

## THE APPLICATION OF MOLECULAR METHODS IN THE IDENTIFICATION OF ISOLATED STRAINS OF PARAINFLUENZA 3 VIRUS OF CATTLE

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**Abstract** - Bovine parainfluenza 3 virus (PI3) causes respiratory infections in cattle and sheep with great economic losses in livestock. The aim of this investigation was to determine the significance of molecular methods in the identification of isolated strains of PI3 virus. Twenty cattle nasal swabs were analyzed for the presence of PI3 using the standard virology method of virus isolation in MBDK cell line and virus neutralization test. The identification of isolated strains was confirmed by RT-PCR and method of direct sequencing with primers for PI3 fusion (F) protein gene. PI3 virus was isolated and identified in four nasal swabs using the standard virology method and RT-PCR. The analysis of nucleotide sequences of isolated PI3 strains showed high similarity with sequences isolated from cattle in Asia. Our results showed that molecular methods are very useful in the diagnosis of PI3 infections as well as for the identification and characterization of PI3 strains in Serbia.

**Key words:** Bovine parainfluenza 3 virus; RT-PCR; direct sequencing

### INTRODUCTION

Bovine parainfluenza 3 virus (PI3) belongs to the genus *Respirovirus*, subfamily Paramyxovirinae and family Paramyxoviridae. The genome consists of a single-stranded RNA molecule, 15-16 kb long, and comprises 6 to 20 genes. Viral RNA is settled in a helicoidal capsid surrounded by an outer envelope. Two glycoprotein antigens hemagglutinin/neuraminidase (HN) and fusion protein (F) form spikes on the surface of the outer envelope. These glycoproteins have an essential role in the process of viral binding to receptors on the surface of host cells, the entrance of virus by fusion and the release of viral particles from infected cells (Murphy et al., 2000).

Parainfluenza 3 virus causes severe infection in cattle in intensive and extensive livestock. It is one of the most common causative agents of respiratory infections in cattle and sheep, especially in youngsters. Primary infection of cattle is usually followed by secondary bacterial infections, which can lead to death. Unfavorable environmental factors such as low temperature and inadequate nutrition may influence the course of the disease in cattle (Oem et al., 2013).

The sources of infection are sick animals infected with PI3 that shed the virus into the environment through secretions. They usually have symptoms such as hyperthermia, apathy, nasal discharge, shortness of breath and cough. In some serious cases,

interstitial pneumonia can develop. The primary infections usually resolve after 3 to 4 days without consequences. Immunity is not long lasting and after a few months, these animals are susceptible to PI3 reinfection (Maidana et al., 2012).

At present, several traditional and molecular methods are in use for the diagnosis of PI3. The gold standard in the laboratory diagnosis of PI3 is the method of virus isolation (cell lines MDBK or VERO), followed by a virus neutralization test. Some other tests can also be used, such as the hemagglutination test (HA test), hemagglutination inhibition test (HI test), ELISA test and direct or indirect immunofluorescence. The molecular methods used for the diagnosis of PI3 are reverse transcription PCR (RT-PCR) and real-time PCR (rt-PCR) (Murphy et al., 2000).

Having in mind the significant economic losses in cattle husbandry caused by PI3 infections, prevention of diseases can be achieved by vaccinations. Different inactivated and attenuated anti-PI3 vaccines are in use and can be administered by intramuscular or intranasal route. Vaccine-induced immunity is short-term and reinfection with the same virus is possible several months after vaccination (Salt et al., 2007).

It is well known that the use of traditional virological diagnostic methods, such as isolation of the virus in cell lines, requires long term and high precision work under sterile conditions. Therefore, the aim of this investigation was to determine the significance of some molecular methods in the identification of isolated PI3 strains originating from cattle in Serbia.

## MATERIALS AND METHODS

### *Samples*

Twenty nasal swabs were collected from calves and beefs with severe respiratory infection in intensive and extensive livestock from different areas in Serbia. Samples were prepared according to standard proce-

dures immediately after arrival at laboratory and inoculated into the MDBK cell line in order to isolate the virus (OIE Manual, 2012, chapter 2.4.13).

### *Isolation of virus in cell culture and virus-neutralization test*

Samples of cattle nasal swabs were inoculated onto a 96-well microtiter plate with a monolayer of MDBK cell line (ATCC CCL-22, Cell line bank, Brescia, Italy). Each pool was inoculated with 100 µl of sample. Plates were incubated for 1 h at a temperature of 37°C and in an environment with 5% CO<sub>2</sub>. After 1 h, 1 ml of minimum essential medium (MEM, Biochrom, Germany) with 2% fetal calf serum (PAA, Austria) was added. Plates were then incubated under the same conditions and observed every day for the appearance of cytopathic effect of the virus (CPE).

Identification of the isolated strains of PI3 was done using a reference SF4 strain of PI3, ATTC-VR 281 (American Bioresearch, Tennessee, USA), titer of 6.25 log<sub>10</sub> TCID<sub>50%</sub> /ml, as a positive control and specific hyperimmune anti-bovine PI3 serum (Scientific Institute of Veterinary Medicine of Serbia, Belgrade). The virus neutralization test was performed according to the OIE Manual (2012, chapter 2.4.13).

### *RT-PCR method*

The extraction of viral RNA from infected MDBK cell lines with 50% CPE was done using a Pure Link RNA Mini Kit (Ambion, USA) according to manufacturer's instruction.

Primers for specific conserved parts of the PI3 genome that encode the F protein of the outer envelope were used for RT-PCR (FW 5'CATTGAATTCATACTCAGCAC3' and REV 5'AGATTGTCGCATTT(AG) CCTC3') (Lyon et al., 1997).

The RT-PCR protocol consisted of reverse transcription at 47°C for 30 min, followed by initial denaturation at 94°C for 2 min. After this step, PCR consisted of 35 repeated cycles of denaturation at 94°C for 45 s, annealing at 51°C for 45 s and elonga-

tion at 72°C for 60 s. The process was finalized with final elongation at 72°C for 5 min. The presence of a specific 400bp band for PI3 was detected by agarose gel electrophoresis and considered as a positive result.

#### *PI3 direct sequencing*

PCR products were purified with a QIA quick Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purified PCR products were subsequently sequenced with a Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) using the PCR primers as sequencing primers. Sequencing reactions were analyzed on an ABI Prism 310 Genetic Analyzer.

The obtained nucleotide sequences were aligned and compared with documented virus sequences available in the Gene Bank database using the BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequence was assigned to a PI3 if it corresponded by more than 95% similarity with a known PI3 strain.

## RESULTS AND DISCUSSION

The gold standard in laboratory diagnosis of PI3 is the method of virus isolation in cell lines, followed by the virus neutralization test. The most frequent cell lines used for the isolation of PI3 are MDBK (Almeida Vaucher et al., 2008) and VERO cell lines (Breker-Klassen et al., 1996).

Conceicao et al. (2007) used the MDBK cell line for the isolation of PI3 and confirmed that this cell line is the most sensitive for propagation of bovine PI3 virus. In our study, 20 nasal swabs were inoculated into the MDBK cell line. After 48 h and 72 h, characteristic cytopathic effects of PI3 appeared in four samples (Figs. 1 and 2). The identification of the four PI3 isolates was done by virus neutralization test.

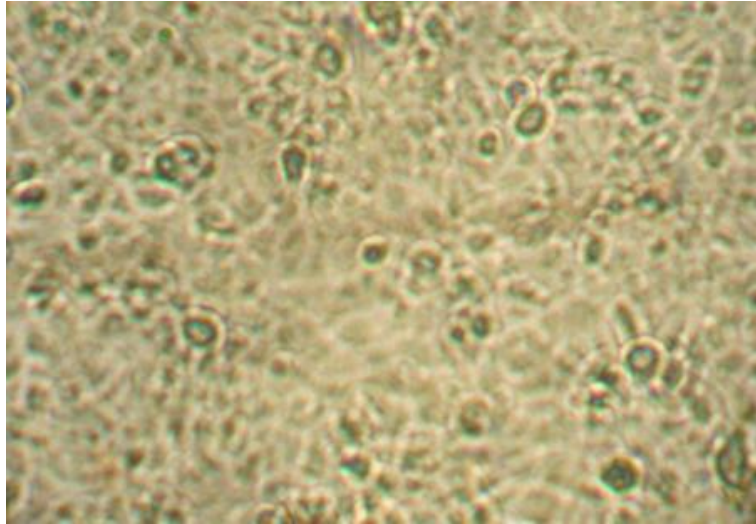
The discovery of molecular methods introduced new, specific and very sensitive methods for the labo-

ratory diagnosis of viral infections. Lyon et al. (1997) used RT-PCR in order to confirm the presence of viral nucleic acids of PI3 in samples collected from cattle, sheep and humans. The RT-PCR method was performed with primers for the F protein gene of PI3. The results obtained in this investigation confirmed the validity of the application of molecular methods in the diagnosis of PI3 infections.

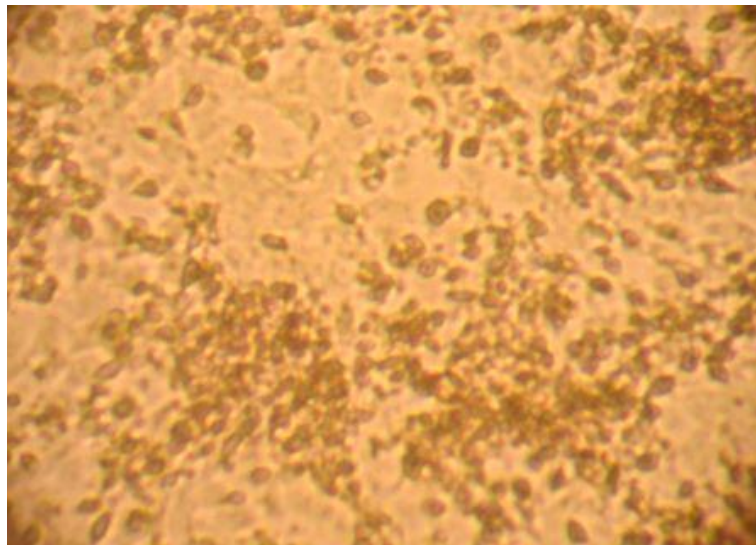
Numerous studies have compared the results obtained by standard virological methods and those obtained by molecular methods of viral diagnostics. Gafer et al. (2009) isolated three strains of PI3 from 205 nasal swabs of sheep and goat collected in seven different regions in Egypt. The procedure of virus isolation was performed in the MDBK cell line, while identification of isolated PI3 strains was performed by the virus neutralization test. The identification of isolated PI3 strains was confirmed by RT-PCR. Similar findings were shown in our investigation, where the identification of all four isolated PI3 strains was confirmed by RT-PCR with primers for the F protein gene (Fig. 3).

Intisar et al. (2010) examined 273 lung specimens from camels with pneumonia collected from slaughterhouses in four different areas in Sudan. In addition, eight samples were collected from outbreaks of respiratory infections in camels. Using antigen detection sandwich ELISA kits, six of the 281 specimens tested were positive for the PI3 antigen. Four specimens yielded a cytopathic virus when inoculated into a MDBK cell culture. However, only two out of four samples that were positive by ELISA and isolation test were positive by RT-PCR with primers for the F protein gene.

Other pairs of primers are also in use for the detection of PI3 virus, such as primers for the conserved parts of a genome that encode hemagglutinin/neuraminidase (HN) or matrix (M) protein of the PI3 virus. Oem et al. (2013) isolated five PI3 strains from nasal swabs collected from cattle in different area of South Korea with clinical symptoms of mild pneumonia. The identification of isolated PI3 strains was confirmed by RT-PCR with primers for the HN protein



**Fig. 1.** MDBK cell line (negative control) Priprema za Blister Plate



**Fig. 2.** MDBK cell line with CPE of PI3 virus 72 h after inoculation.

gene. The nucleotide sequence of the isolated strains showed a high level of similarity with the sequence of PI3 strain SD0835 isolated in China. Almeida Vaucher et al. (2011) used primers for the conserved part of the HN gene in the characterization of bovine PI3 in Brazil as well. Horwood et al. (2008) isolated three PI3 strains from cattle nasal swabs by isolation of the virus in a CRIB-1 cell culture and virus neutralization test. The identification of isolated PI3 strains was done by RT-PCR with primers for the M protein gene of the

virus. The results of the direct sequencing method showed that nucleotide sequences of these three isolated strains had a high level of similarity with the sequence of the referent PI3 strain Kansas/15626/84.

Analysis of four nucleotide sequences of PI3 virus isolated from cattle in Serbia showed that isolated PI3 strains from our country had a high level of similarity with nucleotide sequences of strains isolated in China and South Korea (Fig. 4).

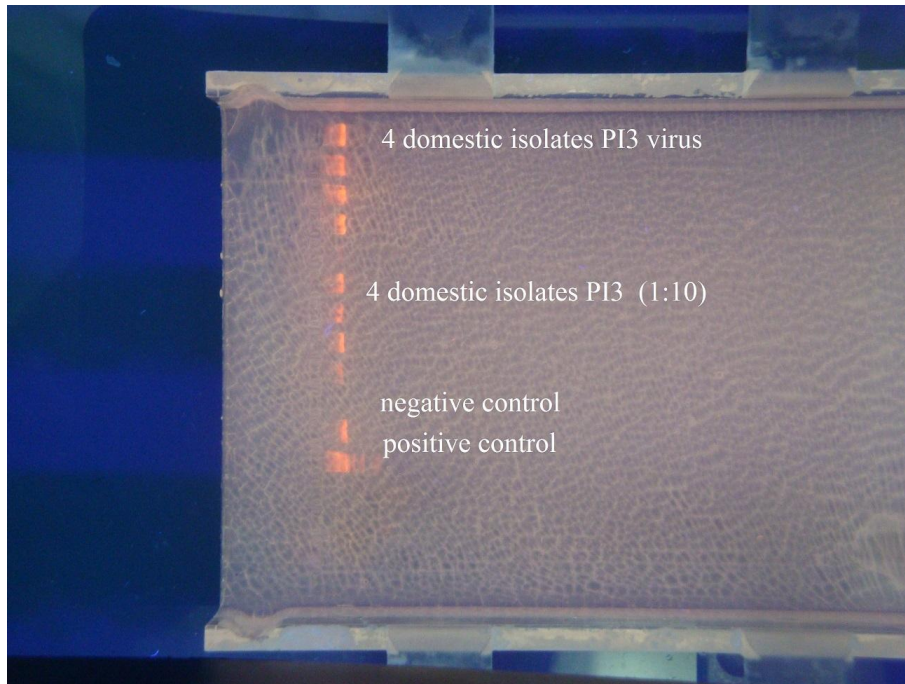


Fig. 3. RT-PCR of isolated PI3 strains.

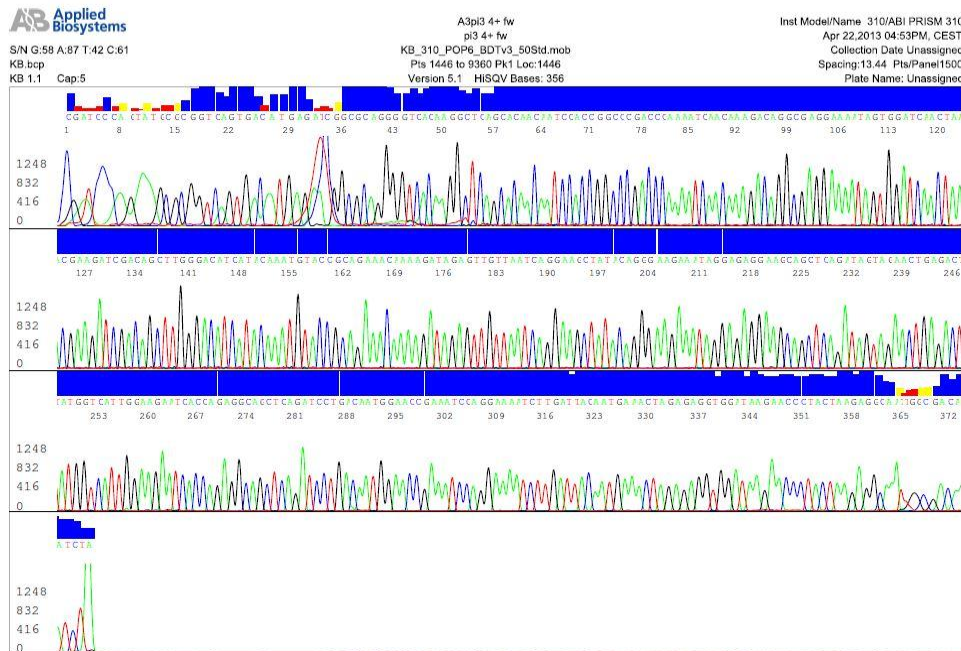


Fig. 4. Nucleotide sequence (electropherogram) of BPI3 4+ isolate from Serbia.

It must be emphasized that F protein gene sequences in GenBank originate mainly from PI3 strains iso-

lated in Asia and that this phenomenon of high degree of concordance of nucleotide sequences of the virus

strains isolated in geographically distant areas is not unique. Zhu et al. (2011) determined the nucleotide sequence of the M protein gene of the PI3 virus. Based on these results, they proved that four strains of PI3 isolated in China had similar nucleotide sequences to the strains of PI3 virus isolated in cattle in Australia.

Maidana et al. (2012) determined the similarity of nucleotide sequences of PI3 strains from Argentina with strains isolated in Australia and China. In their study, sequencing primers for the M protein gene of PI3 were used. The results of their investigation confirmed the presence of bovine PI3 strains, genotypic groups B and C, outside Australia and China.

The results of our investigation showed that molecular methods are very useful in the diagnosis of PI3 infections in cattle in Serbia. Furthermore, the use of a direct sequencing method is very important for the identification and characterization of PI3 strains in Serbia and comparison with sequences of PI3 viruses isolated all over the world.

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