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Validation of Taqman based assays for specific detection and differentiation of wild type and Neethling vaccine strains of LSDV

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Abstract: Lumpy skin disease (LSD) is an important animal disease with significant health and economic impact and is considered as a notifiable disease by the OIE. Attenuated strains of LSDV have been used successfully as vaccines (LAV) but can produce mild or systemic reactions. Vaccination campaigns using LAVs are therefore only viable if accompanying DIVA assays are available. Two DIVA qPCR assays were developed which are able to distinguish Neethling-based LAVs and wild type LSDV. Upon validation both assays were shown to have high sensitivity and specificity with a diagnostic performance comparable to other published DIVA assays. This confirms their potential as reliable tools to confirm infection in animals during vaccination campaigns based on Neethling vaccine strains.

Keywords: Lumpy Skin Disease virus; real-time PCR; DIVA diagnostic protocol; differentiation; field strain; vaccine strain

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1. Introduction

Due to the significant health and economic impact of Lumpy skin disease (LSD), it is considered as an important animal disease which is on the OIE list of notifiable diseases. The etiological agent of the disease is Lumpy skin disease virus (LSDV) which belongs to the family of *Poxviridae* [1] and is highly host specific causing disease in cattle and water buffalo. Together with sheeppox virus (SPPV) and goatpox virus (GTPV) it belongs to the genus of *Capripoxvirus*. These viruses have a DNA genome of about 150,000 bp long and encodes 147 putative genes [2].

Lumpy skin disease was first described in Zambia in 1929 and occurred exclusively on the African continent and the Middle East until 2012 when it began to spread to Turkey (2013), Iraq and Cyprus (2014), Greece (2015), Bulgaria, N. Macedonia, Serbia, Albania, Montenegro, Armenia, Azerbaijan, Kazakhstan and Russia (2016), Namibia, S. Arabia, Mozambique (2017), Bangladesh, China, India, Syria (2019), Bhutan, Nepal, Djibouti, Vietnam, Hong Kong, Myanmar, Sri Lanka, (2020) [3,4,5]. The disease is mainly characterized by the development of nodules on the skin which can develop into skin lesions and scab formation. Mortality is in general low, but exceptions have been reported as well as differences between *Bos taurus* and *Bos indicus* breeds [6,1,7,8,9,10,11].

In LSD control, attenuated strains of LSDV, SPPV, and GTPV have been used successfully as vaccines in infected areas [1]. When using a vaccine-based on the Neethling strain, mild or systemic reactions have been observed in some of the vaccinated animals.

These reactions can cause complications with the control and eradication measures, such as stamping out, as the distinction between vaccinated and infected animals based upon clinical picture become problematic [12,5]. These issues can be addressed by the application of diagnostic assays that quickly and specifically differentiate between wild type strains LSDV and the vaccine strains LSDV.

Molecular assays such as PCR have the advance to be not only quick and highly sensitive~~,~~ but they can target highly specific genomic regions. Although the genomes of capripoxviruses are highly conserved [2], the increasing availability of (full genome) sequences allows the identification of genomic differences between wild type LSDV and vaccine strains. These differences can be used for the development of PCRs which can differentiate infected from vaccinated animals (DIVA). Several of such DIVA PCRs have been developed in the past and included conventional [13] and Real Time PCRs using specific TaqMan probes [14,15,16,17]. An overview of all these tests is described in detail elsewhere [18]. However, the cost and / or the readily availability of the primers, probes and reagents can be a bottleneck, certainly if they make use of modified nucleotides such as LNA's [15]. Therefore, it was the aim of this study to develop and validate an assay with standard real-time PCR primer, probes and reagents suited for rapid, sensitive and specific detection and differentiation of LSDV wild type strains from Neethling based vaccine strains of LSDV. The KV-DIVA qPCR protocol consists from two Taqman probe-based assays. The first assay is specific for wild type strains (KV-2 assay) and is a modified version of a published assay [14]. The second assay is specific for Neethling vaccine strains (KV-VAC) and was developed in 2017 and has not yet been published. Both assays were not developed as a first line diagnostic tool but rather as a DIVA test, in combination with assays specific for all Capripox viruses [19-22], to support control and eradication strategies. Another import aspect of an assay is it robustness. The assay will be implemented in different laboratories with each their peculiarities and proper equipment. This can cause minute changes in test circumstances and potentially influence the assay characteristics. This was evaluated during this study by performing the validation in 2 separate and independent laboratories, namely in VSI "Kraljevo" (Serbia) and in Sciensano (Belgium).

2. Materials and Methods

2.1. Design of KV-2 and KV-VAC assays:

The design of primers and tests for both tests was performed in VSI "Kraljevo" using the online software Primer3 ~~(primer3.ut.ee)~~(primer3.ut.ee) and Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Design of primers and probes for wild type strain specific assay, KV-2, was previously described [14]. After sequencing of twenty LSDV wild type strains obtained from IAEA laboratory in Seibersdorf, Austria, (data not shown), forward and reverse primers were slightly modified compared to the original protocol. Modifications are marked in bold letters and presented in Table 1.

Table 1. Primers and probe for KV-2 assay specific for wild type strains of LSD virus.

Oligo	Sequence (5`- 3`)
KV2_Fmod	TGGGA**Y**GATAACAACGTTTATG
KV2_Rmod	ACATTGTCATC**Y**GGTAATGTA
LSD_KV2_Pro_field	VIC-TTACCACCTAATGATAGTGTTTATGATTTACC-BHQ1

Primers and probes specific for vaccine strains of LSDV (KV-VAC assay) are designed to detect part of the LW008 gene, where the probe (labeled with FAM) binds 100% specifically for part of the genome of vaccine strains, Table 2. In wild type strains of LSDV as well as in SPPV and GTPV strains at the same position in the genome there are 9 nucleotides mismatches, and the probe does not bind to LW008 gene of the mentioned viruses.

Table 2. Primers and probe for KV-VAC assay specific for Neethling based vaccine strains of LSD virus.

Oligo	Sequence (5`- 3`)
LSDV_vacc_5670f	TGCTTGTTTCCATTCTCCACT
LSDV_vacc_5829r	AAAAATGGGCGCAGTAGTATTT
LSDV_vacc_5726_Pro	FAM-CGCTGACATCGTTAGTCCACTCG-BHQ1

2.2. Validation study

The validation study was performed in parallel in Veterinary Specialized Institute Kraljevo (VSI Kraljevo) and at Sciensano (EU Reference laboratory for Capripox viruses).

2.2.1. DNA extraction

Extraction of viral DNA from blood samples, milk, skin scarifications, skin biopsies, internal organs, nasal swabs of animal origin as well as swabs and samples from the environment and insects was performed at VSI by using a commercial MagVet Universal DNA / RNA kit (LSI, Germany) and Bioextract Superball (Biosellal, France) on Kingfisher mL and Kingfisher Flex (Thermo Scientific, Finland) according to the manufacturer's instructions. The extracted DNA was stored at temperatures below -16 ^{0}C until the start of the testing.

Viral DNA was extracted at Sciensano using the NucleoSpin Blood and NucleoSpin tissue kits (Macherey-Nagel, Düren, Germany) as previously described [21].

2.2.2. Samples for analytical sensitivity (Ase), specificity (Asp), efficiency and repeatability

The efficiency of the qPCR test performed at VSI for wild type strains of LSD was performed by examining serial tenfold dilutions of wild type strain of virus Serbia/Bujanovac/2016 [23]. The efficacy of the qPCR test for vaccine strains of LSD was performed by testing serial ten-fold dilutions of a vaccine based on the Neethling strain of LSD (OBP, South Africa, NCBI Accession No. AF409138). The obtained results were processed in AriaMx software.

The analytical sensitivity of the assays was determined at VSI Kraljevo by testing serial dilutions of quantified wild type and vaccine strain DNA obtained from the EU reference laboratory for Capripox viruses, Sciensano, Belgium. The number of copies of the LSDV Bulgaria wild type strain was 1.15 * 10^{6}/ μL and the number of copies of the genome of the LSDV Neethling vaccine strain was 5.63 * 10^{6}/ μL. **Cut-offs** of the assays were determined by testing of 20 replicates of wild type strain Serbia/Bujanovac/2016 and Neethling vaccine strain (OBP vaccine) diluted to a limit of analytical sensitivity and calculating 2 SD from the mean Ct values.

The repeatability was determined at VSI by analyzing eight replications in triplicate. The sample used was OBP vaccine strain (KV-VAC) and the Serbia/Bujanovac/2016 LSDv strain (KV-2). At Sciensano the repeatability and reproducibility was evaluated by spiking negative EDTA-blood with a LSDV wild type (LSDV Bulgaria) and a Neethling vaccine strain. From these 2 samples genomic DNA was extracted and the 2 DIVA qPCRs (KV-2 and KV-VAC) were run in 4-fold (repetitions, equal conditions) in 5 different runs (reproduction, different conditions).

Analytical specificity of the assays was tested on wild type strains of LSDV from Serbia, and also on different wild type LSDV strains obtained from IAEA laboratory in Seibersdorf, Austria and Diagnostic Veterinary Laboratory, Podgorica, Montenegro. The specificity was also checked at the Federal Research Center of Virology and Microbiology, Pokrov, Russia on their collection of LSDV, SPPV, GTPV strains and other viruses, as well as at the Faculty of Veterinary Medicine, Ss. Cyril and Methodius University, Skopje, North Macedonia. At Sciensano the own collections was tested in addition to samples

provided in 2004 by Dr. P. Kitching, at that time work-ing at National Centre for Foreign Diseases, Winnipeg, Manitoba, Canada in collaboration with Dr. P. Mellor, at that time working at the Pirbright Institute (Pirbright, United Kingdom) and in 2010 by Dr H. Yadin, at that time working at the Kimron Veterinary Research Institute (Bet Dagan, Israel). These viruses were isolated over the past 4 decades from sheep, goat and cattle throughout the endemic regions of Africa, Asia and the Middle East. in total 90 capripox virus strains were tested (Supplementry table 1) In addition a panel of 41 other microorganisms (Supplementary table 2) were tested with the KV2 and KV-VAC real-time PCRs.

2.2.3. Samples for diagnostic sensitivity (Dse) and specificity (Dsp)

The diagnostic sensitivity of the KV-2 and KV-Vac was determined at VSI by examining samples originating from naturally infected animals (blood, nasal swabs, skin biopsy and milk), vaccinated animals, insects that may be carriers of this disease and environmental swabs. The number and type of samples used for test validation are given in Table 3.

Table 3. ~~The number~~Number **and type of** clinical **samples** used in assay validation.

Samples collected during LSD outbreak in 2016	
1. Skin biopsy	94
2. Blood	31
3. Nasal swabs	17
4. Milk	2
5. Flies	7
6. Environmental swabs	12
Samples collected prior outbreak (2014) and after the outbreak (2017)	
7. DNA of internal organs originating from cattle taken in 2014	40
8. Blood of animals vaccinated in 2017	40
Total	243

The diagnostic sensitivity was tested at Sciensano using 48 samples (tissue and blood) obtained from animal experiments with LSDV (Table 13). The panel consisted of 18 wild type and 30 for vaccine type samples. Although the complete range of very strong to very weak positive samples were included, the emphasis (regarding number of samples) was placed on weak to very weak positives as these are the most challenging for a PCR.

For the **diagnostic specificity** 34 negative blood samples originated from Belgian cattle were tested at Sciensano.

2.2.3. Samples for comparative analysis

The diagnostic sensitivity and specificity were calculated at VSI comparing results of KV-2 and KV-VAC assays with results obtained with CaPV Bowden assay on 163 samples listed in supplementary table 3. **At Sciensano** the panels for diagnostic sensitivity and diagnostic specificity were used with the KV2 and KV-VAC qPCR, the DIVA Agianniotaki PCR [12] and the pan D5R PCR [21], supplementary table 4.

2.3. Real-time PCR

2.3.1. KV-2 and KV-VAC assays

~~Although the KV-2 and KV-VAC assays can be performed combined in the same tube as "duplex" PCR assay, since the probes are labeled with different fluorophores attached to the 5' end, the performances of the assays were assessed separately, as singleplex reactions.~~

The KV-2 and KV-VAC assays were evaluated as single-plex in both institutes.

The PCR reaction mix at VSI contained 12.5 µl Brilliant III Ultrafast qPCR master mix (Agilent Technologies, USA); 0.2 µl of the forward (50 µM) and reverse primer (50 µM) and 0.1 µl of the probe (50 µM). Water is added to a total volume is 20 µl and 5 µl of template was added. The real-time PCR reaction was performed on AriaMx (Agilent Technologies, USA) and Stratagene Mx3000P (Stratagene, USA) devices. Primers and probe were ordered from Metabion, Germany. The thermal profile for both assays hot start - 3 minutes at 95 ° C, denaturation 15 s at 95 ° C and annealing 30 s at 60 ° C, 45 cycles.

Real-time PCRs at Sciensano were carried out in a total volume of 20 µl consisting of 10.0 µl LightCycler 480 (LC480) Probes Master (Roche, Vilvoorde, Belgium), 2.5 µl DNA template, 1 U FastStartTaq DNA polymerase (Roche, Vilvoorde, Belgium), 0.8 mM MgCl2, final concentration of 1 µM of each primer and 0.35 µM probe, BSA was added additionally to a final concentration of 0.1 µg/µl. The primers and DNA template were denatured separately at 95°C for 3 min before the rest of the mix was added. The thermal cycling profile for both real-time PCRs was: 95°C for 10 min and 45 cycles of 95°C 15s and 60°C 30s. The real-time PCRs were carried out on Roche LightCyclers 480 instrument. Primer and probe were ordered from IDT, Belgium.

2.3.2. Additional real-time PCRs

At VSI a published panCapripox real-time PCR, Bowden assay, was used to confirm the presence of Capripox virus [19].

At Sciensano a panCapripox specific assay D5R [21] was used to demonstrated capripox DNA. The DIVA real-time PCR of Agianniotaki [15] was used to compare the differentiation capacity of the KV-2 and KV-VAC assays.

3. RESULTS

3.1. Results of validation study in VSI Kraljevo

3.1.1. Efficiency of the wild- type strain KV-2 assay

By testing the five tenfold serial dilutions of Serbia/Bujanovac/2016 isolate, using AriaMx software, it was determined that the efficiency of the qPCR reaction, using Brilliant III Ultrafast qPCR kit, is 99%.

3.1.2. Efficiency of the vaccine strain KV-VAC assay

By testing the five tenfold serial dilutions of the OBP vaccine strain of LSD virus (Neethling strain), using AriaMx software, it was determined that the efficiency of the qPCR reaction, using the Brilliant III Ultrafast qPCR kit, was 99.6%.

3.1.3. Analytical sensitivity of the assays

Analysis of the obtained results for 95% probability of detection (20 replicates) determined that limit of detection of the test for the wild type LSDV strains KV-2 is 24 copies of the genome per reaction, while limit of detection of the assay for vaccine strains – KV-VAC is 12 copies of the genome per reaction.

3.1.4. Determination of Cut-off of assays

Based on testing of 20 replicates of positive quantified reference DNA (wild type strain - LSDV Bulgaria and vaccine - Neethling strain) diluted to the limit of analytical sensitivity of both tests (24 copies of genome/reaction for wild type or 12 copies of genome/reaction for vaccine strains of LSDV), cut-off values were determined for both assays to be Ct (Cq) 40. This means that any sample that has a Ct (Cq) value of 40 or lower is considered positive.

3.1.5. Determination of repeatability and reproducibility of the assays

The LSDV wild type strain and the LSDV vaccine strain were correctly identified 20 times with the real-time PCR assays. No cross contamination was found between wild type and vaccine strain. The evaluation of the repeatability of KV-2 and KV-VAC assays is summarized in Table 4 and Table 5. A high reproducibility was found for both real-time PCRs. The inter-run coefficient of variation for KV-2 and KV-VAC type assay was 0.79 %and 0.47 % respectively.

Table 4. Repeatability of KV-2 assay.

	Run 1	**Run 2**	**Run 3**
Mean	35.73125	35.17375	35.43875
SDEV	0.410311	0.292913	0.285329
CV	1.148326	0.832761	0.805132
Inter run Mean	35.44792		
Inter run SDEV	0.278863		
Inter run CV	**0.79**		

Table 5. Repeatability of KV-VAC assay.

	Run 1	**Run 2**	**Run 3**
Mean	35.55375	35.285	35.19625
SDEV	0.227843	0.314234	0.352134
CV	0.640841	0.890559	1.000487
Inter run Mean	35.345		
Inter run SDEV	0.167818		
Inter run CV	**0.47**		

3.1.6. Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity

The results of comparative examination of samples originating from diseased animals, environmental samples and insects using Bowden, KV-2 and KV-VAC assays are given in supplementary table 3. A total of 163 samples, including naturally infected animals, vaccinated animals, flies and environmental swabs taken during 2016 outbreak, were analysed. Using the capripox specific Bowden assay 149 samples scored positive and 14 negative. When these 163 samples were analysed with both new assays 115 (KV-2 assay) and 32 (KV-VAC assay) samples were found to be positive. All samples which were negative on the Bowden assay were also negative on both assays. Three samples (1 blood sample and 2 two environmental swabs) which were positive on the Bowden assay, albeit with Ct value of 36.5 or higher, were negative on both assays. As these samples originated from a non-vaccinated animal (blood sample) or farms where vaccination was not performed (swabs) a signalwas expected in the KV-2 assay. Three samples, two skin and one blood sample, were positive on both wild type and vaccine strain of LSDV.

Using the PCR data of these 163 samples the diagnostic sensitivity and diagnostic specificity of KV-2 and KV-VAC assays were compared to the Bowden assay using MedCalc statistical software. The diagnostic sensitivity of KV-2 assay was 97.46 % with a diagostic specificity of 100 % while the diagnostic sensitivity and specificity of KV-VAC assay was 100 %. The results are summarized in table 6.

Table 6. Diagnostic sensitivity and diagnostic specificity for KV-2 and KV-VAC vs. Bowden assay.

	Diagnostic sensitivity and diagnostic specificity for KV-2 vs. Bowden assay		**Diagnostic sensitivity and diagnostic specificity for KV-VAC vs. Bowden assay**	
Statistic	**Value**	**95% CI**	**Value**	**95% CI**

Sensitivity	97.46%	92.75% to 99.47%	100.00%	89.11% to 100.00%
Specificity	100.00%	92.60% to 100.00%	100.00%	97.22% to 100.00%
Positive likelihood ratio				
Negative likelihood ratio	0.03	0.01 to 0.08	0	
Disease prevalence	95.00%		95.00%	
Positive predictive value	100.00%		100.00%	
Negative predictive value	67.43%	40.38% to 86.35%	100.00%	
Accuracy	97.58%	93.94% to 99.34%	100.00%	97.76% to 100.00%

An additional 40 LSDV negative samples and 40 samples from vaccinated cattle were tested with Bowden, KV-2 and KV-VAC assays. Specificity and sensitivity of KV-2 and KV-VAC assays comparing with Bowden assay was 100%. Results are given in table 7.

Table 7. Additional testing on vaccinated animals and negative animals.

Samples	Number of samples	Bowden positive	Bowden negative	KV-2 positive	KV-2 negative	KV-Vacc positive	KV-Vacc negative
DNA from true negative cattle (samples from 2014)	40	0	40	0	40	0	40
Blood from vaccinated cattle 2017	40	12	28	0	40	12	28

All samples from cattle that are negative on LSDV (n=40) were tested negative using Bowden, KV-2 and KV-VAC assay. From samples that originated from vaccinated animals (n=40), 12 were positive using Bowden and KV-VAC assay and none was positive using KV-2 assay.

3.1.7. Analytical sensitivity and exclusivity

The KV-2 assay, which is specific only for wild type strains of LSDV, successfully detected all 31 wild type strains of LSDV and did not detect any of the three Neethling vaccine strains, 12 SPPV strains and 12 GTPV strains (supplementary table 1). However, the Kenyavac KSGP 0240 strain that is used as a vaccine in some African countries, scored positive on the KV-2 assay. Also, KV-2 assays did not detected recombinant strain Dergachevskyi, the only recombinant strain used in this study.

The LSDV KV-VAC assay specific for vaccine strains detected all three Neethling virus vaccine strains used in this study but did not detect Kenyavac KSGP 0240 as a vaccine strain. This assay did not detect any of the 31 wild type strains of LSDV, and did not detect any of the 12 SPPV strains and 12 GTPV strains. Also, KV-VAC assay did not detected Dergachevskyi - recombinant strain.

An additional exclusivity testing of KV2 and KV-VAC assays with 20 bacterial and 10 viral species (supplementary table 2.) showed no false positive results.

3.1.8. Statistical analysis of VSI Kraljevo validation results

Passing-Bablok regression analysis revealed the following equations between the Ct values obtained by BOW real-time PCR (y) and by the KV2 (x_1) (Figure 1a.) and KV-VAC (x_2) (Figure 2a.) with 95% CI:

$$y = -0.01\ (95\%\ CI\colon -0.36 - 0.39) + 1.06\ (95\%\ CI\colon 1.05 - 1.08)\ x1 \quad (1)$$

$$y = -0.38\ (95\%\ CI\colon -1.11 - 0.20) + 1.02\ (95\%\ CI\colon 1.00 - 1.04)\ x2 \quad (2)$$

The respective Bland-Altman plots are presented in Fig. 1b. and Figure 2b. The average total biases for the comparison of BOW real-time PCR with KV-2 assay were -1.63

cycles (Figure 1b.) and those with KV-VAC assay were -0.05 cycles (Figure 2b.). According to concordance correlati the precision between methods, which measures how far each observation deviates from the best-fit line and represents Pearson correlation coefficient, was 97.05% and 99.68% and when the BOW real-time PCR was compared with the KV-2 assay and with KV-VAC assay, respectively.

The average Ct values obtained by BOW real-time PCR were not significantly different from those obtained with real-time PCR of VAC (mean ± SE: 27.17 ± 0.95 and 27.22 ± 0.96 for the BOW and VAC assay, respectively; $P > 0.05$). However, the average Ct values obtained with the BOW assay were slightly lower than those obtained with the KV2 method but this difference was statistically significant (mean ± SE: 25.39 ± 0.66 and 27.01 ± 0.71 for BOW and KV2, respectively; $P < 0.05$).

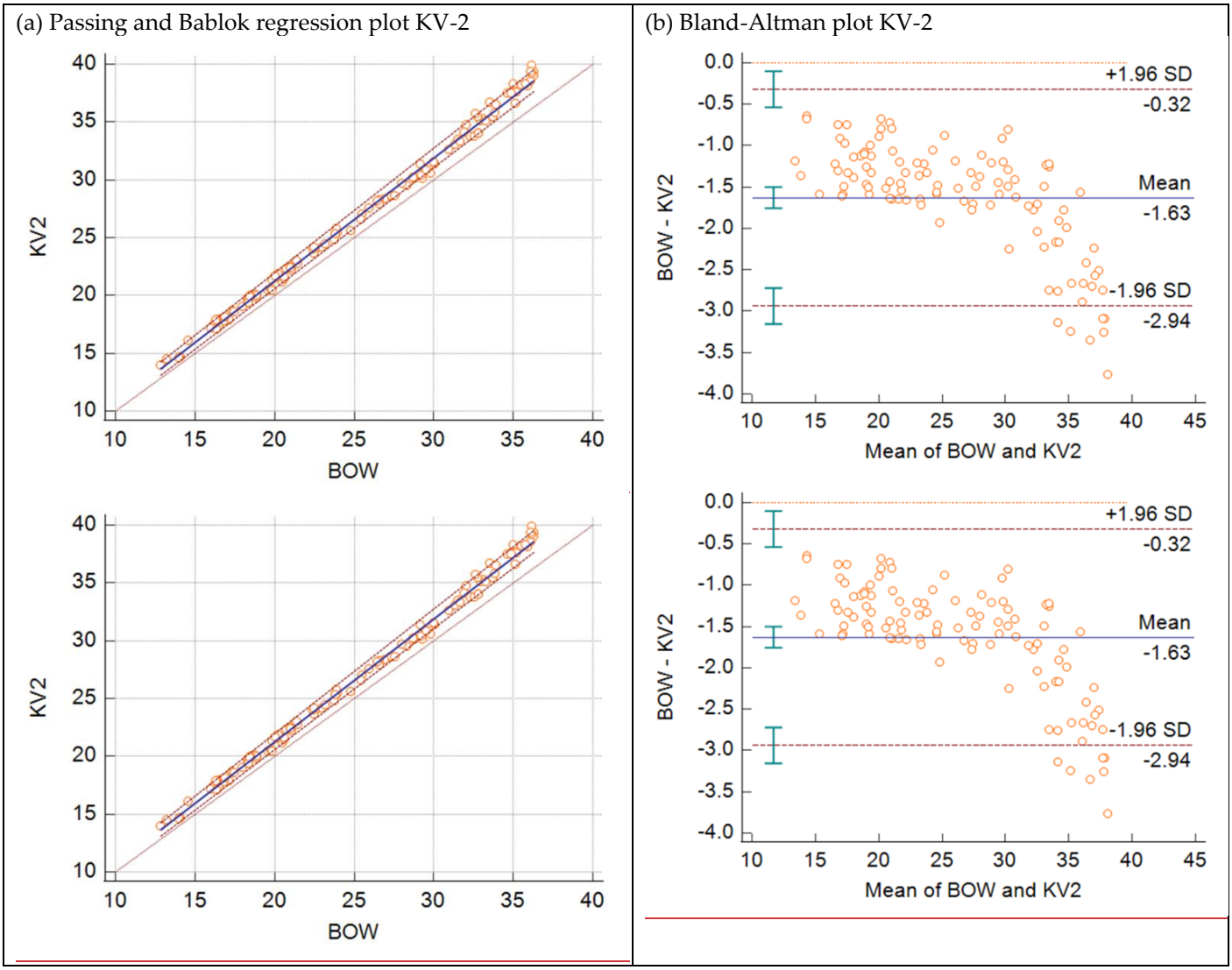

Figure 1. (**a**) Passing-Bablok regression plot: comparison of Ct values obtained with the KV-2 wild type strain assay vs. CaPV specific real-time PCR assay Bowden, with 95% CI in 109 samples. (**b**) Bland–Altman plot: comparison of Ct values obtained with the KV-2 wild type strain assay vs Capripox specific real-time PCR assay Bowden, with 95% CI in 109 samples.

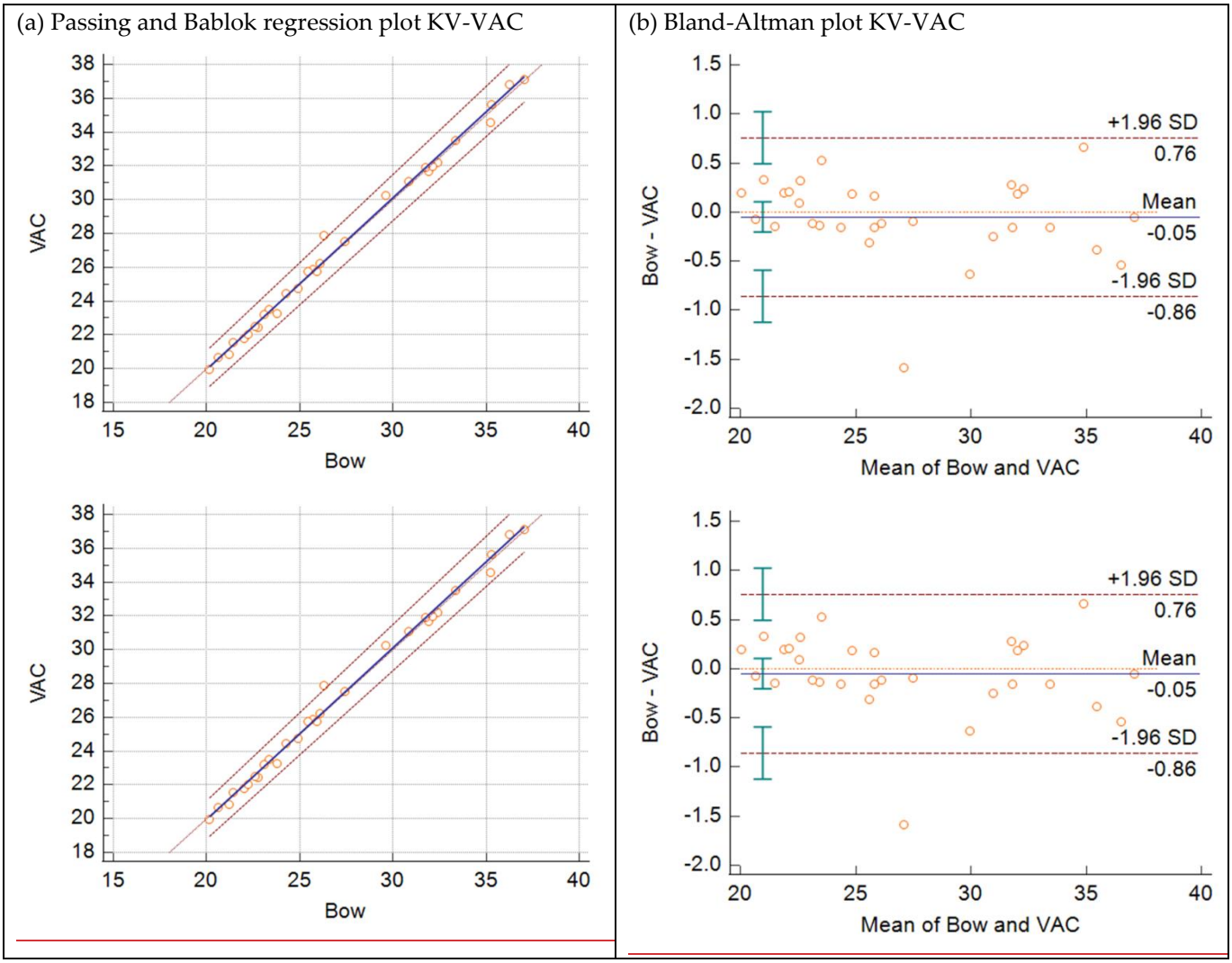

Figure 2. (**a**) Passing-Bablok regression plot: comparison of Ct values obtained with the KV-VAC vaccine strain assay vs capripox specific real-time PCR assay Bowden, with 95% CI in 30 samples. (**b**) Bland–Altman plot: comparison of Ct values obtained with the KV-VAC vaccine strain assay vs Capripox specific real-time PCR assay Bowden, with 95% CI in 30 samples.

3.2. Results of validation study in Sciensano

3.2.1. Determination of repeatability and reproducibility of the assays

The LSDV wild type strain and the LSDV vaccine strain were correctly identified 20 times with the real-time PCRs. No cross contamination was found between wild type and vaccine strain. The total coefficient of variation was 2.16 % for the wild type real-time PCR and 1.98 % for the vaccine type real-time PCR. A high reproducibility was found for both real-time PCRs. The results were shown in Table 8 and 9.

Table 8. Repeatability and reproducibility of the KV-2 assay.

KV-2 (WT)	day 1	day 2	day 3	day 4	day 5
Mean	35.5	34.95	34.68	34.45	34.43
Std. Deviation	0.2449	1.237	0.6397	0.4509	0.5737
Coefficient of variation	0.69%	3.54%	1.85%	1.31%	1.67%
Inter run mean	34.8				
Inter run SD	0.7525				
Inter run CV	**2.16%**				

Table 9. Repeatability and reproducibility of the KV-VAC assay.

KV-VAC (VAC)	day 1	day 2	day 3	day 4	day 5
Mean	32.5	31.1	32.05	31.7	32.13
Std. Deviation	0.383	0.469	0.5686	0.2708	0.5315
Coefficient of variation	1.18%	1.51%	1.77%	0.85%	1.65%
Inter run mean	31.9				
Inter run SD	0.632				
Inter run CV	**1.98%**				

3.2.2. Diagnostic sensitivity and diagnostic specificity:

Diagnostic sensitivity

Forty-eight samples with a positive status for the panDR real-time PCR were analyzed. The KV-VAC real-time PCR detected 25 out of the 30 vaccine samples and all wild type samples (n=18) correctly. The 5 vaccine samples that were negative on the KV-VAC real-time PCR, had a very low viral load as indicated by the pan D5R real-time PCR (Cp-values > 38). The KV2 real-time PCR detected all the wild type samples (n=18) and all vaccine samples (n=30) correctly.

The DIVA Agianniotaki real-time PCR detected 20 out of the 30 vaccine samples and all wild type (n=18) samples correctly. The 10 vaccine type samples that were negative on vaccine channel, had a very low viral load as indicated by the pan D5R real-time (Cp-values > 38). All obtained PCR results are summarized in supplementary table 4.

Diagnostic specificity

All blood samples scored negative in the KV2 and KV-VAC real-time PCR, except 1 sample which had a borderline Cp value of 40 in the KV2 real-time PCR. For DIVA Greece 1 sample had a Cp value of 40 for the wild type channel, another sample for the vaccine channel. For the pan D5R real-time PCR, 1 sample was doubtful in the D5R channel. All the obtained values for internal and external control with the pan D5R real-time PCR were within the acceptance criteria. The obtained Cp values of 40 for the different real-time PCRs could indicate a non-specific reaction.

Diagnostic sensitivity and specificity of KV-2 and KV-VAC assays compared to D5R assay was calculated using MedCalc statistical software and the results are given in table 10. The diagnostic sensitivity and specificity of the KV-2 assay was 100 % while the diagnostic sensitivity of the KV-VAC assay was 85.71% with a specificity of 100%.

Table 10. Diagnostic sensitivity and diagnostic specificity for KV-2 and KV-VAC vs. D5R assay assay.

	Diagnostic sensitivity and diagnostic specificity for KV-2 vs. D5R assay		**Diagnostic sensitivity and diagnostic specificity for KV-VAC vs. D5R assay**	
Statistic	**Value**	**95% CI**	**Value**	**95% CI**
Sensitivity	100.00%	81.47% to 100.00%	85.71%	69.74% to 95.19%
Specificity	100.00%	88.43% to 100.00%	100.00%	81.47% to 100.00%
Positive likelihood ratio				
Negative likelihood ratio	0.00		0.14	0.06 to 0.32
Disease prevalence	95.00%		95.00%	
Positive predictive value	100.00%		100.00%	
Negative predictive value	100.00%		26.92%	14.06% to 45.34%
Accuracy	100.00%	92.60% to 100.00%	86.43%	74.22% to 94.28%

Diagnostic sensitivity and diagnostic specificity of DIVA Agianniotaki assay compared to D5R assay are calculated using MedCalc statistical software and the results are given in table 11. The diagnostic sensitivity and specificity of the Agianniotaki wild type

assay was 100 % while the diagnostic sensitivity of the Agianniotaki vaccine assay was 75% and the specificity was 100%.

Table 11. Diagnostic sensitivity and diagnostic specificity for Agianniotaki vs. D5R assay assay.

	Diagnostic sensitivity and diagnostic specificity for Agianniotaki wild type vs. D5R assay		**Diagnostic sensitivity and diagnostic specificity for Agianniotaki vaccine vs. D5R assay**	
Statistic	**Value**	**95% CI**	**Value**	**95% CI**
Sensitivity	100.00%	81.47% to 100.00%	75.00%	58.80% to 87.31%
Specificity	100.00%	88.43% to 100.00%	100.00%	81.47% to 100.00%
Positive likelihood ratio				
Negative likelihood ratio	0.00		0.25	0.15 to 0.43
Disease prevalence	95.00%		95.00%	
Positive predictive value	100.00%		100.00%	
Negative predictive value	100.00%		17.39%	10.96% to 26.48%
Accuracy	100.00%	92.60% to 100.00%	76.25%	63.26% to 86.44%

3.2.3. Analytical sensitivity and exclusivity:

None (n=34) of the SGPV's, SPPV's and GPV's were detected with the KV2 and KV-VAC real-time PCR, while they were clearly positive with the pan-capripox D5R (Supplementary table 1).

Exclusivity of the assays

None of the samples (n=11) from animals infected with clinically or genetically related pathogens were detected with the KV2 and KV-VAC real-time PCR (Supplementary table 2).

3.2.4. Statistical analysis of Sciensano validation results

Passing-Bablok regression analysis (Fig. 3a, 4a, 5a, 6a) revealed the following equations between the Ct values obtained by D5R real-time PCR (y) and by the KV-2 wild type (x_1), Agianniotaki wild type (x_2), KV-VAC (x_3) and Agianniotaki vaccine (x_4) with 95% CI:

$$y = -1.68\ (95\%\ CI: -29.54 - 17.81) + 1.03\ (95\%\ CI: 0.48 - 1.81)\ x1 \quad (3)$$

$$y = -14.38\ (95\%\ CI: -86.57 - 8.26) + 1.39\ (95\%\ CI: 0.76 - 3.37)\ x2 \quad (4)$$

$$y = 4.08\ (95\%\ CI: -1.62 - 9.03) + 0.89\ (95\%\ CI: 0.76 - 1.05)\ x3 \quad (5)$$

$$y = 5.36\ (95\%\ CI: -3.10 - 16.62) + 0.88\ (95\%\ CI: 0.57 - 1.13)\ x4 \quad (6)$$

The respective Bland-Altman plots are presented in Fig. 3b, 4b, 5b, 6b. The average total biases for the comparison of D5R real-time PCR with KV-2 wild type were 0.8 cycles, D5R real-time PCR with Agianniotaki wild type were -0.2 cycles, D5R real-time PCR with KV-VAC were -0.2 cycles and D5R real-time PCR with Agianniotaki vaccine were -0.9 cycles. According to concordance correlation coefficient, the precision between methods, which measures how far each observation deviates from the best-fit line and represents Pearson correlation coefficient, was 58.70%, 48.83%, 93.98 and 82.35% when the D5R real time PCR was compared with the KV-2 wild type, Agianniotaki wild type, KV-VAC and with Agianniotaki vaccine respectively.

The average Ct values obtained by D5R real-time PCR were not significantly different from those obtained with real-time PCR of KV-2 wild type (mean ± SE: 35.96 ± 0.49 and 35.17 ± 0.5652 for the D5R and KV-2 wild type assay, respectively; $P > 0.05$). The average Ct values obtained by D5R real-time PCR were not significantly different from those obtained with real-time PCR of Agianniotaki wild type (mean ± SE: 35.96 ± 0.49 and 36.17 ± 0.67 for the D5R and Agianniotaki wild type assay, respectively; $P > 0.05$). The average Ct values obtained by D5R real-time PCR were not significantly different from those obtained with real-time PCR of KV-VAC (mean ± SE: 36.88 ± 0.75 and 37.05 ± 0.68 for the D5R and KV-VAC assay, respectively; $P > 0.05$). The average Ct values obtained by D5R real-time PCR were not significantly different from those obtained with real-time PCR of Agianniotaki vaccine (mean ± SE: 36.08 ± 0.84 and 36.95 ± 0.74 for the D5R and Agianniotaki vaccine assay, respectively; $P > 0.05$).

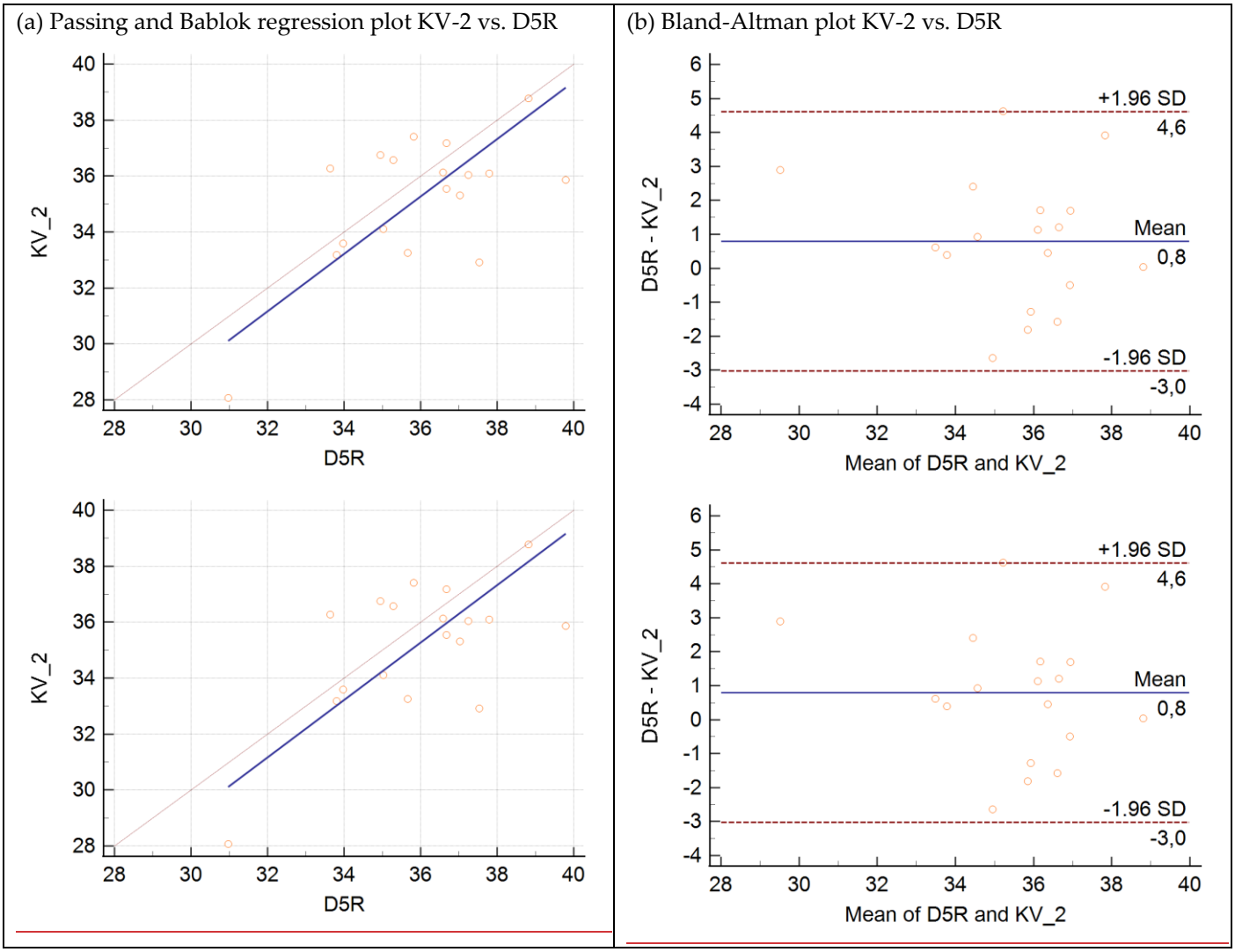

Figure 3. (**a**) Passing-Bablok regression plot: comparison of Ct values obtained with the KV-2 wild type strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 18 samples. (**b**) Bland–Altman plot: comparison of Ct values obtained with the KV-2 wild type strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 18 samples.

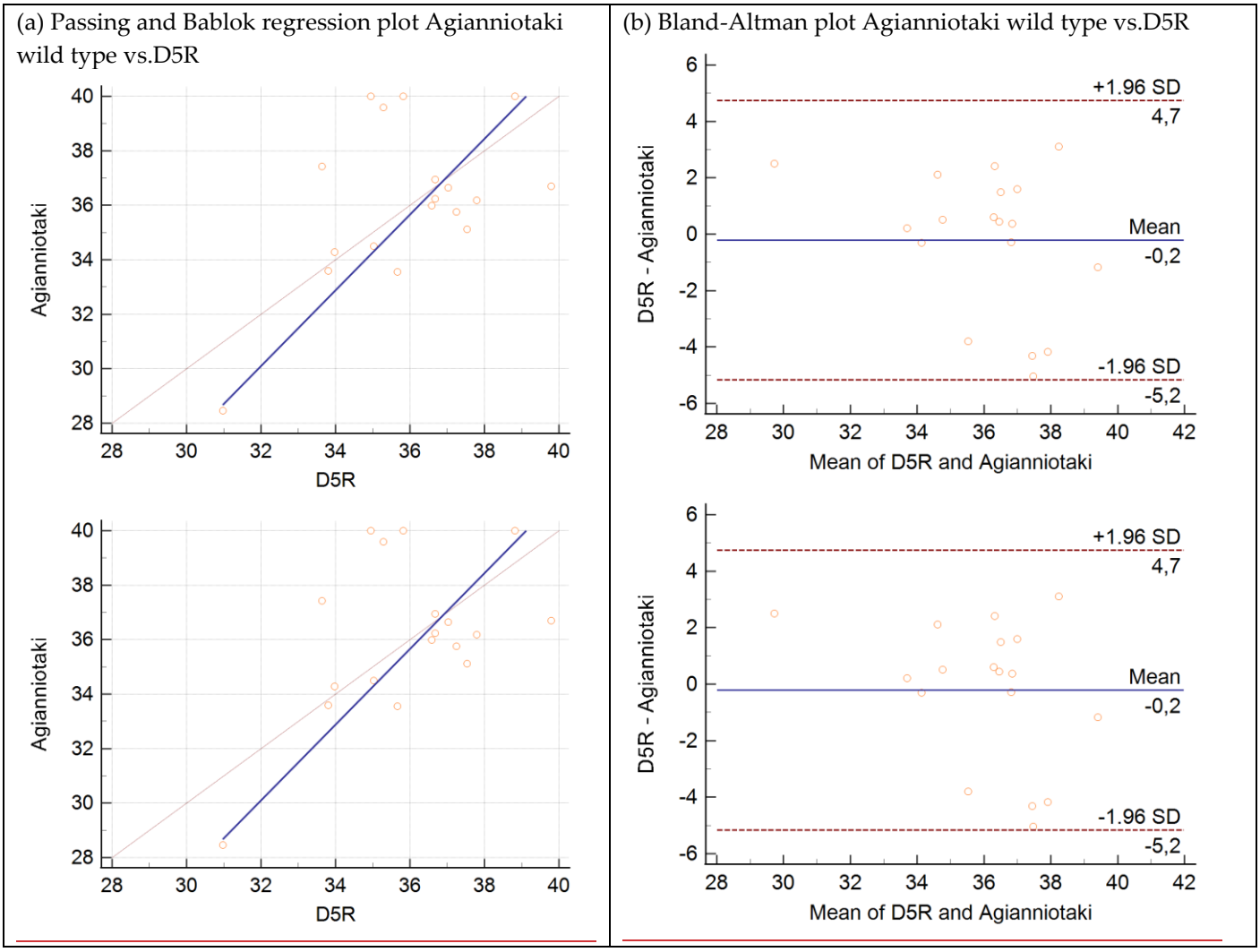

Figure 4. (**a**) Passing-Bablok regression plot: comparison of Ct values obtained with the Agianniotaki wild type strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 18 samples. (**b**) Bland–Altman plot: comparison of Ct values obtained with the Agianniotaki wild type strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 18 samples.

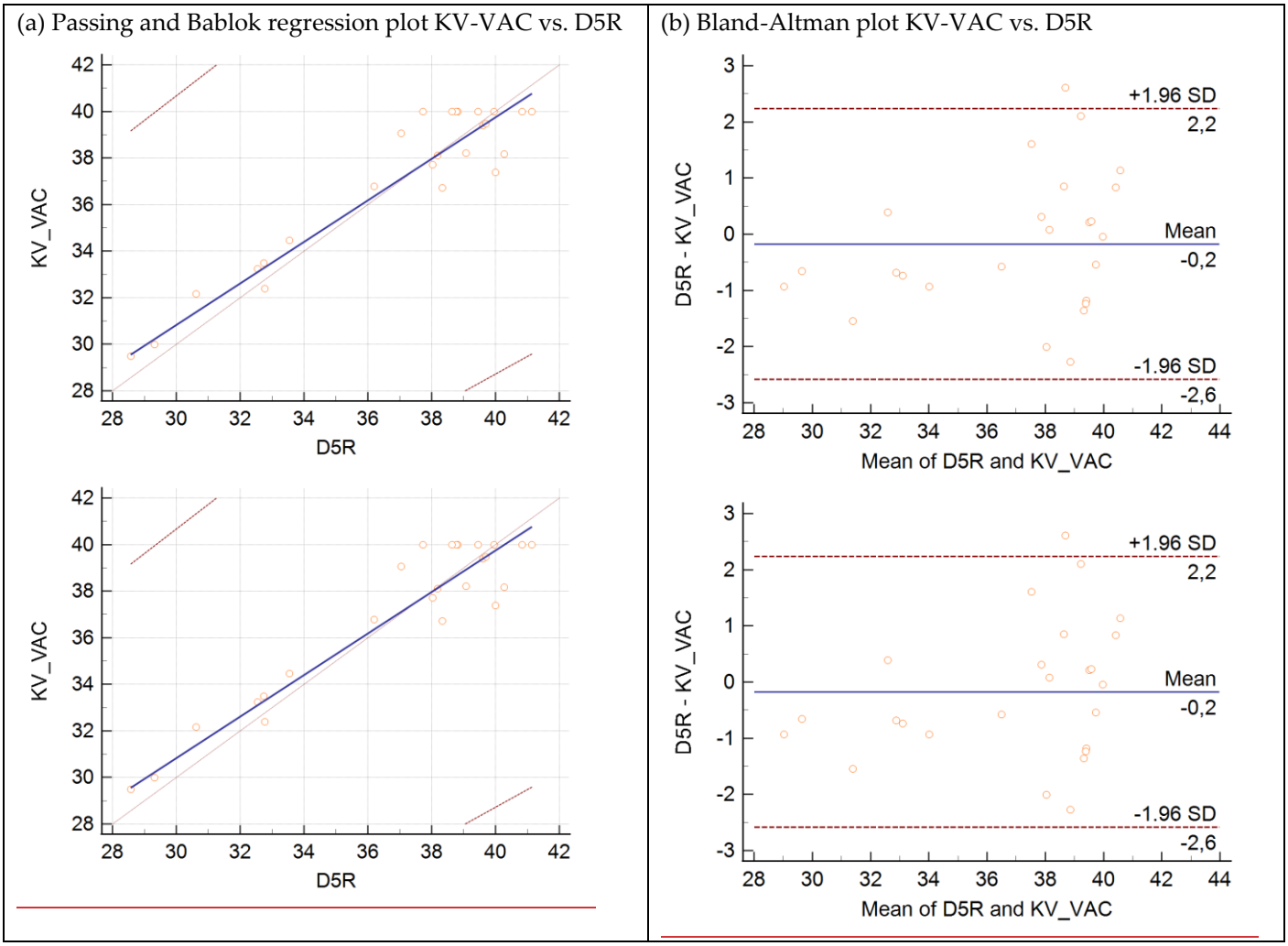

Figure 5. (**a**) Passing-Bablok regression plot: comparison of Ct values obtained with the KV-VAC vaccine strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 25 samples. (**b**) Bland–Altman plot: comparison of Ct values obtained with the KV-VAC vaccine strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 25 samples.

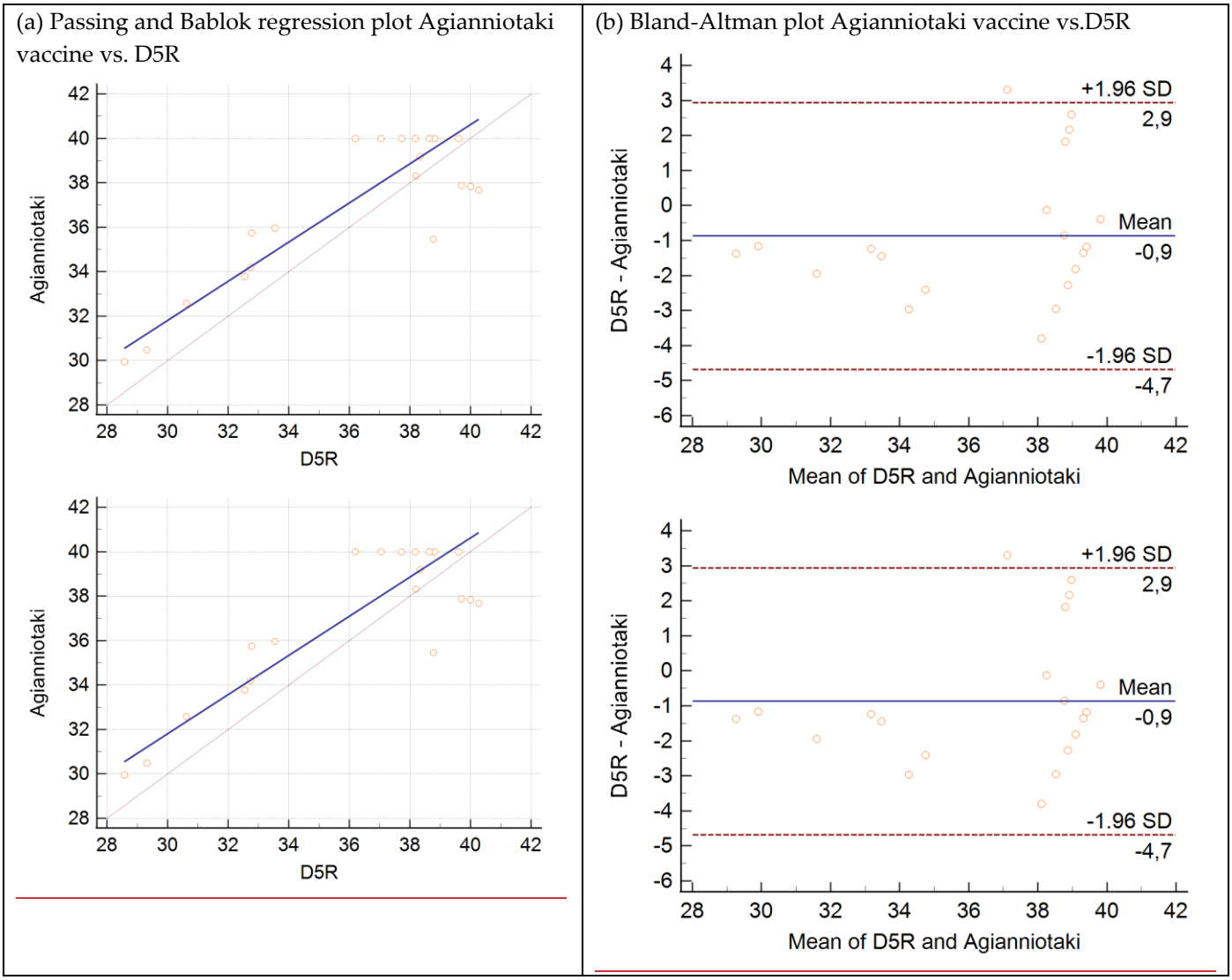

Figure 6. (**a**) Passing-Bablok regression plot: comparison of Ct values obtained with the Agianniotaki vaccine strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 20 samples. (**b**) Bland–Altman plot: comparison of Ct values obtained with the Agianniotaki vaccine strain assay vs capripox specific real-time PCR assay D5R, with 95% CI in 20 samples.

4. Discussion

Lumpy skin disease is an important disease of cattle and a major threat to livestock with severe socio-economic impact. The direct and indirect costs of disease control can cause great economic damage. Experience from the recent outbreak in the Balkan region shows that mass and broad vaccination of animals is an efficient way in eradicating this disease **[24,5]**. Rapid detection and reliable differentiation of wild type from vaccine strains is one of the key factors for the application of timely measures to combat LSD **[14]**. There is a need for diagnostic tests to be constantly improved in order to be able to respond to the ever-changing situation on the field. During the eradication of LSD in epidemic in Serbia in 2016, vaccination began three weeks after the first confirmed case of the disease. A coverage of 99% was achieved three months after the start of the campaign, when the last case was recorded **[24,5]**. The average time between the notification of a suspicion to stamping out was 2.2 days, which, with the application of mass vaccination, had a decisive influence on the rapid suppression of the disease **[5]**. The reason for such a

rapid diagnosis was the development of two TaqMan-based qPCR tests for specific detection of wild type strains of LSDV **[14]**, which allowed that results were obtained in a few hours. This methodology allows a short test duration and high throughput of samples.

The assays presented in this paper are not intended for first-line screening of samples but are intended to be used after examination of samples by another Pan-Capripox essay, such as **[19-22]** with usage of internal amplification control (IAC) to exclude influence of PCR inhibition. KV DIVA assays were validated in Veterinary Specialized Institute Kraljevo, NRL for Capripox viruses in Serbia and Sciensano, European Union Reference Laboratory for Capripox viruses, Belgium.

In VSI Kraljevo validation was performed on 243 samples of blood, skin, nasal swabs and milk originating from naturally infected or vaccinated animals, insects and environmental swabs. Tests have shown a very high sensitivity. The KV2 test for wild type strains showed a sensitivity of 24 copies of the LSDV genome per reaction, while the KV-VAC test, specific for vaccine strains, showed a sensitivity of 12 copies of the LSDV genome, which is comparable to the results obtained by **[15]** who reported sensitivity of DIVA test of 8 genome copies of wild type or vaccine strain LSDV per reaction. Pestova and Sprygin **[17,16]** reported limit of detection of 0.21 lgTCD50/ml and 0.15 lg TCD50/ml for wild type and vaccine assays respectively. The developed KV-VAC assay show very good linear correlation with Bowden assay and obtained Ct values are almost identical, which is expected because the limit of detection of both assays is the same. Wild type strain specific assay KV-2 is somewhat less sensitive than Bowden assay, 24 copies vs. 12 copies, and therefore it is understandable why there is statistically significant difference in Ct values. Observed differences are more noticeably in samples with lower quantity of viral genome, especially in those with Ct above 33 with Bowden assay.

In Sciensano, validation was performed on 48 samples obtained from animal experiment, comparing the performance of KV-2 and KV-VAC assays with DIVA Agianniotaki assay and CaPV D5R assay. When comparing with performance on wild type strains of LSDV with D5R assay, KV-2 assay and DIVA Agianniotaki showed 100% sensitivity. When comparing with performance on vaccine strains of LSDV with D5R assay, KV-VAC assay and DIVA Agianniotaki showed 85.71% and 75% sensitivity respectively. Comparing KV-DIVA assays with DIVA Agianniotaki assay, it can be concluded that KV-DIVA assays have almost identical performances. It must be emphasized that mainly samples with high Ct values were selected for the experiment. There were no statistically significant differences between Ct values obtained with panCapripox D5R assay and KV DIVA assays or Agianniotaki DIVA assay.

The specificity of the tests in VSI Kraljevo was examined on 60 different Capripox viruses, of which 31 belonged to classical wild type strains of LSDV, 4 to vaccine strains of LSDV (three Neethling based and one based on KSGP 0240 strain), one recombinant strain of LSDV, 12 SPPV and 12 GTPV strains. All classical wild type strains of LSDV and all Neethling-based vaccine strains were identified accurately using KV-2 and KV-VAC tests.

Using KV-DIVA assays, the Kenyavac strain KSGP 0240 strain was identified as a wild type strain. Although this strain is used as a vaccine against LSD in Kenya and some other African countries, genome studies have shown that this strain is genetically significantly different from the Neethling based vaccine strains of LSDV. Scientific and clinical trials have led to the conclusion that this strain is not sufficiently attenuated and causes clinical symptoms of disease in vaccinated animals **[25].**

The specificity of the tests in Sciensano was examined on 34 CaPV strains, of which six were wild type strains of LSDV, four were vaccine strains of LSDV, 20 SPPV and four GTPV strains. All wild type and vaccine strains of LSDV were identified accurately while there were no false positive results.

KV2 and KV-VAC assays were developed in 2016 and 2017 when possible recombinant strains had not yet been reported and aimed to correctly distinguish disease causing wild type strains from vaccine Neethling strains of LSD virus. This assays failed to detect

Dergachevsky - recombinant strain, the only recombinant strain available for us for in-vitro testing. Byadovskaya **[26]** conducted an extensive comparative performance test of several commercial and published DIVA tests and found that they were unable to correctly identify recombinant strains. Badhy**[27]** states that the isolates that appeared in 2019 in Bangladesh differ from strains from Africa, Europe, the Middle East, as well as from variant strains from Russia and China **[28,29]**. The occurrence and spread of recombinant strains have not been fully elucidated and their circulation must be monitored.

In the most European countries where the disease appeared in 2015 and 2016, a live attenuated vaccine based on the Neethling strain was used. Therefore, it was very important to use tests in diagnostic laboratories to distinguish wild type LSDV from Neethling vaccine strains. In this paper we presented an extensive validation study, which included 90 different Capripox virus strains and 291 samples originated from naturally infected animals, experimentally infected animals, vaccinated animals, insects and environment samples, conducted in two laboratories. The validation in the 2 laboratories with different equipment not only demonstrated the high sensitivity and specificity of the presented DIVA tests but also their robustness. This guarantees their accurate and cost effectiveness, essential for disease eradication, and demonstrates their flexibility in application under changing laboratories conditions. These DIVA assays are also in routine use for strain typing at Federal Research Center of Virology and Microbiology, Pokrov, Russia and Faculty of Veterinary Medicine, Ss. Cyril and Methodius University, Skopje, North Macedonia. However, in countries using an LSD vaccine based on SpPV or GtPV, a different diagnostic strategy must be applied.

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Data Availability Statement: All the data that is not present in the paper can be obtained upon request to the authors.

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