

**SZENT ISTVÁN UNIVERSITY**

Doctoral Training Programme in Veterinary Science

**Genetic methods in vaccine control procedure**

**Objectives of the PhD thesis**

**By**

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## **I. Scientific background and aims**

To ensure the safety, purity, efficacy and potency of the veterinary vaccines has utmost importance on account of the biological aspects of vaccines, which may affect the health of the farm animal populations, the environment and the public health. For this reason the authority carries out examinations thoroughly to control the quality of vaccines. However, during the examinations the authority should have clear picture about the vaccine strain in the vial, the present methods do not offer sufficient answer to this question. We can be informed about this parameter mostly indirectly, assuming the appropriate quality assurance of the manufacturing procedure stated in the registration dossier.

There are several indications in the literature that in contrary of the safety rules, standards and the quality assurance, the Master Seed Virus can be contaminated by other viruses or can be changed to another virus. In this respect the methods, which can offer effective way to identify and distinguish the strains in the vial, have importance in the state vaccine control procedure.

Applying PCR in the control procedure, we can identify the vaccine strains. For this reason in this study my aim was to apply molecular biological methods in the vaccine control procedure in order to i.) identify the vaccine strains in the vial; ii.) develop method for distinguishing wild and vaccine strains; iii.) reveal the putative role of the vaccine in adverse effects; iv.) reveal the genetic causes leading to adverse vaccine effects.

For these purposes I examined three different problems:

- Applying RFLP-PCR to reveal homologous contamination of live attenuated NDV vaccines. A further aim was to prove that the contamination was produced by the manufacturer.
- The genetic characterisation and phylogenic analysis of the IB strains caused outbreaks in Sweden. The aim was to develop a diagnostic PCR and a phylogenetic PCR. A further aim was to prove the involvement of vaccine strain in the outbreaks.
- The clinical and virological characterisation of a myxoma virus caused aspecific clinical manifestation in a vaccinated rabbit population.

## **II. Material and Methods**

### **Samples**

NDV examination: 12 vaccines, in case of IBV examination trachea and intestinal specimens from natural outbreaks in Sweden. In the myxoma examination, the eyelid, lung and rectum specimens from a carcass died in a natural case.

### **Isolation of RNA**

The RNA was extracted by Trizol™ (13). The precipitated RNA was pelleted at 14 000 x g for 30 min, washed once with 400 µl of 70% ethanol, dried and

dissolved in 50  $\mu$ l of freshly prepared diethyl pyrocarbonate-treated water (DEPC, Fluka Chemi AG, Buchs, Switzerland) as described by Sambrook et al. (1989), and used immediately or stored at  $-70^{\circ}\text{C}$ .

### **The synthesis of cDNA**

The synthesis was performed in 25  $\mu$ l reaction mixtures containing 5  $\mu$ l RNA, 5  $\mu$ l DEPC-treated water and 1  $\mu$ l of random hexamers (0.02 U; Pharmacia Biotech, Uppsala, Sweden). RNA was denatured at  $65^{\circ}\text{C}$  for 5 min. Subsequently, the tubes were placed on ice, and 17  $\mu$ l of a premix containing 1  $\mu$ l of RNAGuard (24 U, Pharmacia), 2.5  $\mu$ l of each dNTP (2 mM; Pharmacia), 5  $\mu$ l of 5x 1st strand reaction buffer (Gibco BRL, Bethesda, MD, USA), and 1  $\mu$ l Moloney murine leukemia virus reverse transcriptase (200 U; Gibco BRL) was added. The reaction mixtures were incubated at  $37^{\circ}\text{C}$  for 90 min followed by inactivation of the enzyme at  $98^{\circ}\text{C}$  for 5 min.

### **PCR and gelelectroforesis**

Several PCR assays were developed or applied for our purposes. For IBV examinations a diagnostic and a phylogenetic PCR assays were developed used nucleocapsid gene and the S1 gene. In case of NDV, an RFLP-PCR method developed by Wehmann et al. was applied. To identify the myxoma isolate (BP04/2001), a PCR based on the envelope gene was used.

For visualization of the PCR products, 5  $\mu$ l of the PCR mixture were electrophoresed in a 2% agarose gel.

After electrophoresis, the gel was stained in ethidium bromide and viewed under UV light.

### **Sequencing and sequence analysis**

The PCR products were sequenced by Genotype GmbH (Germany, Hirschhorn). The DNASTar 5.0 software package was used for sequence analysis.

### **SDS PAGE**

The isolated virus and vaccine virus as control were spreaded on RK-13 cells. The supernatant was treated with lysis buffer. The proteins were electrophorised on SDS PAGE.

### **Electron microscopy**

1 mm<sup>3</sup> organ specimens were fixed with 4% paraformaldehyde (3 h at 4°C) and 0.2% glutaraldehyde. After washing in 0.2 M PBS, they were postfixed with 1% OsO<sub>4</sub> in PBS, dehydrated in an ethanol series, and embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin (40-60 nm) sections were cut with an ultramicrotome (Reichert OM U3), imaged at 80 keV with an electron microscope (JEM JEOL 100S, JEOL Ltd., Akashima, Japan).

## **III. Results and discussion**

In our studies we proved that the homologous contamination of live attenuated NDV vaccines could be revealed with RFLP-PCR by identifying the vaccine strains. We evidenced that the contamination was produced by the manufacturer.

In order to examine effectively and quickly the IB outbreaks in Sweden, a diagnostic PCR assay was developed, whose broad reactivity provides possibility to detect IBV stains spread in flocks.

The wild IB viruses and the vaccine strains was identified and distinguished with the phylogenetic PCR. Massachusetts strains and D274 strain were the causative factors of the outbreaks in Sweden. The nucleotide analysis of the S1 gene revealed further subclasses of the Massachusetts strains in question: strains of natural cases and vaccine origin. The phylogenetic PCR may have an utmost importance to improve the appropriate vaccine use. A given vaccine serotype provides restricted protection against another wild serotype, for this reason the identification of the wild viruses can help to choose the most effective vaccine strain.

During the examination of an aspecific myxomatosis appeared in a vaccinated rabbit population, we reported the first occurrence of the atypical myxomatosis in Hungary. This type of myxomatosis was known only in France and Belgium to date. The virus was isolated (BP04/2001). The genetic and virological examination revealed 97% similarity with the Lausanne reference strain, which is the representative of myxoma virus circulating in Europe. The protein examination showed the absence of a ca. 200 kDa protein in case of the BP04/2001. The aerogen, direct spreading was proved in animal experiments.

#### **IV. Publications**

Farsang, A., Ros, C., Renström, L.H.M., Baule, C., Soós, T. and Belák, S. (2002): Molecular epizootiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain. *Avian Pathology*, 31: 229-236.

Farsang, A., Makranszki, L., Dobos-Kovács, M., Virág, Gy., Fábíán, K., Barna, T., Kulcsár, G., Kucsera, L. and Vetési, F. (2003): Occurrence of atypical myxomatosis in Central-Europe: clinico-virological examinations. *Acta Veterinaria Hungaria*, Accepted.

Farsang, A., Wehmann, E., Soós, T. and Lomniczi, B. (2003): Positive identification of Newcastle Disease Virus Vaccine Strains and Detection of Contamination in Vaccine Batches by Restriction Site Analysis of Matrix Protein Gene. *Journal of Veterinary Medicine B*, Accepted.

### **Conferences**

Poster: A. Farsang, E. Wehmann, T. Soós and B. Lomniczi: „Genetic methods in vaccine against Newcastle Disease”; EFIS2000, Kaziemierz Dolny, Poland, 2000

Poster: A. Farsang, F. Vetési, M. Dobos-Kovács, L. Kucsera, L. Makranszki, G. Kulcsár, T. Barna and T. Soós: „Clinico-pathological examination of an aspecific myxomatosis”; XIIth International Congress of Virology, Paris, France, 2002