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Detection of viral DNA of myxoma virus using a validated PCR method with an internal amplification control

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ABSTRACT

Recognition of myxomatosis is usually based on clinical symptoms, but amyxomatous cases of the disease require the use of laboratory methods. Nowadays PCR assays are routinely employed for detection of MYXV DNA, but none of them have had their diagnostic usefulness conclusively confirmed through validation. The aim of the study was the development and validation of a PCR with an internal amplification control (IAC) for intravital and postmortem detection of viral DNA of myxoma virus. To avoid false negative results a chimeric internal amplification control (IAC) was prepared and incorporated into the PCR and amplified by the same primer set as the target DNA (M071L). The optimal concentration of particular ingredients in the PCR mixture (including IAC concentration and volume of DNA sample) was determined. To minimize the risk of amplicon carry-over contamination, uracil *N*-glycosylase was added to the reaction. Before proper validation the robustness of the IAC-PCR was verified. Validation of the method encompassed the following parameters: the analytical and diagnostic specificity (ASp, DSp) and sensitivity (ASe, DSe) of the assay, repeatability, and intra-laboratory reproducibility. The assay LOD was established at 2 TCIU of the virus particles/0.2 ml tissue homogenate with a 100% capacity to detect different MYXV strains (ASp). The method was characterized by good DSp of 0.955 (0.839–0.999 CI) and DSe of 0.976 (0.914–1.00 CI). In addition, it was repeatable and reproducible and confirmed its suitability for the detection of MYXV in clinical material. The IAC-PCR developed meets OIE validation requirements for virological methods and can be used in diagnostic or epidemiological studies of rabbit myxomatosis.

1. Introduction

Since the 1950s, when the first case of myxomatosis was detected, the disease has spread rapidly in wild and farmed rabbits worldwide (Fenner, 1994; Kerr, 2012; Bertagnoli and Marchandeau, 2015). Constant virus passages through susceptible wild rabbits have resulted in both increased animal resistance to infection and attenuation of circulating virus strains. On the other hand, the emergence of rabbits genetically resistant to disease could also drive the selection of more virulent myxoma virus (MYXV) strains (Dwyer et al., 1990; Boots et al., 2004). For instance, in Spain most circulating wild-type MYXV strains in the rabbit population were found to be highly virulent (Alda et al., 2009). Poxviruses, of which MYXV is one, evolve easily because of a high mutation rate compared to other animal DNA viruses (Kerr et al., 2012). As a consequence, myxomatosis still remains a real problem even on rabbit farms where vaccination has been implemented (Dalton et al., 2015). Myxomatosis is a systemic and lethal infection characterized by swelling and myxomas mainly located on the rabbit's ears, eyelids and face. Besides the typical nodular form of the disease, the amyxomatous form associated with lung infections is often reported in farmed animals (Marlier et al., 2000; Farsang et al., 2003; Pšikal et al., 2003; Kopczewski and Sroka, 2007).

Considering the difficulties of diagnosing the amyxomatous form of the disease, there is a need to establish a fast and reliable molecular technique for identification of MYXV infections when the first symptoms of disease appear. Early detection of disease and subsequent application of the appropriate control measures could limit the spread of the virus in the herd. Several PCR-based assays have been developed and are widely used for rapid detection of MYXV (Dalton et al., 2009; Albini et al., 2012; Duarte et al., 2013). They are able to detect viral DNA in a vast range of clinical samples. Moreover, some methods allow discrimination between vaccinated and naturally infected animals and detection of mixed infections caused by wild-type and vaccine MYXV strains (Cavadini et al., 2010). However they are prone to inhibition which may result in false negative results. Therefore testing of the samples by the use of molecular methods requires application of

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different controls to confirm that the method is operating as intended. Among them is the internal amplification control (IAC) which allows monitoring of reaction inhibition during amplification of the target virus sequence. This control and others warrant that the assay operates correctly. Consistent and reliable results can be obtained only when the assay used has been validated and confirmed to have good analytical and diagnostic performance.

Here, we report the development, optimization and validation of a sensitive PCR-based assay for intravital and postmortem detection of rabbit myxomatosis. To confirm the performance of the assay an IAC was incorporated into the method.

2. Materials and methods

2.1. Reference material, wild-type MYXV strains and swab samples

A cell culture infected with the reference MYXV Lausanne strain (ATCC VR-115), 10% tissue homogenates of rabbit skin containing the virus (Lausanne strain), and equivalent MYXV-negatives were used as control reference material. This material belonged to the collection of virus strains of the National Reference Laboratory for Rabbit Myxomatosis at the National Veterinary Research Institute in Puławy, Poland. Virus suspensions containing $10^{2.0}$ or $10^{4.0}$ tissue culture infectious units (TCIU)/ml of MYXV were used for the optimization of the IAC-PCR and subsequent validation studies. To assess whether the performance of the developed IAC-PCR was adequate, 54 samples of rabbit skin collected from animals which died of nodular myxomatosis in the years 1983 to 2013 in Poland were used (Table 1, Fig. 1). Infections in rabbits were confirmed by AGID assay. In addition, 16 conjunctival swab samples collected from symptomatically infected outbreak rabbits on the same farms on which disease was recognized were used to check the suitability of the method for intravital detection of MYXV infections. All samples were stored at −20 °C until DNA isolation took place.

2.2. Selection of PCR primers

IAC-PCR primers for specific detection of MYXV were designed based on the multiple sequence alignment of the M071 L gene fragment of the Lausanne virus strain (GenBank accession no. AF170726), French vaccine strain (GenBank accession no. GQ409969), and Polish (GenBank accession nos. MK347431-53), British (GenBank accession nos. JX565566, JX565572 and KC660084), Spanish (GenBank accession no. EU552530), Australian (GenBank accession nos. JX565562-65, JX565567-69, JX565571, JX565573-84, KC660079-83 and KC660085), US (GenBank accession no. KF148065) and Brazilian (GenBank accession no. JX565574) wild-type MYXV strains using Primer3 v. 4.0 software. Primers were synthesized by Genomed S.A. and stored at −20 °C until use.

2.3. IAC construction

The IAC was constructed based on DNA fragments of the InvA and InvE genes of *Salmonella* Enteritidis. In order to obtain a 501 bp IAC product, a primer pair (Myxo_M071_IACF-R) composed of nucleotide sequences of the primers used for *Salmonella* and MYXV detection was designed (Table 2). PCRs were carried out in 25 µl reaction volumes under the conditions described in Section 3.1 except for a higher primer annealing temperature (68 °C). The gel-purified amplification product was cloned into the pCR 2.1-TOPO plasmid vector using the TOPO TA Cloning Kit (Invitrogen, Waltham, MA, USA). Plasmid DNA extraction was conducted using a Plasmid Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The presence of the correct sequence of DNA insert was verified by sequencing (Genomed S.A., Warsaw, Poland). Plasmid DNA at a concentration of 50 ng/µl was stored at −80 °C until use.

Table 1
Detailed information on samples collected from MYXV outbreak rabbits, geographical location of outbreaks and year of their occurrence in Poland.

No. of sample	MYXV strain	Province	District	Year	Designation in figure 1
1	2-08	Kujawy-Pomerania	Toruński	2008	1
2	1-11		Toruń	2011	2
3	ZA-83	Lublin	Zamojski	1983	3
4	128-94		Parczewski	1994	4
5-7	17-99, 5-00, 18-09		Puławski	1999, 2000, 2009	5
8, 9	9-91, 112-94	Łódź	Piotrków Trybunalski	1991,1994	6
10, 11	126-94, 1-95		Łódź	1994, 1995	7
12, 13	12-96, 8-99		Łaski	1996, 1999	8
14	45-97		Sieradzki	1997	9
15, 16	3-99, 10-00		Zduńskowolski	1999, 2000	10
17	4-99		Pabianicki	1999	11
18	26-01		Opoczyński	2001	12
19	27-01		Radomszczański		13
20	1-06		Zgierski	2006	14
21	56-93	Małopolska	Tarnów	1993	15
22	13-98		Tarnowski	1998	16
23	2-99		Kraków	1999	17
24-31	159-94, 7-96, 6-99, 7-99, 9-99, 6-00, 7-00, 28-01	Mazovia	Radom	1994, 1996, 1999, 2000, 2001	18
32-36	14-98, 15-98, 16-98, 17-98/ 18-98		Lipski	1998	19
37	24-98		Radomski		20
38	16-99		Kozienicki	1999	21
39	1-00		Grójecki	2000	22
40	8-00		Szydłowiecki		23
41	8-01		Siedlce	2001	24
42	9-95	Podkarpacie	Mielecki	1995	25
43	19-98		Jarosławski	1998	26
44-46	20-98, 21-98, 22-98		Lubaczowski		27
47	23-98		Przeworski		28
48	29-01	Podlasie	Bielski	2001	29
49-51	13-99, 14-99, 15-99	Silesia	Lubliniecki	1999	30
52	1-13	Wielkopolska	Śremski	2013	31
53	1-05	West Pomerania	Policki	2005	32
54	1-08		Łobeski	2008	33

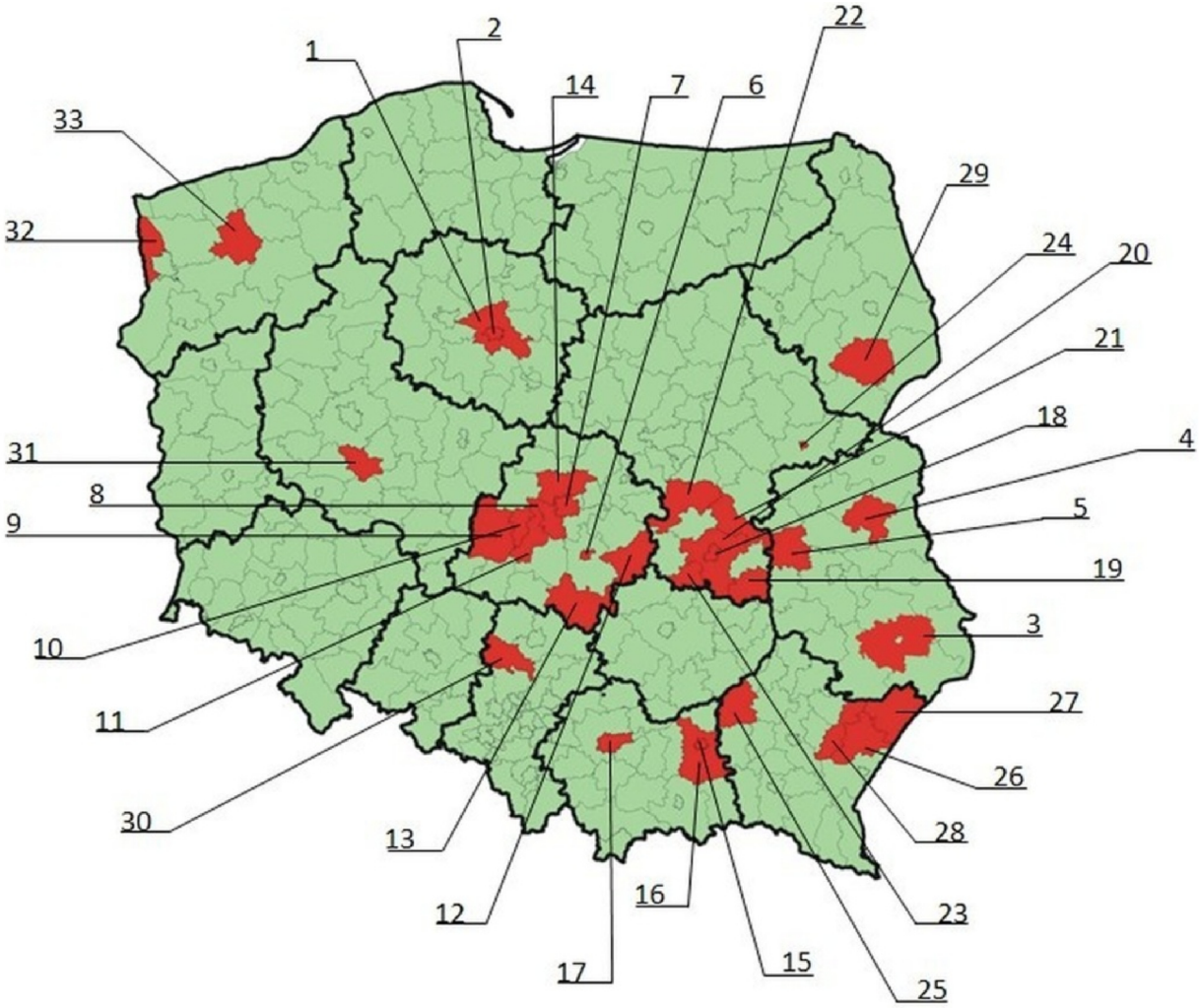

Fig. 1. Geographical distribution of myxomatosis outbreaks detected in the years 1983–2013 in Poland. Numbers (see Table 1) indicate districts (highlighted in red) in which disease occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Development and optimization of IAC-PCR

Viral DNA was extracted from 200 µl of a cell culture suspension containing $10^{2.0}$ TCIU/ml of MYXV using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Subsequently, the PCR was optimized for concentration of the reaction ingredients. The concentrations of the following components of the PCR mixture were tested: primers MyxoM071F and R (from 0.1 to 0.8 µM), magnesium ions (from 0.5 to 2.5 mM), and *Taq* polymerase (Invitrogen) (from 1 to 4 U); addition (from 5 to 40 µg) of bovine serum albumin (BSA) was also evaluated. Finally the volume of DNA sample (from 1 to 5 µl) added to the reaction and annealing temperature of primers were checked. Initial denaturation of nucleic acids was carried out at 95 °C for 5 min followed by 35 cycles consisting of amplification at 94 °C for 30 s, primer annealing from 50.5 °C to 58.5 °C for 30 s, extension at 72 °C for 45 s, and a final extension at the same temperature for 7 min (Supplementary Table S1). To minimize the risk of amplicon carry-over contamination from the previous reaction, heat-killed thermolabile uracil *N*-glycosylase (HK-UNG, Epicentre, Madison, WI, USA) was added to the reaction mixture. The optimal concentration of the enzyme (from 0.1 to 0.5 U), digestion time (15 or 30 min) and temperature of the reaction (37 °C or 50 °C) were also determined experimentally (Supplementary Table S1). For the optimization of IAC concentration in the PCR mixture, tenfold dilutions of plasmid DNA (from 10 pg to 1 fg of DNA) were prepared and added to each reaction mixture containing 2 µl of MYXV DNA extracted from cell culture suspension. The IAC-PCR assay was carried out in a 25 µl reaction volume containing 1 × PCR buffer (Invitrogen), 2 mM of deoxyribonucleotide triphosphate (dNTP) mix (dATP, dCTP, dGTP, and dUTP) (Invitrogen), and molecular grade water to the required volume. The correct performance of the MYXV

Table 2
Primers used for IAC construction and detection of MYXV.

Application	Name of primers	Position	Sequence (5' → 3')	Product size (bp)	References
Virus detection	MyxoM071F MyxoM071R	67231-67252 67565-67586	CCGCCAAGAACCACAGTAGTTT CGTGGAGGAGATCATCAGAACA	356 bp	This work
IAC construction	Myxo_M071_IACF Myxo_M071_IACR	– –	CCGCCAAGAACCACAGTAGTTT**TGCCTACAAGCATGAAATGG**[a] CGTGGAGGAGATCATCAGAACA**AAAACTGGACCACGGTGACAA**[a]	501 bp	This work, Stone et al., 1994

[a] *Salmonella* sequence is printed in bold.

DNA isolation and amplification steps was monitored using the appropriate controls. These were positive (suspension of cell culture containing MYXV Lausanne strain) and negative (water used instead of the sample) DNA extraction controls, reaction mixture control (PCR mixture without a DNA template) and an environmental control (an open tube during the addition of the DNA sample). PCR products were analyzed in 1.7% agarose gel containing SimplySafe dye (EURx, Gdańsk, Poland). Electrophoresis was carried out in 1 × TBE buffer, pH 8.0 at a constant voltage of 100 V (5 V/cm) for 90 min. The size of amplicons obtained was compared to a DNA mass standard (GeneRuler 100 bp DNA Ladder, Thermo Fisher Scientific (Fermentas), Waltham, MA, USA).

2.5. Determination of robustness of the method

Before proper validation of the IAC-PCR, its robustness was verified based on changes introduced into the protocol: i) a change to the purity of the DNA sample by deliberately not washing viral DNA prior to its elution from the column, ii) changing the reaction volume (23 µl ± 1%, 5%, 10%, 15% and 20%) with proper concentration of individual PCR components, and iii) eschewing the optimal annealing temperatures for primers and changing to 54 °C, 55 °C, 57 °C and 58 °C. The influence of the purity of the DNA sample was assessed spectrophotometrically with a NanoPhotometer (Implen, Munich, Germany) by measuring UV light absorbance at 260/280 nm. The impact of other changes introduced on the amplification efficiency of the reaction was also spectrophotometrically determined by measuring the concentration of PCR product obtained for each reaction compared to that of PCR mixture without a DNA template. DNA concentration was established in ng/µl. Reactions were performed in triplicate for each evaluated parameter.

2.6. Validation of the assay

Validation of the IAC-PCR method was performed according to the recommendations described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2018). The following parameters of the method were determined: i) the analytical specificity (ASp) and sensitivity (ASe), ii) the diagnostic specificity (DSp) and sensitivity (DSe) and iii) repeatability and intra-laboratory reproducibility. ASp was assessed based on the method's exclusivity, inclusivity and selectivity, i.e. the ability to detect MYXV DNA in a reaction mixture containing different concentrations of the following inhibitory substances: $CaCl_2$ (1–7.5 mM), KCl (10–100 mM), EDTA (0.1–2.5 mM), SDS (0.0025–0.03%), ethanol (2–6%), heme (0.001–0.1%) and collagen (20–60 ng/µl). Exclusivity was defined as the lack of detection of other lagomorph viruses, bacteria, fungal pathogens or parasites. The assay inclusivity (capacity to detect different MYXV strains) was evaluated *in silico* by BLAST comparison of the nucleotide sequences of IAC-PCR primers and the M071 L gene fragments of Polish and other MYXV strains available in the GenBank database (see Section 2.2). ASe, also known as the detection limit of the method (LOD) was determined using serial tenfold dilutions (from 10^{-1} to 10^{-6}) of MYXV DNA in nuclease-free water extracted from tissue homogenate of rabbit skin. The lowest concentration of viral DNA that tested positive was considered to be the assay LOD. Testing was performed in 10 replicates. The assessment of DSp and DSe was conducted using a panel of 20 negative and 40 positive samples with low ($1 \times 10^{2.0}$ TCIU/ml) and high ($1 \times 10^{4.0}$ TCIU/ml) levels of MYXV contamination. DSp and DSe were estimated using the proportions of positive and negative results to the total number of tested samples with subsequent determination of the confidence intervals using the Clopper-Pearson method (Fleiss, 1981; Gardner et al., 2000). Results were expressed as modes and confidence intervals shown as the percentile values of a ß-distribution with 0.025 for the lower and 0.975 for the upper limit. The method's repeatability was assessed by the testing of the same panel of samples (15 positive and 5 negative) by the same operator 7 days apart and its reproducibility was assessed by different analysts independently using various thermocyclers.

2.7. Virus detection in clinical samples

Viral DNA was extracted from 200 µl of tissue homogenates and swab samples using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The developed IAC-PCR method was used to detect MYXV in clinical material encompassing 54 virus-positive archive skin samples and 16 conjunctival swabs collected from symptomatically infected rabbits from outbreaks of myxomatosis.

3. Results

3.1. Optimization of the IAC-PCR

IAC-PCR MyxoM071F-R primers were designed in the highly conserved region of the M071 L gene of Polish and other MYXV strains available in GenBank. They allowed amplification of a 356 bp DNA fragment of MYXV of which the specificity was confirmed by sequencing. The optimized assay was carried out in a 25 µl reaction volume containing 1 × reaction buffer, 2 mM dNTPs, 2 mM $MgCl_2$, 0.4 µM of each primer (MyxoM071F-R), 1 U Platinum *Taq* DNA polymerase, 10 fg of IAC DNA and 2 µl of DNA sample (Table 3). There was no increase in amplification efficiency observed in mixtures containing the addition of BSA. In order to prevent a carry-over contamination, the reaction mixture was supplemented with 0.2 U HK-UNG. To obtain the highest assay sensitivity and to avoid interference with the amplified target sequence, the concentration of IAC DNA in the reaction was also determined. IAC produced a visible signal when the reaction contained from 10 pg to 1 fg of IAC DNA. At concentrations above 100 fg IAC decreased the efficiency of amplification of MYXV DNA but at concentrations of 10 fg or below it had no adverse effect on amplification of the DNA template, and therefore this was observed to be the optimal concentration range (Supplementary Fig. S1). The following optimal temperature profile was used: incubation of the mixture with the HK-UNG at 50 °C for 30 min, initial denaturation at 95 °C for 5 min and 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 45 s and a final elongation step at 72 °C for 7 min (Table 4).

3.2. Assessment of method robustness and validation parameters

By examining method robustness it was seen that the purity of DNA samples with an A260/A280 ratio above 2.0 inhibited amplification of viral DNA. Findings from this study suggest that the change in reaction volume did not affect the amplification efficiency, whereas changes of primer annealing temperature resulted in its 10% deterioration. As

Table 3
The composition of the optimized PCR mixture.

Ingredient	Volume	Concentration in the mixture
Nuclease-free water	10.8 µl	–
10 x PCR buffer	2.5 µl	1 x
dNTPs[a]	2.5 µl	1(2) mM
Magnesium ions	1.0 µl	2 mM
MyxoM071F	1.0 µl	0.4 µM
MyxoM071R	1.0 µl	0.4 µM
HK-UNG	2.0 µl	0.2 U
Taq polymerase	0.2 µl	1 U
IAC DNA	2.0 µl	10 fg
sample DNA	2.0 µl	–
Total	25.0 µl	–

[a] The 1 mM concentration of dNTPs refers only to the dATP, dCTP and dGTP content. In the case of dUTP a 2 mM concentration is present in the master mix.

Table 4
Temperature profile of the optimized IAC-PCR.

Stage	Temperature (°C)	Time	Cycle number
Incubation with HK-UNG	50	30 min.	–
Initial denaturation	95	5 min.	–
Denaturation	94	30 s	35
Annealing	56	30 s	
Elongation	72	45 s	
Final elongation	72	7 min.	–

demonstrated here the results confirmed the robustness of the IAC-PCR in the examined range of parameters. When assessing the ASp of the method, 100% homology of the IAC-PCR primer sequences with nucleotide sequences of wild-type Polish, Lausanne and other MYXV strains was demonstrated. Moreover, there was no cross-reactivity found with other lagomorph pathogens (the exclusivity test) (Supplementary Fig. S2). The selectivity test showed that various substances could negatively affect the PCR. Their minimal inhibitory concentrations were as follows: $CaCl_2$ 2.5 mM, KCl 50 mM, EDTA 1 mM, SDS 0.01%, ethanol 4%, and heme 0.1%. In the case of the presence of tissue collagen in the examined samples, no reaction inhibition was seen. The assay LOD was established at 10 TCIU/ml i.e. 2 TCIU of the virus/0.2 ml of tested tissue homogenate (Supplementary Fig. S3). The most probable DSe value for the IAC-PCR was 0.976 (0.914–1.00) with a 95% confidence interval, whereas the DSp for negative samples was 0.955 (0.839–0.999) (Table 5). A graphic demonstration of the DSe and DSp results together with the probability distribution is presented in Fig. 2. All positive and negative samples used in repeatability and intra-laboratory reproducibility tests were correctly identified by IAC-PCR.

3.3. Diagnostic application of IAC-PCR

The developed IAC-PCR was able to successfully detect all virus strains in archive tissue samples. Likewise, PCR amplicons of predicted size were obtained for all conjunctival swabs collected from infected rabbits during an outbreak of myxomatosis. The controls used (including IAC) confirmed the correct method performance (Fig. 3).

4. Discussion

Despite the common use of vaccines against myxomatosis, MYXV still causes outbreaks of disease in farmed rabbits (Calvete et al., 2002; Kerr, 2012; Bertagnoli and Marchandeau, 2015; Dalton et al., 2015). Although recognition of the typical nodular form of the disease is straightforward, the infections caused by lower virulence or amyxomatous virus strains could remain unrecognized (Marlier et al., 2000, 2001). In addition, mixed infections with both wild-type and vaccine strains also require the use of laboratory methods for confirmation. To meet this need, an IAC-PCR method for rapid detection of MYXV in clinical samples was established and validated in this study.

Nowadays, PCR-based methods are routinely used in the diagnosis of myxomatosis. Besides identification of the virus, they enable strain characterization (Dalton et al., 2009; Kerr et al., 2010) and differentiation between attenuated, wild-type and vaccine strains (Cavadini et al., 2010). PCR primers are mainly designed in the conserved regions of the MYXV genome such as the M071 L (Cavadini et al., 2010), M034 L (Dalton et al., 2010), M152R (Pérez de Rozas et al., 2008; Albini et al., 2012), M029 L (Belsham et al., 2010) and M000.5 L/R genes (Duarte et al., 2013). In this study, the M071 L gene fragment, which is highly conserved among foreign and Polish MYXV strains, was chosen for the design of the IAC-PCR (unpublished data). Based on genome similarities of wild-type, and vaccine virus strains, it is highly likely that PCR methods currently used in MYXV diagnostics, and the developed IAC-PCR could detect all virus strains.

The diagnostic methods used for detection of animal infectious diseases should be validated (OIE, 2018). Currently only the real-time PCR protocol of Duarte et al. (2013) has been taken through the validation process and has met OIE requirements. Although validation confirmed the high sensitivity and specificity of the method, some validation parameters such as selectivity and robustness were not assessed. Moreover, only a small number of virus strains circulating in a limited geographical area were tested using this assay. The IAC-PCR described in this work was also rigorously validated according to the OIE requirements. The validation parameters confirmed the method's good analytical and diagnostic specificity and sensitivity as well as its repeatability, intra-laboratory reproducibility and suitability for the detection of MYXV in animal tissues and conjunctival swab samples. Moreover, the inter-laboratory comparison trial aiming for the detection of MYXV DNA isolated from skin tissues of outbreak animals confirmed correct method operation (unpublished results). Thorough method optimization and subsequent validation for tolerance of non-ideal circumstances confirmed its robustness to changing reaction conditions and low sensitivity to the presence of sample-derived inhibitors affecting the course of amplification. Direct comparison of the detection limits between validated methods, i.e. qualitative IAC-PCR and quantitative real-time PCRs, would be difficult, however IAC-PCR was able to detect at least 2 TCIU of the virus/0.2 ml of tested tissue homogenate compared to a theoretically more sensitive (2.6 copies of MYXV DNA) real-time PCR (Duarte et al., 2013). The DSe and DSp results for the method should be considered good in relation to generally accepted parameters characterizing diagnostic methods. In fact, when the DSe value was analyzed within the adopted confidence intervals, 8.6% of MYXV-positive and 16.1% of negative samples either remained undetected or were identified as false positive. It is noteworthy that the mode value (the most probable result) was close to the one indicating correct virus detection (100% results concordance) in all tested samples.

Although molecular methods enable rapid identification of the virus and early detection of infection, their use is associated with the risk of obtaining false positive or negative results (Vaneechoutte and Van Eldere, 1997; Burkardt, 2000). Therefore to monitor the course of the reaction, several types of control including an internal control (IC) of amplification should be used during molecular analysis (Hoorfar et al., 2004; Maaroufi et al., 2006). It allows the reaction environment to be monitored, in particular the presence of sample-derived enzyme inhibitors (Sachadyn and Kur, 1998; Rodríguez-Lázaro et al., 2004; Diez-Valcarce et al., 2011). The developed PCR assay contained an IAC constituted of bacterial DNA containing primer sequences complementary to the sequence of the amplified MYXV gene fragment. Until now, in the diagnosis of rabbit viral diseases, an IC has only been used in molecular detection of rabbit hemorrhagic disease virus (Gall et al., 2007; Le Gall-Reculé et al., 2017; Dalton et al., 2018; Hall et al.,

Table 5
DSe and DSp for IAC-PCR with determined confidence intervals.

Parameter	Number of samples (n)		Average (x)	Mode	Confidence interval		Standard deviation (Sd)
	tested	correctly identified			low (0.025)	high (0.975)	
DSe	40	40	0.976	1	0.914	1.0	0.023
DSp	20	20	0.954	1	0.839	0.999	0.043

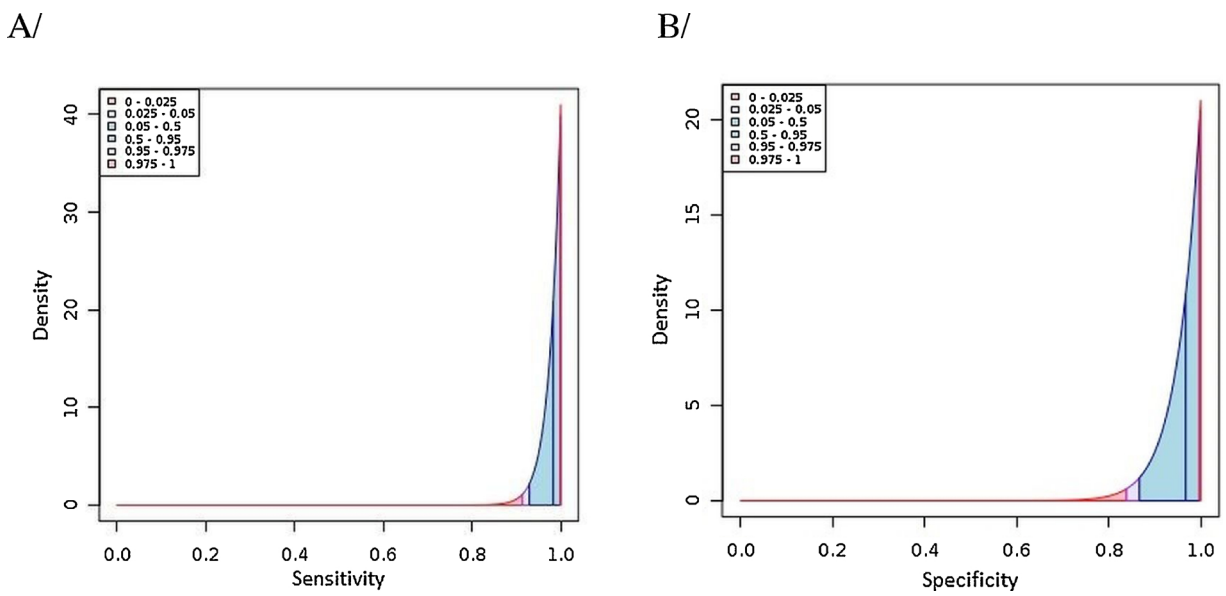

Fig. 2. DSe (A) and DSp (B) of IAC-PCR. Values fitting the confidence interval were marked in blue (0.025–0.975). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

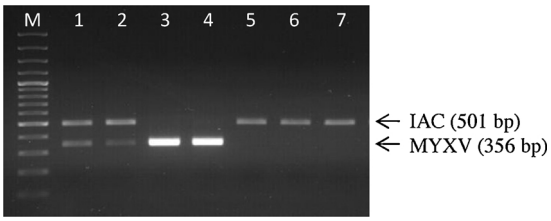

Fig. 3. An example of IAC-PCR results for MYXV detection in archive skin samples collected from rabbits from outbreaks of myxomatosis. M - 100 bp DNA Ladder, 1 - weekly positive sample (MYXV 20–98 strain), 2 - weekly positive sample (MYXV 2–99 strain), 3 - strong positive sample (MYXV 18-09 strain), 4 - strong positive sample (MYXV 1–11 strain), 5 - negative DNA extraction control, 6 - negative control of reaction mixture, 7 - negative environmental PCR control.

2018). Besides IAC, an enzymatic treatment of the PCR mixture with HK-UNG could be employed to prevent PCR carry-over contamination with a specific product of the previous amplification (Pang et al., 1992; Rys and Persing, 1993; Mohamed et al., 2004). Its usefulness in suppressing sample cross-contamination events has previously been proved in a real-time qPCR for MYXV detection (Belsham et al., 2010). Similarly, in our studies, supplementation of the reaction mixture with HK-UNG eliminated the risk of false positive results.

Although the IAC-PCR was able to detect a broad number of wild-type virus strains circulating over the years in the Polish rabbit population, complete knowledge of the method's detection capability could only be obtained if atypical virus strains were tested using this method. Nevertheless, the comparative sequence analysis of wild-type and reference virus strains encouraged the assumption that the developed IAC-PCR should allow the detection of atypical MYXV strains. The method is characterized by high diagnostic sensitivity and specificity and confirmed its suitability for early detection of MYXV DNA in animal tissues and in conjunctival swabs collected from rabbits at different phases of disease. The IAC-PCR developed in the present study is suitably equipped with different types of controls monitoring correct performance of the method. Therefore this method fulfills the requirements for a robust molecular tool to be used in official monitoring programs or surveillance studies of myxomatosis, a notifiable rabbit disease. Only a validated assay can provide reliable results on disease occurrence, and therefore implementation of disease control measures should rely on such an assay.

The OIE has established requirements for virological methods used for diagnostic or epidemiological studies of infectious animal diseases. The developed IAC-PCR method for the detection of MYXV infections in rabbits was validated according to these requirements. It is characterized by good diagnostic sensitivity and specificity, and can be used for intravital and postmortem detection of myxomatosis.

Authors' contributions

EK collected samples, performed the molecular assays, compiled and analysed results and drafted the manuscript. ZO performed statistical analyses, interpreted the results and prepared their graphical presentation. AR conceived the original idea, interpreted the results and corrected the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113709.

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