applied microbiology

MDPI

Article

The Development of a Real-Time PCR Assay for Specific Detection of the NISKHI Sheep Pox Vaccine Virus Strain DNA

Alexander Sprygin [1,*], Ali Mazloum [1], Antoinette Van Schalkwyk [2,3], Alena Krotova [1], Kseniya Shalina [1], Marko Dmitric [4], Olga Byadovskaya [1], Larisa Prokhvatilova [1] and Ilya Chvala [1]

1 Federal Center for Animal Health, 600901 Vladimir, Russia
2 Agricultural Research Council, Onderstepoort Veterinary Institute, 100 Old Soutpan Road, Onderstepoort 0110, South Africa
3 Department of Biotechnology, University of the Western Cape, Robert Sobukwe Road, Bellville 7535, South Africa
4 Department of Food Safety, Veterinary Specialized Institute Kraljevo, Zicka 34, 36000 Kraljevo, Serbia
* Correspondence: spriginav@mail.ru

Abstract: Sheep pox (SPP) constitutes a global animal health scourge, despite the numerous efforts targeting the eradication of the disease implemented in affected countries. An efficient control and eradication strategy incorporates the use of live attenuated vaccines, which in turn requires a method for differentiation between vaccinated and infected sheep. The NISKHI live attenuated SPP vaccine (LAV) is abundantly used in Russia, Kazakhstan and other Central Asian countries. This study describes the development and evaluation of a real-time PCR with a high-resolution melting assay, capable of differentiating the NISKHI vaccine virus from circulating virulent field strains. The RNA polymerase subunit RPO132 gene contains a unique single nucleotide polymorphism (SNP) capable of altering the melting curves of amplicons from LAV and virulent field isolates circulating in the region. The melting temperature (Tm) of field isolates ranged from 75.47 °C ± 0.04 to 75.86 °C ± 0.08, while the vaccine strain averaged 76.46 °C ± 0.12. Subsequent evaluation of this assay demonstrated that the recent SPP outbreaks in central Russia may be attributed to virulent field isolates. This robust assay was proven to consistently and differentially detect the NISKHI LAV strain when analyzing clinical samples from affected sheep.

Keywords: sheep pox virus; vaccine; NISKHI; sequences; HRM

Citation: Sprygin, A.; Mazloum, A.; Van Schalkwyk, A.; Krotova, A.; Shalina, K.; Dmitric, M.; Byadovskaya, O.; Prokhvatilova, L.; Chvala, I. The Development of a Real-Time PCR Assay for Specific Detection of the NISKHI Sheep Pox Vaccine Virus Strain DNA. *Appl. Microbiol.* **2022**, *2*, 956–964. https://doi.org/10.3390/applmicrobiol2040073

Academic Editor: Fulvio Marsilio

Received: 19 October 2022
Accepted: 7 November 2022
Published: 15 November 2022

1. Introduction

Sheep pox virus (SPPV) is the etiological agent of the important sheep pox (SPP) disease, notifiable to the World Organization for Animal Health (OIE). It belongs to the poxvirus genus *Capripoxvirus* [1], which additionally includes the lumpy skin disease virus (LSDV) and goat pox virus (GTPV). The SPPV genome is approximately 150 kilobase pairs (kbp) with 147 open reading frames and exhibits 96% nucleotide identity with GTPV, and 97% nucleotide identity with LSDV across the entire length. Homologs of all the SPPV and GTPV genes are present in LSDV [1]. Despite observations of LSD in antelopes and giraffes, it predominantly infects cattle and buffaloes [2], while SPPV and GTPV can cross the species barrier and infect both sheep and goats [3]. Sheep pox causes considerable economic losses to milk, wool and animal production due to severe clinical signs, high mortality rates and diminished earnings from exports [4]. Clinical signs include conjunctivitis, rhinitis and skin lesions on the muzzle and other wool-free areas [5].

Sheep pox is transmitted through direct contact with an infected animal, but indirect contact transmission via objects contaminated with scabs and nasal secretions containing high viral loads has been reported [6].

The geographic distribution of SPP predominantly includes regions in the Northern Hemisphere with intensive sheep production. Regions where the disease is endemic include the Middle East, North Africa, the Indian subcontinent, large regions of central

Asia, Turkey, Iraq, Iran, Afghanistan and southeastern Europe [5]. Recently outbreaks have been reported in Kazakhstan, Mongolia, Azerbaijan, Turkey, Greece, Bulgaria and Russia, where sporadic cases were reported in the Far East and the Caucasus. Since 2016, the frequency of outbreaks outside of the endemic regions, in the central part of Russia, has increased dramatically [7].

SPPV and GTPV vaccines could be used in cattle as a heterologous vaccine against LSD, but the safety and efficacy of these vaccines must be proved in controlled experimental challenge studies [8]. In countries affected by SPP, vaccination with a homologous SPPV vaccine is an effective strategy for controlling the spread of the disease. However, the use of live attenuated vaccines (LAV) requires specialized methods to differentiate between infected and vaccinated animals. In the absence of serological assays capable of differentiating between LAV and wild-type SPPV infections, molecular assays have been developed but are inhibited by the dearth of genomic data on circulating field strains [9].

Commercial vaccines based on different LAV strains are globally available. The Yugoslavian RM65 is widely used in the Middle East, Asia and in the Horn of Africa, while the Romanian Fanar is used in India and the Maghreb countries [10]. In contrast, the Russian Federation (RF) and various former Soviet Union countries use the NISKHI strain in vaccination campaigns [11].

A molecular assay capable of discriminating between SPP vaccines derived from the commonly used Romanian and the Yugoslavian RM/65 strains and virulent SPPV field isolates has been published [9]. Yet a similar assay does not exist capable of discriminating between the NISKHI vaccine strain widely used in Russia and Asia and virulent wild-type SPPVs.

The aim of this study was to develop and evaluate a real-time PCR assay, with a high-resolution melting profile, capable of differentiating between the NISKHI vaccine strain and the virulent wild-type sheep pox virus isolates.

2. Materials and Methods

2.1. Samples and Viruses

Eight virulent SPPV strains, isolated from outbreaks in the RF, were used to develop the real-time PCR assay in addition to the NISKHI LAV strain. Metadata pertaining to these isolates are provided in Table 1.

Table 1. A brief description of the SPPV isolates used in the design of this study.

Isolate/Strain	Region	Isolation Year	Type of Material	GenBank Accession Number
Amur	Amurskaya oblast	2018	Scabs	Not applicable
Moscow 2018 (M18)	Moscow region	2018	Scabs	ON961655
Tula	Tula region	2018	Scabs	ON961657
Pskov	Pskov region	2019	Scabs	Not applicable
Moscow 2019 (M19)	Moscow region	2019	Scabs	ON961656
Tver	Tver region	2019	Scabs	Not applicable
Kaluga	Kaluga Region	2020	Scabs	Not applicable
Dagestan	Republic of Dagestan	2022	Scabs	Not applicable
ARRIAH	"NISKHI"	1996	Cell culture	AY077834

The newly designed high-resolution melt (HRM) real-time PCR assay was validated by analyzing 55 field samples of suspected SPP outbreaks submitted to the Federal Center for Animal Health in Vladimir, Russia, for laboratory confirmation of the disease. Whole blood samples from SPP outbreaks submitted between 2018 and 2022 from the RF, were processed and analyzed as described below.

2.2. DNA Extraction

Viral genomic DNA was extracted following the phenol–chloroform extraction protocol previously published [12]. Briefly, the samples were treated with sodium dodecyl-sulfate

(SDS) and proteinase K, in order to enzymatically digest the proteins and non-nucleic acid cellular components. A mixture of organic phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0, is added to promote the partitioning of lipids and cellular debris into the organic phase, leaving the DNA in the aqueous phase. Absolute, ice-cold ethanol was added to the aqueous phase at a ratio of 2:1 (v/v) along with 5M sodium acetate (pH 5.5) at a ratio of 1:30 (v/v) and the DNA was precipitated by incubation for 1 h at −20 °C. The precipitated DNA was collected by centrifugation at 16,000 g for 30 min, air-dried and resuspended in 50 µL TE buffer [12].

2.3. Primer Design and PCR Protocol

A unique single nucleotide polymorphism (SNP) was identified in the gene encoding the RNA polymerase subunit RPO132, capable of differentiating between virulent wild-type SPPVs and the NISHKI LAV strains (Supplementary Table S1; Figure 1). A set of primers was designed to amplify this region, including the aforementioned SNP (Table 2). The primers were designed using CLC Qiagen Workbench v.21, to amplify a 109 bp region (Figure 1). The primer sequences and additional characteristics are presented in Table 2, while their binding positions in relation to the different isolates are indicated in Figure 1.

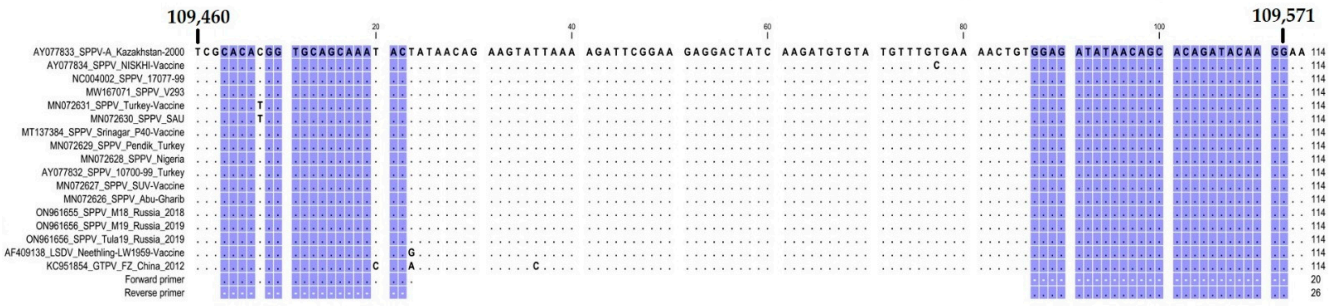

Figure 1. Alignment of specific primers to different SPPV isolates, LSDV Neethling and GTPV China 2012. The primer binding regions are indicated in blue.

Table 2. Characteristics of primers designed for the HRM assay.

Name	Sequence (5′->3′)	Template Strand	Calculated Tm (°C)	Length
Forward primer	CACACGGTGCAGCAAATACT	Plus	60.6	20
Reverse primer	CCTTGTATCTGTGCTGTTATATCTCC	Minus	59.6	26

Reactions volumes of 20 µL contained 1.0 µL each of forward and reverse primers at a final concentration of 0.5 mmol per primer, 10.0 µL of 2× SsoFast EvaGreen master mix, 5.0 µL of DNA template (contains 200 ng) and nuclease-free sterile double distilled water. Negative, or no template, and positive controls were included for each reaction.

PCR was performed in a Rotor-Gene Q5 plex HRM thermocycler using the commercial SsoFast™ EvaGreen® Supermix kit with an initial denaturation step at 98 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The PCR product was then denatured at 95 °C (held for 30 s), cooled to 65 °C (held for 60 s) and melted from 65 °C to 90 °C with a 0.1 °C temperature increment every 2 s with continuous data acquisition. The amplification plots and melting graphs were analyzed using the Rotor-Gene Q5 plex, and the corresponding curves were displayed as negative first-derivative plots of fluorescence with respect to temperature. Normalized melt curves and differences in curves were acquired by analyzing the active melt region separately for each virus by designating the corresponding pre- and post-melt regions.

2.4. Assay Validation on Clinical Samples

Eight SPPVs isolated from the RF between 2018 and 2022 were used in the design and validation of the new HRM assay (Table 1). Viral genomic DNA was extracted and the RPO132 gene sequence was determined in order to use the isolates as appropriate controls in the validation of the assay. Serial 10-fold dilutions were prepared with gDNA from the aforementioned samples as well as the NISKHI vaccine strain. The HRM assay was performed on all nine strains in triplicates and the results were expressed as mean (M) and standard deviation (SD) at ($p \leq 0.05$).

Additionally, 55 samples of suspected SPP cases were submitted for laboratory confirmation. Following DNA extractions, the samples were simultaneously subjected to PCR using the OIE-recommended primers as well as the newly developed HRM assay.

2.5. Assessment of Limit of Detection

In order to determine the limit of detection of the newly developed HRM assay, a 10-fold serial dilution of the NISKHI vaccine strain DNA was used. The virus amount was expressed in TCID 50/mL and was 2.5×10^6. Dilutions were tested in triplicate and the highest positive 10-fold dilution was subsequently diluted 2-fold to reach the maximum virus amount possible to detect [13].

2.6. Assessment of Competitiveness

In order to determine the capacity of the new assay in differentiating between co-infected samples (vaccine strain and field isolate), combinations of diluted and undiluted samples were prepared containing the gDNA of the SPPV isolate Tula (Table 1) and the NISKHI vaccine strain. Ten combinations of wild-type vaccine combinations were prepared and tested in triplicate, with the optimal melting temperature reported as the mean (M) and SD of the three replicates.

2.7. Statistical Analysis

Each HRM reaction was performed in triplicate and the results were used to perform statistical analyses and calculate the mean (M), grand mean (GM) and standard deviation (SD) based on percentage and Fisher's exact test at a 95% confidence interval at ($p \leq 0.05$) using the Statistica (version 10.0) program.

3. Results

3.1. Assay Design

The complete genome sequence of the SPPV NISKHI LAV strain was compared to all the available sequences in Genbank, representing isolates from the Russian Federation, the Middle East and North Africa (Supplementary Table S1). The RNA polymerase subunit PRO132 gene was selected, based on a unique SNP, as the target region in the design and amplification of a 109 bp amplicon (Figure 1).

The sequence alignment of the amplified region, flanked by the newly designed primers, indicated a unique "T" to "C" substitution in the NISKHI vaccine strain compared to the wild-type SPPV isolates (Figure 1). Additional SNPs were detected in the LSDV and GTPV sequences, indicating that the newly designed assay could be used to differentiate between SPPV and GTPV, but not LSDV (Figure 1).

The newly designed HRM assay was evaluated using serially diluted templates of eight samples listed in Table 1. The calculated average melting temperatures obtained from the triplicate reactions of each of the SPP-positive samples are indicated in Table 3.

Based on the results obtained in Table 3, the average melting temperature for field isolates ranged from 75.47 ± 0.04 to 75.86 ± 0.08 °C (75.65 ± 0.04 across all field samples tested), while the melting point of the vaccine strain averaged at 76.46 ± 0.12 ($p < 0.05$) (Figure 2). Based on these results, it is possible to use this assay for practical application during the differential diagnosis of field SPPV isolates from the NISKHI SPPV vaccine

strain. The reaction was still capable of detecting eight of the nine sample targets at a 10^{-4} dilution (Table 3).

Table 3. HRM testing results for field SPPV isolates (n = 3) and the NISKHI vaccine strain (ARRIAH) (n = 3) using 200 ng DNA and 10-fold dilutions.

Isolate/Strain	Original Sample (200 ng) (M/SD)	Dilution 10^{-1} (M/SD)	Dilution 10^{-2} (M/SD)	Dilution 10^{-3} (M/SD)	Dilution 10^{-4} (M/SD)	GM/SD	GM/SD
NISKHI (ARRIAH)	76.44 ± 0.07	76.57 ± 0.11	76.66 ± 0.2	76.41 ± 0.02	76.225 ± 0.21	76.46 ± 0.12	76.46 ± 0.12
Amur	75.44 ± 0.04	75.79 ± 0.04	75.77 ± 0.02	75.62 ± 0.04	75.43 ± 0.096	75.61 ± 0.05	
Pskov	75.81 ± 0.01	75.78 ± 0.02	75.75 ± 0.02	75.63 ± 0.04	75.41 ± 0.06	75.68 ± 0.03	
Kaluga	75.71 ± 0.01	75.69 ± 0.02	75.65 ± 0.05	75.59 ± 0.01	ND	75.66 ± 0.02	
Tula	75.32 ± 0.03	75.67 ± 0.03	75.62 ± 0.07	75.57 ± 0.03	75.18± 0.09	75.47 ± 0.04	
M19	75.74 ± 0.04	75.68 ± 0.02	75.67 ± 0.02	75.59 ± 0.03	75.51 ± 0.01	75.64 ± 0.02	
Tver	75.66 ± 0.05	75.72 ± 0.03	75.73 ± 0.04	75.57 ± 0.04	75.56 ± 0.02	75.65 ± 0.04	
Dagestan	75.5 ± 0.07	75.79 ± 0.04	75.84 ± 0.04	75.68 ± 0.04	75.62 ± 0.09	75.69 ± 0.06	
M18	75.89 ± 0.1	75.9 ± 0.07	75.88 ± 0.08	75.78 ± 0.06	ND	75.86 ± 0.08	

Not detected (ND) referred to a reaction that failed to produce a detectable fluorescent signal.

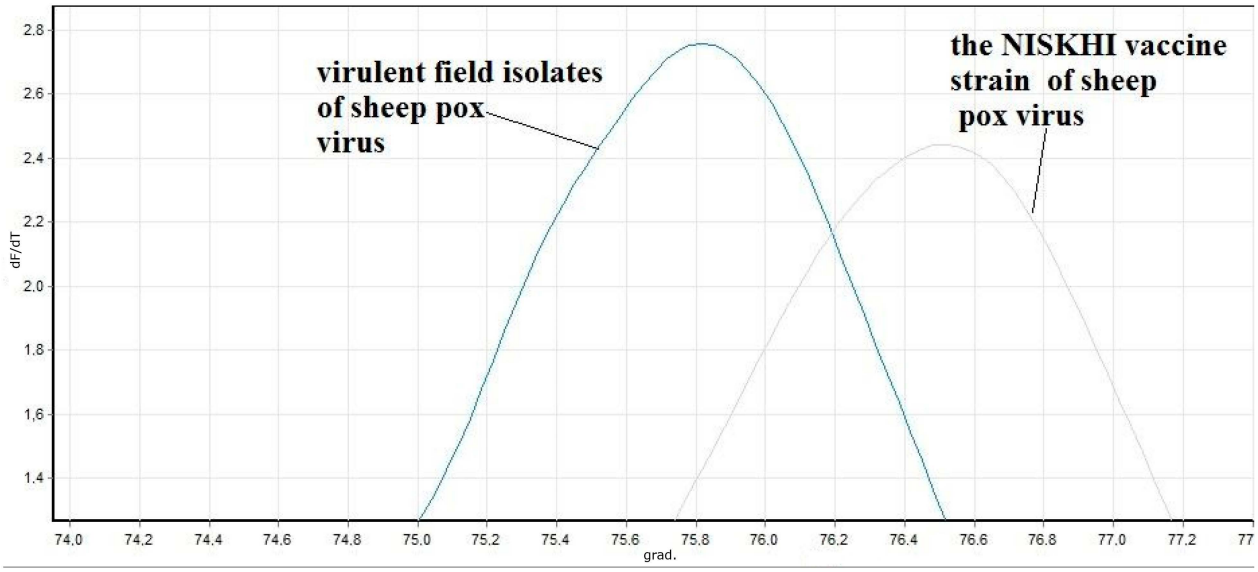

Figure 2. Melting curve variance of SPPV field isolates and the NISKHI vaccine strain.

The differences in melting temperature and curves between the field isolates and the NISKHI vaccine strain are indicated in Figure 2. Based on the melting curves, a clear differentiation of 0.8 °C between the virulent field isolates and the NISKHI vaccine strain was observed.

In order to evaluate the binding and amplification competitiveness between the field and vaccine strain targets, DNA obtained from one target was serially diluted in a constant background of the genomic DNA of the other target (Table 4). The opposite reactions were subsequently performed, where dilutions of the vaccine template were analyzed in the presence of a constant concentration of the field virus DNA template (Table 4).

The data presented in Table 4 demonstrate the capability of the HRM assay to detect both wild-type and vaccine templates in a single reaction. It was observed that both targets were amplified only if they were present at equal concentrations. If the concentrations between the two targets differ by more than 10-fold, the more abundant target outcompetes the other (Table 4). The results are indicated based on a constant concentration of field isolate to a different dilution of the vaccine strain, followed by a constant concentration of vaccine strain to a different dilution of field genomic DNA (Table 4).

Table 4. HRM testing results for constant backgrounds of one target and serial dilutions of the other target (n = 3).

Field Isolate (Constant)/Vaccine Strain (Variable)	**M/SD**	**M/SD**
01:01	75.64/0.14	76.17/0.08
$1{:}10^{-1}$	75.58/0.01	Neg
$1{:}10^{-2}$	75.52/0.12	neg
$1{:}10^{-3}$	75.4/0.03	neg
$1{:}10^{-4}$	75.43/0.07	neg
Vaccine strain (Constant)/Field Isolate (Variable)	**M/SD**	**M/SD**
01:01	76.4/0.04	75.61/0.03
$1{:}10^{-1}$	76.43/0.01	neg
$1{:}10^{-2}$	76.27/0.03	neg
$1{:}10^{-3}$	76.08/0.06	neg
$1{:}10^{-4}$	76.71/0.01	neg

3.2. Performance of the HRM Assay on Clinical Samples

Clinical samples (n = 55) from suspected SPP outbreaks were investigated using the OIE-approved PCR assay and positive samples were submitted to subsequent testing and differentiation based on the new HRM assay. The results indicated that of the 55 samples submitted for laboratory confirmation of SPP, 47 were PCR-positive and all of them belonged to the field or wild-type strain, being thus unrelated to the NISKHI vaccine strain. The optimal Tm of the isolates clustered within the established range previously determined (75.47 $\pm$ 0.04 to 75.86 $\pm$ 0.08) (results not shown). The remaining eight samples were confirmed as SPPV-negative, by subjecting them to the OIE-prescribed pan-capripoxvirus PCR assay (data not shown). The newly described assay will in the future be applied only to SPPV PCR-positive samples for subsequent discrimination between wild-type and vaccine strains.

4. Discussion

The use of LAVs necessitates a strategy of differentiating between infected and vaccinated animals (DIVA) and the introduction of a DIVA strategy could be applied as an alternative to the stamping out strategy that disease-free countries follow to control the outbreaks [14]. Currently, all the commercially available LAVs are derived from subjecting circulating field isolates to multiple passages in cell culture in order to obtain an attenuated phenotype [15]. The design and construction of next-generation capripoxvirus vaccines necessitates the inherent ability to serologically discriminate between vaccine and wild-type virus infection. Considering the worldwide spread of capripoxviruses and their economic impact, such a DIVA approach is in demand.

The most efficient strategy against SPP is vaccination; however, outbreaks do sporadically occur despite continuous vaccination campaigns [16]. In order to successfully control the spread of the virus, it is essential to identify whether an animal was infected with the field strain or if the vaccine failed to provide sufficient protection [17,18]. Vaccines could cause post-vaccine reactions, which could inhibit veterinary scientists from successfully diagnosing the disease [19]. Additionally, rare cases of vaccine revertance, or the adaptation of an attenuated strain to regain virulence, have been described to significantly impede the subsequent capabilities of diagnostic and DIVA assays [20–22].

Considering the wide use of LAV SPP-based vaccines in endemic countries with sporadic occurrences of the disease [18], this study developed a high-resolution melting PCR (HRM-PCR) assay capable of differentiating between the NISKHI vaccine strain and virulent SPPV isolates. Recently the phylogenetic relationship between SPPV isolates circulating in Russia has indicated the high percentage sequence identity amongst virulent field strains, clustering them into a different sub-lineage with the NISKHI vaccine strain [18].

This differential sub-clustering enables the selection of informative SNPs as possible targets for molecular differentiation assays.

Since the NISKHI vaccine strain was originally produced through the serial passage of a virulent circulating isolate [16], HRM-PCR could be a powerful, fast, high-throughput post-PCR method for the detection of SNPs. This differentiating assay is based on a single SNP detected between the full genome sequence of the field isolate and the NISKHI LAV strain [18]. The SNP (T>C substitution) at position 109,536 of the NISKHI genome is located within the DNA-dependent RNA polymerase subunit gene (Figure 1) and was the target of the newly designed high-resolution melting analysis. The melt peak of the vaccine genotype was centered around 76.46 ± 0.12, compared to the virulent genotype, which had a range of 75.47–75.86. Since the vaccine and field strain melting curves differ by more than one degree ($p < 0.05$), it provides a reliable, real-time picture of the characteristics of the NISKHI vaccine and field DNA under investigation. This assay is, to the best of our knowledge, the first capable of specifically detecting the NISKHI vaccine strain DNA in a single PCR.

Importantly, the use of the NISKHI vaccine is currently restricted to ex-Soviet Union countries, while the original strain was obtained from an active outbreak in Kazakhstan in 1994 [11]. This HRM assay would complement the current SPPV diagnostic reaction, especially where it is a priority to differentiate the NISKHI strain from wild-type isolates.

In addition to the NISKHI vaccine, the Yugoslavian RM65 vaccine strain is widely used in the Middle East, Asia and the Horn of Africa, and the Romanian Fanar vaccine strain is used in India and the Maghreb countries [23,24]. The epidemiological situation of SPP in those regions necessitated an assay to assist in the adequate control and DIVA strategy implementation. For this reason, to meet the challenge of investigating SPP outbreaks when using live attenuated sheep pox-based vaccines, Chibssa et al., 2019 reported a conventional PCR assay employed for the differentiation between SPP vaccine (derived from the Romanian or the Yugoslavian RM/65 strains) and the virulent SPPV field isolates circulating in Ethiopia, the Middle East and Mongolia. This assay was based on the 84 bp deletion in the DNA ligase gene and the VARV B22R homolog gene. [8]. This assay is limited to the panel of the strains tested since no SPPV isolates from any ex-Soviet Union countries were included. More importantly, analysis of the NISKHI vaccine strain with this aforementioned assay results in the classification of NISKHI as a field strain [9].

In a subsequent study by Chibssa et al., 2019, an HRM assay for the differentiation of SPPV vaccines from SPPV field isolates, and additional classification of capripoxviruses into SPPV, GTPV or LSDV was developed [9]. Neither the NISKHI strain nor any other ex-Soviet Union isolates were evaluated, which greatly restricts the range of tools to aid in the study of the global SPP epidemiology [9].

The combination of the HRM assay developed herein and the one by Chibssa et al., 2019 can constitute a good complementary strategy to cover the currently used vaccines against SPP [9].

This study is the first description of a reliable PCR assay capable of distinguishing the NISKHI vaccine strain from other SPPV isolates obtained from clinical samples submitted during active outbreaks. The assay was validated on field samples, indicating no link between the field isolates to the vaccine strain. This laboratory tool is a good supplement to diagnostic programs targeting the control and eradication of sheep pox where the live attenuated NISKHI vaccine strain is administered.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/applmicrobiol2040073/s1, Table S1: Sheeppox virus isolates from GenBank used for the genome analysis and primers design.

Author Contributions: Conceptualization, A.S., A.M. and A.V.S.; data curation, A.S.; formal analysis, M.D., O.B. and L.P.; funding acquisition, A.S., A.M. and I.C.; investigation, A.M., A.V.S., A.K. and K.S.; methodology, A.S. and A.K.; writing—original draft, A.S., A.M., A.V.S., M.D., O.B., L.P. and

I.C.; writing—review and editing, A.S., A.M., A.V.S. and I.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grant no. 075-15-2021-1054 from the Ministry of Science and Higher Education of the Russian Federation to implement objectives of the Federal Scientific and Technical Program for the Development of genetic technologies during 2019–2027.

Institutional Review Board Statement: Ethics approval and consent to participate. The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to. Samples used in this study were those submitted to FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), for SPP diagnosis. Ethical approval was not required.

Data Availability Statement: The datasets generated and/or analyzed during the present study are available in the GenBank repository, under accession numbers ON961655—ON961657 and AY077834, https://www.ncbi.nlm.nih.gov/nuccore/AY077834, accessed on 18 October 2022.

Acknowledgments: The authors thank Zinyakov Nikolay for his excellent technical assistance.

Conflicts of Interest: The authors declare they have no competing interests.

References

1. Tulman, E.R.; Afonso, C.L.; Lu, Z.; Zsak, L.; Sur, J.H.; Sandybaev, N.T.; Kerembekova, U.Z.; Zaitsev, V.L.; Kutish, G.F.; Rock, D.L. The genomes of sheeppox and goatpox viruses. *J. Virol.* **2002**, *76*, 6054–6061. [CrossRef]
2. Dao, T.D.; Tran, L.H.; Nguyen, H.D.; Hoang, T.T.; Nguyen, G.H.; Tran, K.V.D.; Nguyen, H.X.; Van Dong, H.; Bui, A.N.; Bui, V.N. Characterization of Lumpy skin disease virus isolated from a giraffe in Vietnam. *Transbound. Emerg. Dis.* **2022**, *69*, e3268–e3272. [CrossRef] [PubMed]
3. Tuppurainen, E.S.M.; Venter, E.H.; Shisler, J.L.; Gari, G.; Mekonnen, G.A.; Juleff, N.; Lyons, N.A.; De Clercq, K.; Upton, C.; Bowden, T.R.; et al. Review: Capripoxvirus Diseases: Current Status and Opportunities for Control. *Transbound. Emerg. Dis.* **2017**, *64*, 729–745. [CrossRef] [PubMed]
4. Babiuk, S.; Bowden, T.R.; Boyle, D.B.; Wallace, D.B.; Kitching, R.P. Capripoxviruses: An emerging worldwide threat to sheep, goats and cattle. *Transbound. Emerg. Dis.* **2008**, *55*, 263–272. [CrossRef] [PubMed]
5. OIE Terrestrial Manual 2008: Sheep Pox and Goat Pox, Office International Des Epizooties Paris. Chapter 2.7.14. pp. 1058–1068. Available online: https://www.woah.org/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/SHEEP_GOAT_POX.pdf (accessed on 18 October 2022).
6. CFSPH. The Center for Food Security Public Health, Lowa State University, College of Veterinary Medicine and Institution of International Cooperation in Animal Biologics, an OIE Collaborating Center. 2008. Available online: https://www.cfsph.iastate.edu/diseaseinfo/disease/?disease=sheep-pox-and-goat-pox&lang=en (accessed on 18 October 2022).
7. World Organisation for Animal Health. WAHIS. Available online: https://wahis.woah.org/#/event-management (accessed on 30 October 2022).
8. World Organisation for Animal Health. *OIE Terrestrial Manual: Lumpy Skin Disease [Internet]*; World Organisation for Animal Health: Paris, France, 2018; Available online: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.0 (accessed on 27 December 2020).
9. Chibssa, T.R.; Settypalli, T.B.K.; Berguido, F.J.; Grabherr, R.; Loitsch, A.; Tuppurainen, E.; Nwankpa, N.; Tounkara, K.; Madani, H.; Omani, A.; et al. An HRM Assay to Differentiate Sheeppox Virus Vaccine Strains from Sheeppox Virus Field Isolates and other Capripoxvirus Species. *Sci. Rep.* **2019**, *9*, 6646. [CrossRef] [PubMed]
10. Uzar, S.; Sarac, F.; Gulyaz, V.; Enul, H.; Yılmaz, H.; Turan, N. Comparison and efficacy of two different sheep pox vaccines prepared from the Bakırköy strain against lumpy skin disease in cattle. *Clin. Exp. Vaccine Res.* **2022**, *11*, 1–11. [CrossRef]
11. Kurchenko, F.P.; Ivanyushchenkov, B.N.; Ufimtsev, K.P.; Alyokhin, A.F.; Gononov, Y.M.; Seitkassymov, B.K.; Kutumbetov, L.B.; Safonov, G.A.; Tatarintsev, N.T. Effectiveness of the cultural virus vaccine based on NISKhI strain against sheep pox. *Veterinariya* **1991**, *10*, 21–23. (In Russian)
12. Sambrook, J.; Russell, D.W. *Molecular Cloning Laboratory Manual*; Science Press: Beijing, China, 2002; pp. 69–71.
13. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622. [CrossRef]
14. Singh, A. Modern Vaccines with Diva Capability: An Overview. *RRJology* **2015**, *5*, 1–12.
15. Wolff, J.; Moritz, T.; Schlottau, K.; Hoffmann, D.; Beer, M.; Hoffmann, B. Development of a Safe and Highly Efficient Inactivated Vaccine Candidate against Lumpy Skin Disease Virus. *Vaccines* **2020**, *9*, 4. [CrossRef] [PubMed]
16. Wondimu, A.; Tassew, H.; Gelaye, E.; Hagos, Y.; Belay, A.; Teshome, Y.; Laiju, S.; Asebe, G. Outbreak Investigation and Molecular Detection of Pox Virus Circulating in Sheep and Goats in Selected Districts of West Gojjam and Awi Zones Northwest, Ethiopia. *Vet. Med.* **2021**, *12*, 303–315. [CrossRef] [PubMed]

17. Kali, K.; Kardjadj, M.; Touaghit, N.; Yahiaoui, F.; Ben-Mahdi, M.H. Understanding the epidemiology of sheep-pox outbreaks among vaccinated Algerian sheep and post vaccination evaluation of the antibodies kinetics of the commercially used vaccine. *Comp. Immunol. Microbiol. Infect. Dis.* **2019**, *65*, 128–131. [CrossRef] [PubMed]
18. Krotova, A.; Shalina, K.; Mazloum, A.; Kwon, D.; Van Schalkwyk, A.; Byadovskaya, O.; Sprygin, A. Genetic characterization of sheeppox virus strains from outbreaks in Central Russia in 2018–2019. *Transbound. Emerg. Dis.* **2022**, 1–6. [CrossRef]
19. Haegeman, A.; Zro, K.; Sammin, D.; Vandenbussche, F.; Ennaji, M.M.; De Clercq, K. Investigation of a Possible Link Between Vaccination and the 2010 Sheep Pox Epizootic in Morocco. *Transbound. Emerg. Dis.* **2016**, *63*, e278–e287. [CrossRef] [PubMed]
20. Hanley, K.A. The double-edged sword: How evolution can make or break a live-attenuated virus vaccine. *Evolution* **2011**, *4*, 635–643. [CrossRef] [PubMed]
21. Bull, J.J. Evolutionary reversion of live viral vaccines: Can genetic engineering subdue it? *Virus Evol.* **2015**, *1*, vev005. [CrossRef] [PubMed]
22. Sprygin, A.; Pestova, Y.; Bjadovskaya, O.; Prutnikov, P.; Zinyakov, N.; Kononova, S.; Ruchnova, O.; Lozovoy, D.; Chvala, I.; Kononov, A. Evidence of recombination of vaccine strains of lumpy skin disease virus with field strains, causing disease. *PLoS ONE* **2020**, *15*, e0232584. [CrossRef] [PubMed]
23. Chaudhary, S.S.; Pandey, K.D.; Singh, R.P.; Verma, P.C.; Gupta, P.K. A vero cell derived combined vaccine against sheep pox and Peste des Petits ruminants for sheep. *Vaccine* **2009**, *27*, 2548–2553. [CrossRef] [PubMed]
24. Abutarbush, S.M.; Tuppurainen, E. Serological and clinical evaluation of the Yugoslavian RM65 sheep pox strain vaccine use in cattle against lumpy skin disease. *Transbound. Emerg. Dis.* **2018**, *65*, 1657–1663. [CrossRef] [PubMed]