

### РОЗДІЛ 2. Моніторинг та діагностика SECTION 2. Surveillance and diagnostics

DOI: 10.31073/onehealthjournal2024-IV-03

#### PCR-BASED PROTOCOL TESTING FOR RABBIT MYXOMATOSIS VIRAL DNA DETECTION

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**Abstract.** Myxomatosis is an acute viral disease of rabbits caused by Myxoma virus, poxvirus belonging to the genus *Leporipoxvirus*. The natural hosts of this pathogen are tapeti (*Sylvilagus brasiliensis*), and brush rabbits (*Sylvilagus bachmani*) in South and Central America, as well as in North America, respectively. The virus of myxoma develops only a mild disease in those species, but in European rabbits (*Oryctolagus cuniculus*) occurred the fatal disease.

The disease is endemic in many European, Asian and American countries, but the agent is still recognized as an emergent infection, associated with mass losses and high mortality in European rabbits.

One Health Scientific and Research Institute, PSI in collaboration with SRI 'Veterinary Biotechnologies', LLC were developed the methodical approach for detection of the DNA of MYXV using Real-Time PCR due the reason of enhancement of myxomatosis control measures in Ukraine.

**Keywords:** rabbit myxomatosis, virus, risk analysis, epidemiology, diagnostics, rabbits, DNA, PCR.

**Introduction.** Myxomatosis is an acute viral disease of rabbits caused by *Myxoma virus* (MYXV), poxvirus belonging to the genus *Leporipoxvirus*. The natural hosts of this pathogen are tapeti (*Sylvilagus brasiliensis*), and brush rabbits (*Sylvilagus bachmani*) in South and Central America, as well as in North America, respectively. The virus of myxoma develops only a mild disease in those species, but in European rabbits (*Oryctolagus cuniculus*) occurred the fatal disease (Bertagnoli, 2015, Águeda-Pinto, 2019).

Myxomatosis is essentially a disease of European rabbits. Brown hares (*Lepus europaeus*) are also susceptible to infection, but rarely develop generalised disease. Large outbreaks of myxomatosis in Spanish hares (*Lepus granatensis*) were caused by a variant strain. *Sylvilagus* spp. are quite resistant and may act as healthy carriers. There is no health risk to humans (Kerr, 2015, 2021, Jenckel, 2022).

Depending on the strain of the virus, it can take between one to three weeks for symptoms of myxomatosis to show. If your rabbit has been exposed to the virus, their behaviour and eating habits may change. When the virus takes hold, your rabbit's eyes, nose and genitals are usually the first parts of the body to be affected (Abade Dos Santos, 2022).

Two forms of the disease are observed: the nodular (classical) form, characterised by gross myxomatous skin lesions, and the amyxomatous (respiratory) form, in which signs are mainly respiratory, skin nodules being few and small. MYXV strains with different degrees of pathogenicity circulate in the field. The nodular form is caused by virulent MYXV strains, is naturally transmitted by biting insects during summertime, and is mainly observed in wild and pet rabbits and in small-scale rabbitries (Silvers, 2006, Kerr, 2022).

During the infection the copious viral immunomodulatory proteins progressively induce the collapse of the host immune system. This favours bacterial infections in the respiratory tract that contribute to the death of the animal. Mild and attenuated strains of virus can cause the amyxomatous (respiratory) form of the disease especially in farmed animals (Kerr, 2022).

Wild rabbits act as reservoirs while mosquitoes and fleas can transmit the virus to domestic rabbits, but in the case of close proximity between rabbits (i.e. in farmed animals), virus may also be transmitted by direct contact. Introduced semen may also pose a risk. There is no age or sex predilection (Calle, 2022, Kerr, 2012).

The diagnosis of myxomatosis is based on the isolation and identification of the virus or the demonstration of its antigens or genome. When skin lesions are present, the viral antigen may be demonstrated by several rapid diagnostic methods such as agar gel immunodiffusion (AGID), negative-staining electron microscopy (nsEM), fluorescent antibody test (FAT).

Polymerase chain reaction (PCR), and histopathology are also valuable assays in this circumstance. Monolayer cell cultures of rabbit kidney inoculated with lesion material will show the characteristic cytopathic effects of poxviruses. The presence of virus can be confirmed by immunoperoxidase monolayer assay, FAT, PCR and nsEM.

Polymerase Chain Reaction (PCR) is a molecular biology technique used to amplify specific DNA/cDNA sequences, and it's a valuable tool in detecting myxoma virus (MYXV) in infected rabbits. First, samples are collected from suspected cases of the infection. These samples can include tissue samples (such as liver or spleen), blood and others. Conjunctival swabs could be also examined by PCR for vital diagnostics of the disease (Bertagnoli, 2015).

PCR-based methods offer several advantages for MYXV detection, including high sensitivity, specificity, and rapid turnaround time. These assays are crucial for early diagnosis, surveillance, and control of disease outbreaks in rabbits.

Polymerase chain reaction (PCR) (Cavadini, 2010, Kwit, 2020) or real-time PCR (Albini, 2012, Belsham, 2010, Duarte, 2014), can be used to amplify genome fragments of MYXV in diagnostic material including eyelid, ear and nasal myxomas, crusts and/or lung lesions, nasal and conjunctival swabs or semen. All these methods are also able to detect the recombinant "MYXV Toledo/ha-MYXV" strain found in the Iberian Hares (Garcia-Bocanegra, 2019). A specific PCR method to detect this agent was set up by Dalton et al. (2019).

One Health Scientific and Research Institute, PSI in collaboration with SRI 'Veterinary Biotechnologies', LLC were developed the methodical approach for detection of the DNA of MYXV using Real-Time PCR due the reason of enhancement of myxomatosis control measures in Ukraine.

**The aim** of the presented research is to study sensitivity, specificity, repeatability and reproducibility of PCR-based protocol for detection of the DNA of MYXV Using Real-Time PCR.

#### **Materials and methods.**

During laboratory testing trials evaluated appearance, activity, specificity, sensitivity PCR-based protocol for detection of the DNA of MYXV using Real-Time PCR and reproducibility.

The *in house* kit samples from batch 1, control evaluation 1 were used, produced in SRI 'Veterinary Biotechnologies' LLC, in May, 2024.

The following samples panel has been used for specificity and sensitivity study of the test-system:

- reference panel DNAs (n = 3) of the MYXV, received from partner lab, and its 1 to 10, 1 to 100, 1 to 1000 and 1:10000 dilutions;
- DNA samples isolated from the livers of infected rabbits (n = 5).

## Section 2

- DNA samples isolated from the livers of the intact animals (n = 10).
- positive control of the amplification (PC);
- negative control of the amplification (NC);
- non-template control (NTC).

Following heterologous templates were used to demonstrate the kit's species-specific specificity (genetic material of other cattle viruses):

- *Avian poxvirus* DNA (vaccine);
- *Capripoxvirus* DNA (vaccine);
- *Bovine herpesvirus* type 1 DNA (IBR virus);

The polymerase chain reaction was managed under the recommended conditions described in the PCR-based protocol for detection of MYXV DNA Using Real-Time PCR. All testing was done without changes of the recommended features of reaction. To estimate the repeatability of testing this has been performed three times.

Table 1

**Amplification cycle for MYXV DNA detection**

Stage	Temperature	Duration	Step	Number of cycles
1	94°C	3 min.	Activation of the DNA-polymerase	1
2	94°C	10 sec	DNA denaturation	50
3	58°C	45 sec	Primer annealing/ elongation	

### Results.

Tests were conducted to verify the commission sensitivity and specificity of the test system and reproducibility obtained when using the results.

At the beginning of the commission were checked for completeness of PCR-based laboratory in house test-systems. All components necessary for operation and instruction were available.

In assessing the appearance found that the test system consists of the following components:

DNA extraction kit (kit # 1):

- «extraction buffer» (lysis bufer) – 1 flask - 15 (30) ml, transparent non-coloured liquid;

- sorbent solution – 1 (2) tube - 1,5 ml, opalescent fluid with white color;

- «Washing buffer» - 1 flask – 50 (100) ml, transparent non-coloured liquid;

- «Washing buffer ethanol» - 1 flask – 50 (100) ml, transparent non-coloured liquid;

- «solution 4» for the final washing - 1 flask – 30 (60) ml, transparent non-coloured liquid;

- TE-buffer – 1 flask – 20 (40) ml, transparent non-coloured liquid.

Kit for PCR amplification (kit # 2):

- «Virus PCR MasterMix» – 1 (2) tubes, 0,5 ml, transparent non-coloured liquid;

- SYBR Green solution — 1 tube, 0,05 or 0,1 ml, transparent light green liquid;

- primer solution (20 pM/□) —1 tube of each (4 vials, 2- for RHDV detection and 2 for RHDV-2 differentiation), 0,125 (0,25) ml, transparent non-coloured liquid;

- deionized water — 1 (2) tubes - 1,25 ml, transparent non-coloured liquid;

- positive control template (for 5 or 10 reactions) – 1 tube, 0,05 – 0,1 ml, transparent non-coloured liquid.

The PCR master mix was prepared using number of samples amount plus one under proportion per sample:

n/n	Component	Final concentration	1 x for reaction (□)
1	Water for PCR		6,7

2	Virus PCR Master Mix- Path ID	<b>1X</b>	<b>9</b>
3	SYBR Green	<b>n/a</b>	<b>1</b>
4	Primer <b>RMV_F</b> (20 pM/□)	<b>400nM</b>	<b>0,4</b>
5	Primer <b>RMV_R</b> (20 pM/□)	<b>400nM</b>	<b>0,4</b>
	DNA (template or control)		<b>2</b>

The results of the sensitivity testing is the ability to identify all encrypted obviously positive samples, it was found that the test system is able to detect DNA of MYXV in particular in both positive reference material samples, including their dilutions 1 to 10 – 1 to 10000 with Ct value 15.2-19.2. This is equal to the minimum titre of virus in the rabbit organism, we recorded an infected animal (in the liver and blood samples) in our previous research.

The specificity of the test system proved amplicon in the absence of any size of Ct value or Ct value over 38 in samples of rabbit origin tissues from non-infected animals.

Used primers didn't hybridize with DNA samples from other viruses, including DNA extracts from *Bovine herpesvirus* of types 1, avian and sheep originated poxviruses (Table. 2).

It was also marked by complete coincidence test results developed MYXV DNA detection protocol in three repetitions under similar conditions and using different Thermocyclers same type.

Table 2

**Testing results for PCR-based protocol for detection of the DNA of MYXV detection using Real-Time PCR with the panels of positive, negative and heterogenic samples**

#	Material	1st repeat result	2nd repeat result	3rd repeat result
1.	<i>Sample 1</i>	Ct 15.2	Ct 15.3	Ct 15.6
2.	<i>Sample 1 1:10</i>	Ct 16.2	Ct 16.9	Ct 16.9
3.	<i>Sample 1 1:100</i>	Ct 17.4	Ct 18.1	Ct 17.2
4.	<i>Sample 1 1:1000</i>	Ct 18.3	Ct 18.4	Ct 17.9
5.	<i>Sample 1 1:10000</i>	Ct 19.2	Ct 19.1	Ct 19.0
6.	<i>Sample 2</i>	Ct 15.2	Ct 15.4	Ct 15.2
7.	<i>Sample 2 1:10</i>	Ct 16.7	Ct 16.8	Ct 16.7
8.	<i>Sample 2 1:100</i>	Ct 17.2	Ct 17.4	Ct 17.2
9.	<i>Sample 2 1:1000</i>	Ct 18.5	Ct 17.9	Ct 18.1
10.	<i>Sample 2 1:10000</i>	Ct 19.2	Ct 19.2	Ct 19.1
11.	<i>Sample3</i>	Ct 16.6	Ct 16.8	Ct 16.9
12.	<i>Sample 3 1:10</i>	Ct 17.7	Ct 17.6	Ct 17.8
13.	<i>Sample 3 1:100</i>	Ct 18.1	Ct 18.3	Ct 17.6
14.	<i>Sample 3 1:1000</i>	Ct 18.8	Ct 19.2	Ct 18.9
15.	<i>Sample 3 1:10000</i>	Ct 19.2	Ct 19.2	Ct 19.1
16.	<i>Liver 1</i>	Ct 19.2	Ct 19.3	Ct 19.2
17.	<i>Liver 2</i>	Ct 16.2	Ct 16.8	Ct 16.4
18.	<i>Liver 3</i>	Ct 16.6	Ct 16.8	Ct 16.9
19.	<i>Liver 4</i>	Ct 18.2	Ct 18.4	Ct 18.3
20.	<i>Liver 5</i>	Ct 17.2	Ct 17.2	Ct 17.6
21.	<i>Liver (intact) 1</i>	N/d	N/d	N/d
22.	<i>Liver (intact) 2</i>	N/d	N/d	N/d
23.	<i>Liver (intact) 3</i>	N/d	N/d	N/d
24.	<i>Liver (intact) 4</i>	N/d	N/d	N/d

25.	<i>Liver (intact) 5</i>	N/d	N/d	N/d
26.	<i>Liver (intact) 6</i>	N/d	N/d	N/d
27.	<i>Liver (intact) 7</i>	N/d	N/d	N/d
28.	<i>Liver (intact) 8</i>	N/d	N/d	N/d
29.	<i>Liver (intact) 9</i>	N/d	N/d	N/d
30.	<i>Liver (intact) 10</i>	N/d	N/d	N/d
31.	IBRV	N/d	N/d	N/d
32.	CPV	N/d	N/d	N/d
33.	APV	N/d	N/d	N/d

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## ПРОТОКОЛ ПЛР-ТЕСТУВАННЯ ДЛЯ ВИЯВЛЕННЯ ДНК ВІРУСУ МІКСОМАТОЗУ КРОЛІВ

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**Резюме.** Міксоматоз - гостра вірусна хвороба кролів, що викликається вірусом міксоми, поксвірусом, який належить до роду *Leporipoxvirus*. Природними господарями цього збудника є тапеті (*Sylvilagus brasiliensis*) і щиткові кролики (*Sylvilagus bachmani*) в Південній і Центральній Америці, а також у Північній Америці відповідно. Вірус міксоми розвиває лише легку форму захворювання у цих видів, але у європейських кролів (*Oryctolagus cuniculus*) спостерігається смертельна хвороба.

Захворювання є ендемічним у багатьох країнах Європи, Азії та Америки, але збудник все ще визнаний як емерджентна інфекція, пов'язана з масовими втратами і високою смертністю серед європейських кролів.

З метою посилення заходів боротьби з міксоматозом кролів в Україні Науково-дослідним інститутом єдиного здоров'я у співпраці з ТОВ НДІ «Ветеринарні біотехнології» було розроблено методичний підхід до виявлення ДНК МУХV за допомогою ПЛР у режимі реального часу.

**Ключові слова:** міксоматоз кролів, вірус, аналіз ризику, епізоотологія, діагностика, кролі, ДНК, ПЛР.

DOI: 10.31073/onehealthjournal2024-IV-03