying and quantifying the virus, within one hour, for Matrix protein and H5 gene. Because of the very high sensitivity of the test, PCR for H5 was performed in duplicate, undiluted and 1:10 dilution, due to the inhibition effect of high concentrations of viral RNA. Conventional RT-PCR on ICycler, Bio-Rad, US was performed for Influenza virus A N1 identification, using WHO recommended primers.

"RNA Master Hybridization Probes" kits, Roche (Germany) and "One step RT-PCR" kits, Qiagen (US) were used for reverse-transcription and amplification for Matrix and H5 and N1 genes, respectively; the amplicons were sequenced on ABI PRISM 310 sequencer in the cleavage site area of hemaglutinine gene, using "Big Dye Terminator v.1.1/v.3.1 Cycle Sequencing Kit" -Applied Biosystem, US. The Romanian sequences, compared with reference ones from Gene Bank were analyzed with BioEdit Sequence Alignment Editor Company – Isis Pharmaceuticals Inc. and ClustalW Multiple Alignment software. All the positive PCR samples were further analyzed by virus isolation test.

**Conventional RT-PCR test, for HA (H5 identification)** we used „ OneStep RT-PCR MIX" - Qiagen (USA), RNasin - Promega (USA) - 40 U/µl/per sample, primers from TibMolBiol (50pmol/µl). In this mix add 5 µl RNA in to each reaction tub.

The protocol for RT-PCR reaction was: $50^0$C – 30', $94^0$C – 15', ($95^0$C – 45'', $50^0$C – 45'', $72^0$C – 2') x 45 cycles, $72^0$C – 10', $4^0$C - ∞.

We used fallowing primers:

P 1 = 5'-GAT AAA TTC TAG CAT GCC ATT CC -3'

P 2 = 5'-TTT TGT CAA TGA TTG AGT TGA CCT TAT TGG -3'

The mix for RT-PCR reaction was:

| Reagent | Volume per reaction |
|---|---|
| Water (Qiagen) | 29,6 µl |
| OneStep RT-PCR Buffer 5x (Qiagen) | 10 µl |
| dNTPs 10 mM (Qiagen) | 2 µl |
| P 1 (50 pmol/µl) (TibMolBiol) | 0.6 µl |
| P 2 (50 pmol/µl) (TibMolBiol) | 0.6 µl |
| RNasin 40U/µl (Promega) | 0.2 µl |
| MIX – enzymes OneStep RT-PCR (Qiagen) | 2 µl |
| RNA | 5 µl |

For identification specific amplicons (300-320 bp) we made electrophoresis in agarose gel (1.5% in TBA buffer); the results were presented in the fig 2.

**Real-Time RT-PCR, for Matrix protein quantification**

We used „RNA Master Hybridization Probes" kit (Roche), RNasin - Promega (USA) -10 U/µl/per sample, primers and probe from TibMolBiol (10µM). In this mix add 5 µl RNA in to each reaction capillary tub.

RT-PCR reaction was made in LightCycler (Roche) with fallowing profile:

| Type of program | Number of cycles | Temperature | Time | Mode |
|---|---|---|---|---|
| RT | 1 / none | $61^0$C | 20’ | none |
| Denaturation | 1 / none | $95^0$C | 30’’ | none |
| Amplification | 45 / Quantification | $95^0$C | 1’’ | none |
| | | $58^0$C | 10’’ | none |
| | | $72^0$C | 15’’ | single |
| Cooling | 1 / none | $40^0$C | 30’’ | none |

In Real Time RT-PCR test we used primers and probe from „TibMolBiol” (Germany) with fallowing sequencing:

P 1 = 5'-CTT CTA ACC GAG GTG GAA ACG TA -3'

P 2 = 5'-CAC TGG GCA CGG TGA GC -3' ;

Al-Mp-TqMn1 = 5′- 6 FAM – CTC AAA GCC GAG ATC GCG CAG A-XT-PH.

The mix for RT-PCR reaction was:

| Reagent | Volume per reaction |
|---|---|
| Water (Roche) | 5,6 µl |
| $Mn(OAc)_2$ 50mM | 0,6 µl |
| Primer 1 Forward (10µM) (TibMolBiol) | 0,4 µl |
| Primer 2 Reverse (10µM) (TibMolBiol) | 0,4 µl |
| RNasin 10U/µl (Promega) | 0,2 µl |
| Probe Al-Mp-TqMn1 (TibMolBiol) | 0,3 µl |
| Buffer MIX x 2,7 (Roche) | 7,5 µl |
| RNA | 5 µl |

The results are presented in the fig 1.

## Results and Discussion

Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, kidney, brain, liver and heart may also be collected and processed either separately or as a pool.

The presence of influenza virus have been confirmed by the use of reverse-transcription polymerase chain reaction (RT-PCR) using nucleoprotein-specific or matrix-specific conserved primers. Also, the presence of subtype H5 or H7 influenza virus has been confirmed by using

H5- or H7-specific primers.

The real application of direct RT-PCR tests may be on rapidly identifying subsequent outbreaks once the primary infected premises has been detected and the virus characterized.

All Romanian isolates were H5N1 HPAI viral strains and they have been confirmed by CRL for Avian Influenza in Weybridge, United Kingdom. The concordance between PCR tests and virus isolation test was of 98%. The number of samples and the performed PCR tests is presented in Table 1. The results for Matrix, H5 and N1 are presented in fig.1, 2 and 3 respectively. The analysis of the Romanian sequences in comparison with similar sequences from Gene Bank is presented in fig. 4. The amino acids sequences at the cleavage site of the hemaglutinine gene are presented in fig. 5.

**Table 1. Number of specimens and PCR tests**

| | |
|---|---|
| Specimens number | 6480 |
| PCR tests for Matrix | 3280 |
| PCR tests for H5 | 6400 |
| PCR tests for N1 | 3200 |

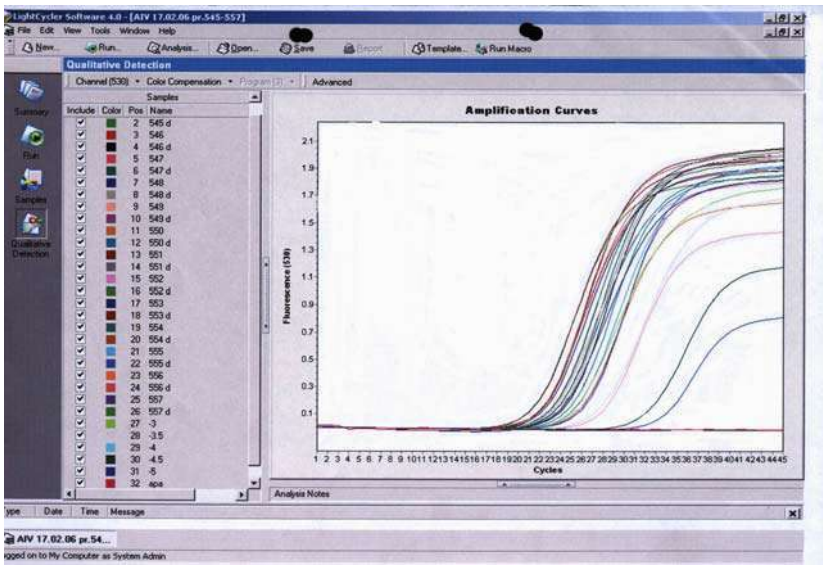


**Fig 1. Real-Time PCR for 10 pool samples from 10 dead birds**

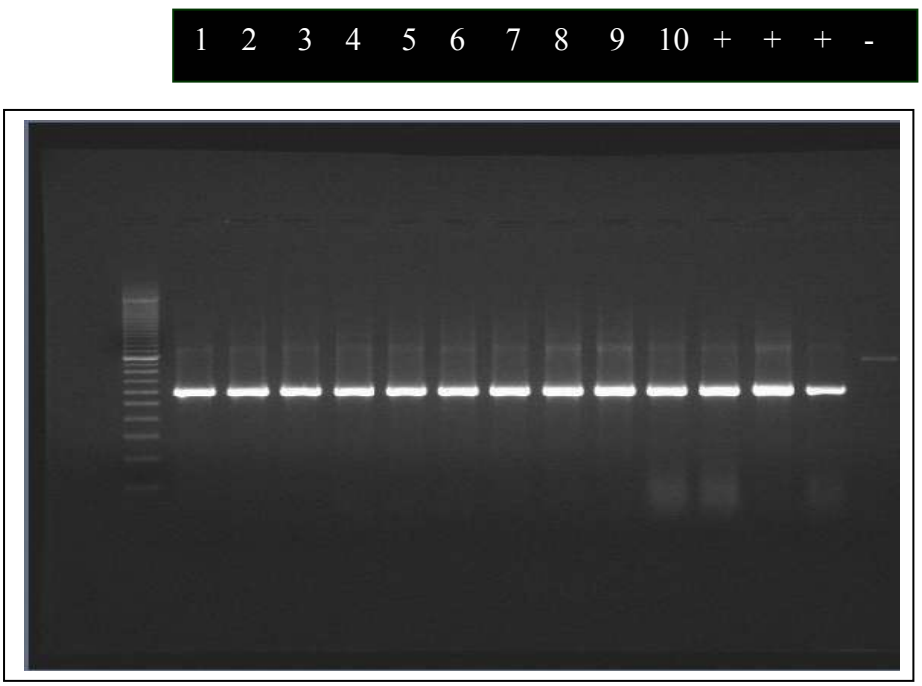


**Fig 2. RT-PCR for 10 pool samples from 10 dead birds, positive control($10^{-5}$, $10^{-4}$, $10^{-3}$ HAU), negative control**

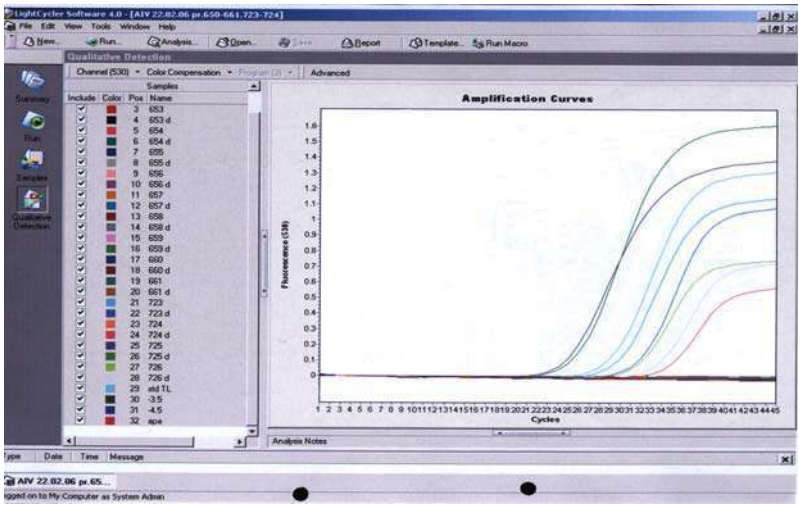


**Fig 3. Real-Time PCR for oropharyngeal swabs from 10 dead birds**

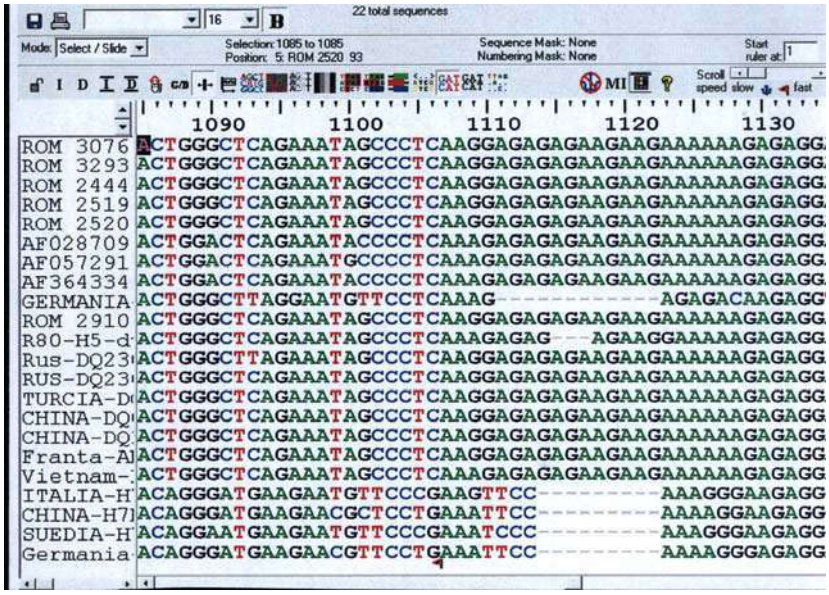


**Fig 4. Romanian sequences in comparison with similar sequences from Gene Bank**


**Fig 5. The amino acids sequences at the cleavage site of the hemaglutinine gene**

## Conclusions

- Romanian molecular diagnosis methodology fulfilled all the technical requirements of CRL Weybridge.
- The availability of Real-Time PCR tests in routine diagnosis considerably reduced the necessary time for virus confirmation.

# DIFERENCIJACIJA IZRAZITO PATOGENIH SOJEVA H5N1 AIV KORIŠĆENJEM REAL TIME PCR U RUMUNIJI

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## Rezime

Brza dijagnoza ptičijeg gripa je preduslov za ograničavanje izbijanja ove bolesti. Dijagnoza uključuje/podrazumeva diferencijaciju virulentnih i nevirulentnih virusa ptičjeg gripa. Nakon PCR snimanja za dobijanje matrica proteina nakon čega je usledilo identifikovanje prisustva H5 i N1 gena, metodologija dijagnostikovanja u Rumunskom institutu za dijagnozu i zdravlje životinja je bila fokusirana ka brzim molekularnim testovima za otkirvanje virulentnih i nevirulentnih sojeva.

Tokom perioda od oktobra do decembra 2005. godine, preko 3400 bioloških uzroaka iz 16 pogođenih gazdinstava iz istočne Rumunije je testirano korišćenjem Real-Time PCR, u prvom izbijanju bolesti u Rumuniji. Preko 3000 uzoraka je testirano u 2006. godini, tokom drugog izbijanja bolesti u Rumuniji kada su bile pogođene i komercijalne farme iz centralne i južne Rumunije.

**Ključne reči:** ptičiji grip, RT-PCR, Real–Time PCR, pojava bolesti u Rumuniji

## References

ALTMULLER A., KUNERL M., MULLER K., HINSHAW V.S., FITCH W.M., SCHOLTISSEK C. (1991): Genetic relatedness of the nucleoprotein (NP) of recent swine, turkey and human influenza A virus (H1N1) isolates.

Virus Res., 22, 79-87.
ARRIOLA J.M., FARR W., URIBE G., ZURITA J. (1999): Experiencias de campo en el uso de vacunos contra influenza aviar. *In;* Proceedings Curso de Enfermedades Respiratorias de las Aves, Asociacion Nacional de Especialistas en Cienvias Avicelase, 3-13.Bankowski R.A. (1982). Proceedings of the First International Symposium on Avian Influenza, 1981. Carter Comp., Richmond, USA.CAPUA I., ALEXANDER D.J. (2004): Avian influenza: recent developments. Avian Pathol, 33, 393-404.
CAPUA I., CATTOLI G., .MARANGON, S., BORTOLOTTI L., ORTALI G. (2002): Strategies for the control of avian influenza in Italy. Vet. Rec., 150,223.
CAPUA I., MARANGON S. (2000): Review article: The avian influenza epidemic in Italy, 1999-2000. Avian Pathol., 29, 289-294.
EUROPEAN UNION (EU) SCIENTIFIC COMMITTEE ON ANIMAL HEALTH AND ANIMAL WELFARE (SCAHAW) (2003): Food Safety: Diagnostic Techniques and Vaccines for Foot and Mouth Disease, Classical Swine Fever, Avian Influenza and some other important OIE List A Diseases. Report of the Scientific Committee on Animal Health and Animal Welfare.
FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED (FAO) (2004): FAO, OIE & WHO Technical consultation on the Control of Avian Influenza. Animal health special report.
KOCH G. (2003): Laboratory issues: Assessment of the sensitivity and specificity of PCR for NDV on cloacal and tracheal swabs compared to virus isolation. Proceedings of the Joint Seventh Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of Countries of the European Union, Padova, Italy.
MUNCH M., NIELSEN L., HANDBERG K., JORGENSEN P. (2001): Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR -ELISA. *Arch. Virol.,* 146, 87-97.
SPACKMAN E., SENNE D.A., MYERS T.J., BULAGA L.L., GARBER L.P., PERDUE M.L., LOHMAN K., DAUM L.T., SUAREZ D.L. (2002): Development of a real-time reverse transcriptase PCR asssy for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.,*40,3256-3260.
STARICK E., ROMER-OBERDORFER A., WERNER O. (2000): Type- and subtype-specific RT-PCR ssays for avian influenza viruses. *J. Vet. Med.* [*B*],47,295-301.
SUAREZ D. (1998): Molecular diagnostic techniques: can we identify

influenza viruses differentiate subtypes and determine pathogenicity potential of viruses by RT-PCR? Proceedings of the Fourth International Symposium on avian Influenza, Athens Georgia. US Animal Health Association,KennettSq.,PA,USA,318-325.
WORLD HEALTH ORGANIZATION EXPERT COMMITTEE (1980): A revision of the system of nomenclature for influenza viruses: a WHO Memorandum. *Bull. WHO,* 58, 585-591.