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ORIGINAL ARTICLE

Experimental lumpy skin disease virus infection of cattle: comparison of a field strain and a vaccine strain

Janika Möller[1] · Tom Moritz[1] · Kore Schlottau[1] · Kiril Krstevski[2] · Donata Hoffmann[1] · Martin Beer[1] · Bernd Hoffmann[1]

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Abstract

Lumpy skin disease virus (LSDV) infections can cause massive clinical signs in cattle and have great economic impact due to severe trade restrictions. For LSDV control, only live attenuated vaccines are commercially available, but they currently are not authorized in the European Union. Moreover, these vaccine virus strains can induce substantial side effects with clinical signs similar to infections with virulent LSDV. In our study, we compared clinical symptoms, viremia, and seroconversion of cattle inoculated either with a virulent field strain from North Macedonia isolated from diseased cattle in 2016 or with the attenuated LSDV vaccine strain "Neethling". Using specimens from the field and from experimental inoculation, different diagnostic tools, including a pan-capripox real-time qPCR, newly developed duplex real-time qPCR assays for differentiation between virulent and attenuated LSDV strains, and several serological methods (ELISA, indirect immunofluorescence test and serum neutralization test [SNT]) were evaluated. Our data show a high analytical sensitivity of both tested duplex real-time qPCR systems for the reliable distinction of LSDV field and vaccine strains. Moreover, the commercially available capripox double-antigen ELISA seems to be as specific as the SNT and therefore provides an excellent tool for rapid and simple serological examination of LSDV-vaccinated or infected cattle.

Introduction

Together with sheeppox virus and goatpox virus, lumpy skin disease virus (LSDV) forms the genus *Capripoxvirus* within the family *Poxviridae* [1]. Lumpy skin disease (LSD) is an emerging, transboundary, viral pox disease affecting cattle [2, 3] and is listed as a notifiable disease under the OIE guidelines [4].

For LSD, a wide range of clinical courses have been described, which vary from subclinical through mild to acute [1]. The incubation period varies from 4-14 days under experimental conditions [5–7] to 1-4 weeks in field outbreaks [7]. After the incubation period, the first clinical signs, including fever, lymphadenopathy and lachrymation, can occur [3, 5, 8]. Subsequently, infected animals can develop characteristic nodular skin lesions, which are found particularly on sparsely haired regions of the body but also may generalize [3, 5, 8–10]. In severely affected animals, lesions can also be observed in the mucous membranes of the eyes [8] and in the respiratory as well as in the gastrointestinal tract. In some cases, these nodules become ulcerative as the disease progresses, leading to an increased risk of myiasis [3]. Additionally, decreased milk yield [9, 11] and growth rate [12, 13] as well as abortions and temporary or permanent infertility in bulls may be observed [9, 11–13]. In addition to its significant impact on national production, an outbreak causes restrictions to the global trade in live animals and animal products [8], and 14 months to three consecutive years without an outbreak are required to regain an official disease-free status [14]. Moreover, control management and eradication measures are cost-intensive, leading to

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✉ Bernd Hoffmann
Bernd.Hoffmann@fli.de

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany

[2] University "Ss. Cyril and Methodius" Skopje, Faculty of Veterinary Medicine, Lazar Pop Trajkov 5-7, 1000 Skopje, Macedonia

an estimated production loss of 45-65% [8]. Therefore, LSD is of great economic importance. During LSD outbreaks, the morbidity rate ranges from 3 to 85% [8, 15, 16], whereas mortality is usually low (1-3%) [8]. However, mortality rates of 40-75% have been reported in naïve populations [8, 17].

LSDV is mainly transmitted mechanically via arthropod vectors [18–21], whereas direct contact between infected and uninfected animals does not seem to be a very effective route of transmission [18]. Nevertheless, transmission via shared use of drinking troughs by severely infected and naïve animals has been observed [22].

LSDV was described for the first time in Zambia in 1929 [15, 23]. In the following years, the virus spread across the African continent and became endemic in sub-Saharan Africa [8]. The first transcontinental spread of LSDV, from Africa to Israel, was reported in 1989 [8, 24, 25]. Between 1990 and 2012, only a few outbreaks of LSDV occurred in the Middle East [8]. In the European Union, LSDV was first reported in 2014, when the virus was found in Cyprus, followed by case reports from Greece in 2015 [26]. LSDV is also endemic in Turkey, where it has been found since 2013 [12], and it has spread across different Balkan countries since 2016 [26].

For control of LSDV, quarantine [9, 12, 27], movement restrictions, and culling of affected as well as in-contact animals are often combined with ring vaccinations [3, 8, 9, 27]. Until now, only live attenuated vaccines originating from field isolates have been commercially available [1, 12, 28]. Attenuation of these vaccines was achieved by multiple passages in cell culture [1, 29] and in the chorioallantoic membranes of embryonated chicken eggs [1, 30]. Unfortunately, usage of these vaccines can result in various adverse events, which are similar to clinical signs induced by LSDV, named "Neethling disease" [31]. Moreover, adverse reactions and local reactions at the injection site have been observed after application of particular vaccines [2, 13]. However, when a herd immunity of over 80% is accomplished, these vaccines provide good protection against LSDV [12]. Due to the fact that usage of live vaccines could compromise the disease-free status of a country, preventive use of these vaccines is not recommended for countries that are free from LSD [8, 12].

Since all capripoxviruses share common major antigens, it is not possible to distinguish them via serological tests [32]. Nevertheless, sequence analysis has revealed major amino acid sequence differences and even some frameshifts between virulent LSDV field strains and the attenuated vaccine strains, whereas comparison of two different virulent strains has shown only minor amino acid sequence differences [33]. Until now, only a few molecular tools for differentiation of LSDV field and LSDV vaccine strains have been available. In addition to gel-based systems combined with enzymatic digestion using restriction enzymes, a real-time qPCR followed by melting curve analysis has been described for distinguishing between virulent and vaccine strains (for an overview of methods, see Agianniotaki et al., [35]). Additionally, two TaqMan probe-based real-time qPCR methods have been proposed. Vidanović et al. described two different assays for specific detection of virulent LSDV field strains in 2016, using primers and probes recognizing the extracellular enveloped virion (EEV) gene region. In the LSDV vaccine strain, this region contains a 27-bp deletion when compared to virulent field strains, and LSDV vaccine strains are therefore not detected by these assays [34]. Another method is a multiplex DIVA real-time PCR developed by Agianniotaki et al. that specifically detects wild-type LSDV and Neethling/SIS vaccine viruses. Here, the binding sites for the primers and probe are located in the GPCR gene [35].

During our study, we examined the clinical reaction of Holstein-Friesian cattle inoculated with either an LSDV vaccine strain or an LSDV field strain from the Republic of North Macedonia in 2016 (hereafter referred to as the LSDV-Macedonia 2016 field strain). For molecular and serological evaluation, EDTA blood, serum, and nasal and oral swabs were taken at specific time points after experimental infection. Additionally, we measured body temperature and determined the clinical score for each animal to compare clinical disease and possible adverse events of vaccination. We also established two different, independent duplex real-time qPCR systems, both of which are able to distinguish between LSDV field strains and the LSDV vaccine strain "Neethling". In addition to designing new primers and probes for the detection of either LSDV field strains or LSDV vaccine strains, we also optimized the assay published by Vidanović et al. [34] by adding a second primer-probe mix for specific detection of LSDV vaccine strains.

Materials and methods

Animals

Two groups of six Holstein-Friesian cattle between nine and ten months old were housed in the facilities of the Friedrich-Loeffler-Institut, Insel Riems, Germany, under biosafety level 3 conditions. The respective experimental protocols were reviewed by the state ethics commission and approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany; Ref. No. LALLF M-V/TSD/7221.3-2.1-022/10).

Experimental infection and collection of samples

The animals were inoculated with either the LSDV-Neethling vaccine strain (in TE buffer, pH 8.0, $10^{7.8}$ $TCID_{50}$/ml

on MDBK cells [FLI cell culture collection number CCLV-RIE0261]) or the LSDV-Macedonia 2016 field strain (in TE buffer, pH 8.0, $10^{7.4}$ $TCID_{50}$/ml on MDBK cells). Therefore, each animal received 3 ml of the virus suspension intravenously (i.v.) and 1 ml subcutaneously (s.c.). For molecular and serological analysis, EDTA blood for the analysis of cell-associated viremia, serum for evaluation of cell-free viremia as well as detection of antibodies, and nasal and oral swabs were taken at -1, 3, 5, 7, 10, 12, 14, 17, 21, and 28 dpi. Body temperature was measured from 4 dpi until the end of the study; the clinical score [5] was recorded from 3 dpi to 28 dpi. After necropsy, a panel of various organs (Supplementary Tables S2 + S3) was analyzed using the pan-capripox real-time qPCR [36].

DNA extraction and pan-capripox real-time qPCR analysis

Organ samples were homogenized in serum-free medium using a TissueLyser II tissue homogenizer (QIAGEN, Hilden, Germany). DNA was extracted from homogenized organ samples, EDTA blood, serum, and nasal and oral swab samples using a NucleoMag Vet kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions using the KingFisher Flex System(Thermo Scientific, Darmstadt, Germany). Additionally, an internal control DNA (IC2-DNA) was added as a means of assessing whether the extraction was successful [37]. Subsequently, the pan-capripox real-time qPCR [36] was performed using the PerfeCTa qPCR ToughMix from Quanta BioSciences (Gaithersburg, USA), supplemented with a modified Capri-p32 TaqMan probe according to the protocols described by Dietze et al. [38] (Supplementary Table S1).

Serological analysis of samples

For serological analysis, we compared the commercially available ID Screen Capripox Double Antigen (DA) ELISA (ID.vet, Montepellier, France), the indirect immunofluorescence test (iIFT), and the serum neutralization test (SNT).

The ID Screen Capripox DA ELISA was performed according to the manufacturer's instructions. Samples with an S/P% ratio of ≥ 30% were defined as positive.

For the detection of antibodies against LSDV by the iIFT method, Vero76 cells (FLI cell culture collection number CCLV-RIE0228) at > 80% confluence were infected with 100 µl of the LSDV-Neethling vaccine strain ($10^{3.8}$ $TCID_{50}$/ml). After incubation for 48 h at 37 °C and 5% CO_2, fixation of the cells was performed using ice-cold acetone-methanol (1:2). Saponin blocking buffer (0.2% w/v BSA, 0.1% w/v NaN_3, 0.05% w/v saponin in 1X PBS) was used for blocking nonspecific binding sites and for dilution of serum samples and the secondary antibody. To prevent nonspecific reactions, serum samples were diluted 1:40, 1:80 and 1:160. For the secondary antibody reaction, FITC-labeled anti-bovine IgG antibody (Sigma-Aldrich, Darmstadt, Germany) was used at a 1:400 dilution. Fluorescence signaling was analyzed using an Axio Vert.A1 microscope (Zeiss, Oberkochen, Germany) with an HXP 120V fluorescent light source.

For the detection of neutralizing antibodies, an LSDV-specific SNT was performed. For this, serum samples were inactivated at 56 °C for 30 min, and log2 dilution series in serum-free minimal essential medium from 1:10 to 1:1280 were prepared in triplicate using a 96-well format. The LSDV-Neethling vaccine strain with a titer of $10^{3.3}$ KID_{50}/ml was used. After incubation for 2 h at 37 °C and 5% CO_2, MDBK cells at a concentration of approx. 30,000 cells/100 µl were added. After incubation for 7 days at 37 °C and 5% CO_2, the titer of neutralizing antibodies was determined using a light microscope (Nikon Eclipse TS-100) by the Spearman-Kärber method [39, 40].

Differentiating LSDV duplex real-time qPCR

For differentiation of LSDV field and vaccine strains, two independent duplex real-time qPCRs were developed (for detailed information, see supplementary Table S1). For the first assay (LSDV DIVA 1), the already published "FLI assay" [34], hereafter called LSD-Field-Mix2-FAM, employing the primers LSDfield for (5'-GTGAAGAAA ATTTAATTTGGGAC-3') and LSDfield rev (5'-CTCTAT GTTTGAAACATTGTCA-3'), and probe LSDfield FAM (5'-FAM-ACCACCTAATGATAGTGTTTATGATT TAC-BHQ1-3'), was supplemented with a primer-probe mix for specific detection LSDV vaccine strains (LSDvac-Mix1-HEX comprising primers LSDvac for [5'-GTCGTTTAA TAAACACAGATGGA-3'] and LSDvac rev [5'-CCTTTT ATAGATGATAATACTGAC-3'], and probe LSDvac HEX [5'-HEX-CGTTCGATTCTGTATTATCGACTGTAA-BHQ1-3']). In this case, the LSD-Field-Mix2-FAM probe recognizes the region encoding the extracellularly enveloped virion (EEV) glycoprotein, in which LSDV vaccine strains display a deletion of 27 bp [34], whereas the LSDvac-Mix1-HEX probe recognizes a region within the interferon gamma receptor gene. The second assay (LSDV DIVA 2) consists of LSDvir-Mix4-FAM, comprising primers LSDvir for (5'-ATCAGTTTCGTAATTTCCAAAAACT-3'), LSDvir rev (5'-CAATTAAATATGAAGTTGATGAACAG-3'), and probe LSDvir FAM (5'-FAM-TCCTGAGACCCRAATTCA ACAATACAT-BHQ1-3'), also recognizing the interferon gamma receptor gene, but in a region specific for virulent LSDV strains, and LSDvac-Mix5-HEX (consisting of primers LSDvac for [5'-TCTTGGACAACTTTGATGCATC-3'] and LSDvac rev [5'-CTTCATAGCCTATTCCGAGAG-3'] and probe LSDvac HEXas [5'-HEX-ACTTGCGTAACT

AATTCCACCCACAA-BHQ1-3'] located in the Kelch-like protein-encoding gene). Duplex real-time qPCRs were performed using a QuantiTect Multiplex PCR NoROX Kit (QIAGEN, Hilden, Germany) with 12.5 µl of reaction mix (1.75 µl of water, 6.25 µl of 2x QuantiTect Multiplex PCR NoROX Master Mix, 1 µl of each primer probe mix, 2.5 µl of DNA) for each sample. Here, the following cycling conditions were used: 15 min at 95 °C, followed by 45 cycles of 45 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C). The cutoff for both LSDV DIVA assays was defined as Cq > 40.

Results

Clinical response

Substantial clinical differences were observed after inoculation with either the LSDV-Neethling vaccine strain or the LSDV-Macedonia 2016 field strain. All cattle inoculated with the LSDV field strain developed fever by 11 dpi. In contrast, the body temperature of the six cattle inoculated with the vaccine strain remained in the normal range during the experiment. The clinical scores were also clearly different between and within the inoculation groups. Whereas three animals in the LSDV-Macedonia 2016 field strain group reached a clinical score of 10 and had to be removed from the animal trial for ethical reasons, only one animal in the LSDV-Neethling vaccine strain group had a maximum clinical score of 7 (Fig. 1).

In addition to fever, inoculation with the LSDV-Macedonia 2016 field strain led to strong ocular and nasal discharge as well as generalized skin lesions in three of six animals (Fig. 2). These cattle (R/136, R/137, R/145) had to be euthanized before the end of the study due to severe clinical signs. In contrast, the other three animals in this group (R/200, R/285, and R/286) developed only mild clinical symptoms. Compared to the field strain, the Neethling vaccine strain caused substantially less-dramatic clinical signs: only two out of six cattle displayed massive reactions at the s.c. inoculation site (Fig. 1), and no generalization of skin lesions was observed in any of the six cattle.

Virus replication and shedding

In order to investigate both viral replication and shedding (Tables 1 and 2), EDTA blood, serum samples, and oral and nasal swabs were taken at different time points during the animal experiment.

In both groups, the first positive results were obtained with EDTA blood at 3 dpi (R/124, LSDV-Neethling vaccine strain; R/136, LSDV-Macedonia 2016 field strain) (Tables 1 and 2). Cattle inoculated with the LSDV-Neethling vaccine strain showed only limited viral replication. At 3 dpi, one out of six cattle was positive in a single sample (R/124, EDTA-blood) with a high Cq value of 36.0. From 5 dpi to 7 dpi, five of six animals tested positive in at least one sample, but with low viral loads and Cq values ranging from 35.5 (R/129, EDTA-blood, 7 dpi) to 38.3 (R/193, EDTA-blood, 7 dpi). Samples taken from animal R/128 were negative for the capripoxvirus genome during the entire animal trial. At 12 dpi, all animals tested negative for the capripoxvirus genome. Therefore, later than 12 dpi, only serum samples were taken for further analysis.

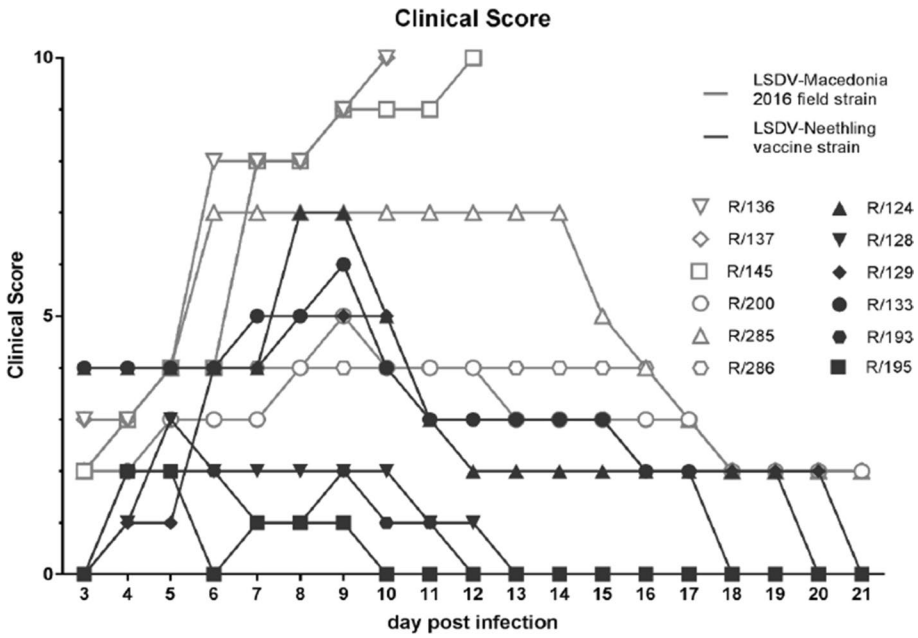

Fig. 1 Clinical scores of cattle infected with the LSDV-Neethling vaccine strain and LSDV-Macedonia 2016 field strain, respectively. Light symbols represent the cattle inoculated with the LSDV-Macedonia 2016 field strain, whereas dark symbols represent animals inoculated with the LSDV-Neethling vaccine strain

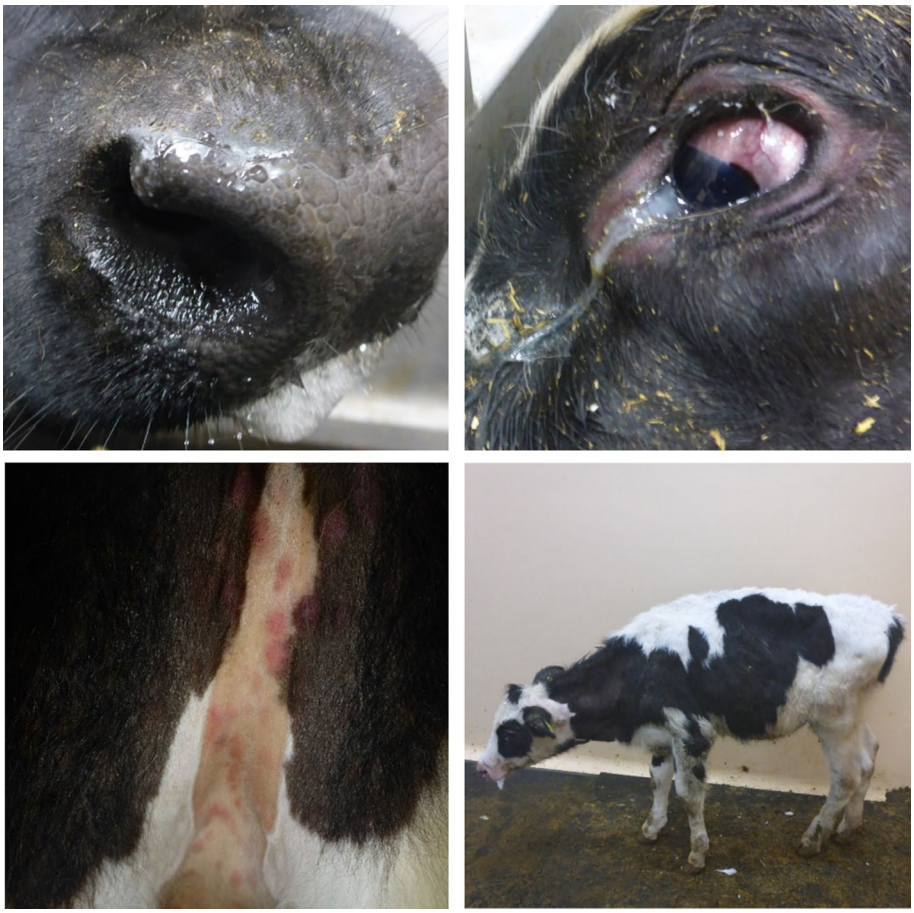

Fig. 2 Clinical reactions after inoculation with the LSDV-Macedonia 2016 field strain. Three of six cattle inoculated with the virulent LSDV field strain from Macedonia in 2016 developed severe clinical signs including nasal and ocular discharge, nodule-like lesions typical for LSDV infection, and massive respiratory problems. These three animals had to be euthanized for ethical reasons

In the LSDV-Macedonia 2016 group, two different phenotypes could be distinguished. When samples from cattle with mild clinical signs were tested, pan-capripox real-time qPCR gave results similar to those obtained with samples from the LSDV-Neethling vaccine group. In these animals, the first positive results for the capripoxvirus genome were obtained at 5 dpi in EDTA blood (Cq 37.1) and serum (Cq 36.1) of R/285. Until 12 dpi, the capripoxvirus genome was detected only sporadically with high Cq values ranging from 35.2 (R/200, nasal swab, 12 dpi) to 39.8 (R/286, serum, 10 dpi) in samples taken from R/200, R/285 and R/286. No capripoxvirus genome could be detected after day 12 pi in these three animals. Therefore, after 14 dpi, only serum samples were collected, and they were analyzed only serologically.

In contrast, the remaining three animals inoculated with the wild-type virus displayed high viral loads in combination with generalized symptoms and progressive disease (R/136, R/137, and R/145). In detail: at 3 dpi, cattle R/136 and R/137 were positive for the capripoxvirus genome in EDTA blood, at first with high Cq values (37.6). In addition, capripoxvirus DNA could be detected in serum of R/136 (Cq 38.1). The first capripoxvirus genome positivity was ascertained in EDTA blood and serum from R/145 at 5 dpi. At this time, R/136 and R/137 were already positive in three out of four sample matrices with Cq values of approximately 31 in EDTA blood, 36 in serum and 34 and 35 in nasal and oral swabs, respectively. From 7 dpi onward, EDTA blood, serum, and nasal and oral swabs from these three animals were positive for the capripoxvirus genome until euthanasia. During this time, viral genome loads increased continuously with peak values at the day of euthanasia (R/136 and R/137 10 dpi, R/145 12 dpi). In detail, very low Cq values ranging from 16.0 (R/136) through 17.0 (R/145) to 17.1 (R/137) were obtained with nasal swabs, whereas lower virus loads were detected in EDTA blood, serum and oral swabs from these three animals (Table 2).

Viral genome loads in different organs

During necropsy, different organs were sampled and analyzed with regard to capripoxvirus genome loads (Supplementary Tables S2 and S3).

In the LSDV-Neethling vaccine group, viral-DNA-positive results were obtained only sporadically when using the pan-capripox real-time qPCR. In detail, mandibular (R/124, Cq 36.3, and R/133, Cq 36.1) and cervical (R/129, Cq 36.5) lymph nodes, as well as liver tissue (R/193, Cq

Table 1 Results for samples taken from cattle inoculated with the LSDV-Neethling vaccine strain examined by pan-capripox qPCR

LSDV-Neethling vaccine strain	Capri-p32-Mix 1-Taq-FAM									
	-1 dpi	3 dpi	5 dpi	7 dpi	10 dpi	12 dpi	14 dpi	17 dpi	21 dpi	28 dpi
R/124_EDTA-blood	no Cq	36.01	38.18	no Cq	no Cq	no Cq	–	–	–	–
R/124_serum	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	n.a.	n.a.
R/124_nasal swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/124_oral swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/128_EDTA-blood	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/128_serum	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	n.a.	n.a.
R/128_nasal swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/128_oral swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/129_EDTA-blood	no Cq	no Cq	36.06	35.47	no Cq	no Cq	–	–	–	–
R/129_serum	no Cq	no Cq	34.81	no Cq	37.89	no Cq	no Cq	no Cq	n.a.	n.a.
R/129_nasal swab	no Cq	no Cq	no Cq	32.8	no Cq	no Cq	–	–	–	—
R/129_oral swab	no Cq	no Cq	37.71	no Cq	no Cq	no Cq	–	–	–	–
R/133_EDTA-blood	no Cq	no Cq	37.50	no Cq	no Cq	no Cq	–	–	–	–
R/133_serum	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	n.a.	n.a.
R/133_nasal swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/133_oral swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/193_EDTA-blood	no Cq	no Cq	no Cq	38.34	no Cq	no Cq	–	–	–	–
R/193_serum	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	n.a.	n.a.
R/193_nasal swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/193_oral swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/195_EDTA-blood	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–
R/195_serum	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	n.a.	n.a.
R/195_nasal swab	no Cq	no Cq	no Cq	37.31	no Cq	no Cq	–	–	–	–
R/195_oral swab	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–	–	–

38.5) and oral mucosa (R/193, Cq 36.8) were affected with low genome load levels (Supplementary Table S2). Moreover, in skin samples from the udder and rumen of R/133, low viral genome loads (Cq 37.5 and 39.4, respectively) could be detected (data not shown). Interestingly, no capripoxvirus genome was found at the inoculation site of the skin in any of the six animals (Supplementary Table S2).

Within the group of cattle inoculated with LSDV-Macedonia 2016, viral genome loads in the organs differed. Like the DNA load in *intra vitam* samples, viral genome loads in the organs of the three animals that displayed only mild clinical signs (R/200, R/285, and R/286) were markedly lower than in those of the three animals with severe disease (R136, R/137, and R/145). For R/200, R/285 and R/286, most of the organs were tested negative for the capripoxvirus genome. Only in skin samples could viral genomes be detected in these three animals, albeit with high Cq values. Especially in skin samples taken from the inoculation site, the capripoxvirus genome was detected with Cq values from 30.3 (R/286) through 34.1 (R/285) to 38.7 (R/200). In contrast, every organ examined from the three severely affected cattle was tested positive for the capripoxvirus genome. Furthermore, in skin samples from the inoculation site, Cq values of 15.2 (R/137), 17.0 (R/145) and 17.7 (R/136) were obtained. The other organs displayed Cq values between 19.4 (R/137, tonsil) and 35.3 (R/145, salivary gland) (Supplementary Table S3). Interestingly, the capripoxvirus genome could also be found in parts of the gastrointestinal tract and parts of the brain of these three animals (data not shown), clearly demonstrating a generalized infection.

Serological responses

Serological responses after inoculation with the LSDV-Neethling vaccine strain and the LSDV-Macedonia 2016 field strain were examined using the ID Screen Capripox DA ELISA, the indirect immunofluorescence assay (iIFT), and the serum neutralization test (SNT) (Tables 3 and 4). Since three animals were removed from the trial at 10 dpi (R/136, and R/137) and 12 dpi (R/145), respectively, only data for the nine animals that survived until the end of the study were used for evaluation of the serological tests.

Overall, inoculation with both LSDV strains caused a serological response in most animals. Furthermore, the ELISA and SNT results were in general agreement regarding positive/negative results. However, in two of nine animals,

Table 2 Results for samples taken from cattle inoculated with the LSDV-Macedonia 2016 field strain examined by pan-capripox real-time qPCR

LSDV-Macedonia 2016 field strain	Capri-p32-Mix 1-Taq-FAM									
	-1 dpi	3 dpi	5 dpi	7 dpi	10 dpi	12 dpi	14 dpi	17 dpi	21 dpi	28 dpi
R/136_EDTA-blood	no Cq	37.59	31.14	27.79	25.91	†	†	†	†	†
R/136_serum	no Cq	38.13	35.81	31.27	25.50	†	†	†	†	†
R/136_nasal swab	no Cq	no Cq	33.81	28.54	16.04	†	†	†	†	†
R/136_oral swab	no Cq	no Cq	no Cq	30.95	25.44	†	†	†	†	†
R/137_EDTA-blood	no Cq	37.60	32.53	27.48	24.76	†	†	†	†	†
R/137_serum	no Cq	no Cq	36.07	29.62	25.66	†	†	†	†	†
R/137_nasal swab	no Cq	no Cq	no Cq	28.03	17.10	†	†	†	†	†
R/137_oral swab	no Cq	no Cq	35.28	31.01	26.24	†	†	†	†	†
R/145_EDTA-blood	no Cq	no Cq	30.93	26.20	25.00	23.44	†	†	†	†
R/145_serum	no Cq	no Cq	37.27	31.69	26.31	23.28	†	†	†	†
R/145_nasal swab	no Cq	no Cq	no Cq	23.86	19.97	17.01	†	†	†	†
R/145_oral swab	no Cq	no Cq	no Cq	30.67	28.38	32.70	†	†	†	†
R/200_EDTA-blood	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–
R/200_serum	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	n.a.	n.a.
R/200_nasal swab	no Cq	no Cq	no Cq	37.86	38.24	35.18	no Cq	no Cq	–	–
R/200_oral swabs	no Cq	no Cq	no Cq	37.02	no Cq	36.17	no Cq	no Cq	–	–
R/285_EDTA-blood	no Cq	no Cq	37.14	no Cq	no Cq	no Cq	no Cq	no Cq	–	–
R/285_serum	no Cq	no Cq	36.11	no Cq	38.49	37.79	no Cq	no Cq	n.a.	n.a.
R/285_nasal swabs	no Cq	no Cq	no Cq	37.17	35.77	36.18	no Cq	no Cq	–	–
R/285_oral swabs	no Cq	no Cq	no Cq	no Cq	37.18	34.74	no Cq	no Cq	–	–
R/286_EDTA-blood	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	no Cq	–	–
R/286_serum	no Cq	no Cq	no Cq	no Cq	39.78	no Cq	no Cq	no Cq	n.a.	n.a.
R/286_nasal swabs	no Cq	no Cq	no Cq	no Cq	no Cq	36.96	no Cq	no Cq	–	–
R/286_oral swabs	no Cq	no Cq	no Cq	no Cq	34.86	–	no Cq	no Cq	–	–

positive results were obtained at an earlier time point with the ELISA than with the SNT. In addition, the iIFT seems to be more sensitive than both ELISA and SNT, since serum samples from particular animals were scored positive days before the ELISA or the SNT showed any positive reactivity.

The ELISA reactions first were positive at 14 dpi in six of nine animals (four of the LSDV-Neethling group and two of the LSDV-Macedonia 2016 group). At 21 dpi, one additional animal (R/193, LSDV-Neethling vaccine strain) had specific antibodies, and at 28 dpi, serum from R/286 (LSDV-Macedonia 2016) scored positive, showing that eight of nine animals had seroconverted by 28 dpi. Analysis using the SNT revealed neutralizing antibodies starting from 14 dpi in six cattle (three of the LSDV-Neethling group and all three surviving animals of the LSDV-Macedonia 2016 group). For R/195, which was positive in the ELISA from day 14 pi onward, neutralizing antibodies were found at 28 dpi, whereas for R/193, no neutralizing antibodies were detected by SNT. However, iIFT gave antibody-positive results for eight of nine animals at 7 dpi and for all animals at 14 dpi, which could be explained by a higher sensitivity of the iIFT compared to the ELISA and the SNT (Tables 3 and 4). Animal R/128, which was positive in the iIFT from 7 dpi onward, and negative in the ELISA and the SNT during the whole study, might be a so-called "low-responder". Among others, this phenomenon has been described for humans vaccinated with vaccinia virus [41] and could possibly explain the inconsistent results of the iIFT and ELISA as well as the SNT. Additionally, although the serum was diluted to reduce the probability of nonspecific reactions in the iIFT, nonspecific positivity cannot be fully excluded.

Evaluation of the LSDV field/vaccine duplex real-time qPCR

Two independent duplex real-time qPCR systems for differentiation of attenuated LSDV vaccine strains and virulent LSDV field strains were developed and analyzed regarding analytical sensitivity and specificity. The assays are "LSDV DIVA 1", which consists of LSD-Field-Mix2-FAM and LSDvac-Mix1-HEX, and "LSDV DIVA 2" consisting of LSDvir-Mix4-FAM and LSDvac-Mix5-HEX. Here, each system consisted of one assay detecting virulent LSDV strains specifically and one assay for the specific detection of attenuated LSDV vaccine strains.

Both integrated duplex systems showed 100% analytical sensitivity and specificity for all of the LSDV vaccine strains and LSDV field strains analyzed (Supplementary Table S4).

Table 3 Serological data for cattle inoculated with the LSDV-Neethling vaccine strain analyzed using double-antigen ELISA (ID.vet, France), iIFT and SNT. For the ELISA, S/P% >30 is defined as positive. Minus signs (-) indicate no reaction in the iIFT. The strength of fluorescence is represented semi-quantitatively from mild (+) through moderate (++) to strong (+++). A titer ≥13 in the SNT is defined as seropositive

LSDV-Neethling vaccine strain group	Serological examination		
	ELISA (S/P%)	iIFT	SNT (ND_{50}/ml)
R/124_-2 dpi	13	-	<1:10
R/124_7 dpi	4	++	<1:10
R/124_14 dpi	99	+++	18
R/124_21 dpi	168	+++	57
R/124_28 dpi	196	+++	45
R/128_-2 dpi	-4	-	<1:10
R/128_7 dpi	9	+	<1:10
R/128_14 dpi	-1	++	<1:10
R/128_21 dpi	-6	++	7
R/128_28 dpi	22	++	7
R/129_-2 dpi	-1	-	<1:10
R/129_7 dpi	10	+	<1:10
R/129_14 dpi	34	++	18
R/129_21 dpi	49	+++	45
R/129_28 dpi	101	+++	71
R/133_-2 dpi	3	-	<1:10
R/133_7 dpi	16	+	<1:10
R/133_14 dpi	37	++	13
R/133_21 dpi	36	+++	45
R/133_28 dpi	129	+++	180
R/193_-2 dpi	4	-	<1:10
R/193_7 dpi	14	+	<1:10
R/193_14 dpi	17	++	<1:10
R/193_21 dpi	43	++	<1:10
R/193_28 dpi	36	++	7
R/195_-2 dpi	-1	-	<1:10
R/195_7 dpi	12	+	<1:10
R/195_14 dpi	38	++	<1:10
R/195_21 dpi	50	++	<1:10
R/195_28 dpi	88	++	13

Table 4 Serological data for cattle inoculated with the LSDV-Macedonia 2016 field strain analyzed using double-antigen ELISA (ID.vet, France), iIFT and SNT. For the ELISA, S/P% >30 is defined as positive. Minus signs (-) indicate no reaction in the iIFT. The strength of fluorescence is represented semi-quantitatively from mild (+) through moderate (++) to strong (+++). A titer ≥13 in the SNT is defined as seropositive

LSDV-Macedonia 2016 field strain group	Serological analysis		
	ELISA (S/P%)	iIFT	SNT (ND_{50}/ml)
R/200_-2 dpi	3	-	<1:10
R/200_7 dpi	13	+	<1:10
R/200_14 dpi	71	+++	28
R/200_21 dpi	169	+++	28
R/200_28 dpi	196	+++	90
R/285_-2 dpi	7	-	<1:10
R/285_7 dpi	20	+/-	<1:10
R/285_14 dpi	42	++	28
R/285_21 dpi	65	+++	57
R/285_28 dpi	136	+++	90
R/286_-2 dpi	13	-	<1:10
R/286_7 dpi	0	+	<1:10
R/286_14 dpi	-3	++	14
R/286_21 dpi	12	+++	36
R/286_28 dpi	71	++	45

In addition to the five LSDV field strains and the five attenuated LSDV live vaccine strains, we tested both duplex qPCR systems with two different members of the genus *Capripoxvirus*, namely goatpox virus (GTPV) and sheeppox virus (SPPV). LSDV DIVA 1 did not react with SPPV or GTPV isolates. In contrast, the LSDV DIVA 2 assay did show a cross-reaction with both SPPV strains and one of two GTPV strains (Supplementary Table S4).

For further validation, both assays were tested using a DNA dilution series of one virulent LSDV field strain and one attenuated LSDV vaccine strain, and the results were compared to those obtained using the pan-capripox qPCR. In this sensitivity test, three of four assays produced Cq values similar to those obtained with the pan-capripox assay (Supplementary Table S5). Only the LSD-Field-Mix2-FAM yielded Cq values that were clearly higher than those obtained by pan-capripox real-time qPCR.

Taken together, the data show that both duplex real-time qPCR systems are able to detect and differentiate virulent LSDV field strains and attenuated LSDV vaccine strains specifically up to a reference Cq value of approximately 35 (Supplementary Table S5).

Discussion

Pathogenesis in cattle after experimental inoculation with an LSDV field strain and a vaccine virus strain

In general, very clear differences in the pathogenesis of the LSDV-Neethling vaccine strain and the LSDV-Macedonia 2016 strain could be observed during our study. Inoculation with the field strain led to increased body temperature in all six cattle starting around 4 dpi and lasting until 10-12 dpi, which is similar to observations after inoculation with a virulent South African field strain [9]. Furthermore, three animals developed severe clinical symptoms (Fig. 1), leading to premature euthanasia. In contrast to typical mortality rates,

which are usually between 1% and 3% [8], a high mortality rate of 50% was observed in the LSDV field strain group in our study. However, since it is known that high-producing dairy cattle like Holstein-Friesian cattle are more susceptible to LSDV [12] and mortality rates of 40%-75% in naïve populations have been described previously [8, 17], this finding is not unexpected. Compared to the virulent Macedonian LSDV field strain, the attenuated LSDV vaccine strain caused only some mild adverse events in some of the animals (Fig. 1), and fever was not observed at any time during the study. As reported for the South African Onderstepoort Neethling vaccine strain [13], two of six animals showed clear local skin reactions at the site of inoculation with the LSDV-Neethling vaccine strain. However, generalization or systemic symptoms were not detected, which is consistent with previous reports [2]. High virus DNA loads were recorded starting at day 3-5 pi in all four sample matrices taken during the study and in multiple organs analyzed after necropsy in these animals, which were severely infected with LSDV-Macedonia (Table 2 and Supplementary Table S3). As described previously [6], the highest load of viral DNA was detected in samples taken from nodule lesions of the skin, especially those from severely affected animals. However, substantially less viral DNA was detected in the skin samples of non-diseased cattle from the field strain group, whereas nearly all skin samples taken from cattle of the LSDV vaccine group, including the sample from the inoculation site, tested negative for viral DNA (Supplementary Table S2). Interestingly, this finding contrasts with results obtained by Bedekovic et al., who successfully re-isolated LSDV from skin nodules after preventive vaccination with Lumpyvax/SIS-Type [42]. Moreover, most of the samples taken at necropsy from the nine surviving animals showed negative results for LSDV viral DNA (Table 1). In contrast to a study by Tuppurainen et al. in 2005 that showed no correlation between the degree of severity of clinical symptoms and the length of viremia after inoculation with a virulent South African field strain of LSDV [9], our data indicate a correlation between viral DNA detection in swab samples, representing virus shedding, and the degree of severity of clinical symptoms. Seroconversion started around 14-21 dpi, and all animals that survived until the end of the study developed antibodies against LSDV, which were detectable by ELISA, SNT or both (Tables 3 and 4). Here, no difference between the two inoculation groups could be found regarding time point or strength of the antibody reaction. Furthermore, for R/128 (LSDV-Neethling vaccine strain group), no antibodies could be detected using ELISA or SNT. Additionally, iIFT gave positive results for all nine surviving cattle. This disparity in the results for R/128 could possibly be explained by the individual being a low-responder, as it has been observed for humans vaccinated with vaccinia virus [41].

Evaluation of molecular and serological methods for the detection of LSDV infections

For analysis of virus replication and shedding, we used the pan-capripox real-time qPCR assay described by Bowden et al. [36] in combination with a modified TaqMan probe, as described by Dietze et al. [38] (Supplementary Table S1). Also used previously in various studies [6, 16, 42], the primer of the Bowden assay allows rapid and reliable amplification of capripoxvirus genomes [36]. In contrast to Bowden et al., Dietze et al. used a classical TaqMan probe with a dark quencher (BHQ1) instead of a minor grove binder (MGB). Moreover, the modified probe consists of a few more nucleotides. Like the original method, the modified assay showed high sensitivity, as demonstrated by the dilution series results (Supplementary Table S5). Since we were able to detect fewer than 10 copies of the viral genome per reaction, the TaqMan-BHQ1 probe provides a good alternative to the original MGB probe. In general, TaqMan probes are cheaper than MGB probes and can be synthesized by different companies worldwide, and thus, this type of real-time PCR probe is preferred by labs in developing countries.

We also developed two independent duplex real-time qPCR systems for genomic differentiation of virulent LSDV field strains and attenuated LSDV vaccine strains. Compared to the pan-capripox assay, both duplex systems gave similar Cq values, indicating good analytical sensitivity (Supplementary Table S5). Additionally, both assays provided 100% analytical sensitivity and specificity when tested with five LSDV field strains and five attenuated live vaccine strains, but LSDvir-Mix 4-FAM (LSDV-DIVA 2), also detects DNA of SPPV and GTPV strains (Supplementary Table S4). This cross-reaction has to be considered when analyzing samples containing capripoxvirus DNA, and differentiation between the three capripoxviruses has to be performed before using our real-time qPCR systems to rule out the presence of DNA from SPPV or GTPV. However, since the assay was developed as a DIVA technique for LSDV and not for differentiation between the three capripoxviruses, the detection of DNA of GTPV and SPPV with LSDvir-Mix 4-FAM is of minor relevance to the original purpose of the two LSDV DIVA assays. Additionally, GTPV or SPPV have never been reported in areas where LSDV is endemic [43], and there has been only one confirmed occurrence of LSDV in sheep [43–45]. For these reasons, cross-reaction of LSDvir-Mix 4-FAM with SPPV and GTPV is of little importance for the central question of distinguishing between virulent LSDV field strains and attenuated live vaccine strains. Additionally, we found that using nasal swabs resulted in better sensitivity than using EDTA blood, serum, or oral swabs (Table 2), and they are also easy to handle. Therefore, collection of

nasal swabs appears to be the preferred non-invasive sampling technique for use during natural outbreaks.

Serological examination was performed using three different tools: the newly licensed ID Screen Capripox double antigen (DA) ELISA, the indirect immunofluorescence test (iIFT), and the serum neutralization test (SNT) (Tables 3 and 4). We compared the sensitivity of these three methods for analysis of seroconversion after inoculation with LSDV. In the iIFT, a very slight antibody reaction was detected as early as 7 dpi, which is earlier than with ELISA and SNT. However, strong immunofluorescence signals were observed with a sample from one animal (R/128) that was negative in both SNT and ELISA. This can possibly be explained by a low antibody response against the infection based on the specific antigens in the ELISA and the proteins responsible for virus neutralization in the SNT. In addition, nonspecific reactions in the iIFT cannot be completely ruled out. Currently, the virus neutralization test (VNT) is the gold standard for serological examination of capripoxvirus infections [1, 9] due to its high specificity [3]. However, there are some disadvantages of using the VNT, such as high cost, long time requirement, low sensitivity, and especially, the need to use live virus [3]. In our study, the results for the DA ELISA from ID.vet were comparable to those obtained with the SNT. Furthermore, no difference could be found when the ELISA results of the cattle inoculated with LSDV-Neethling vaccine strain were compared to those for the cattle of the virulent field strain group. Therefore, we conclude that the ELISA is as sensitive as the SNT and permits rapid and simple serological analysis of cattle infected or vaccinated with LSDV.

During our study, half of the cattle inoculated with the virulent LSDV field strain from Macedonia developed severe clinical symptoms, and high virus loads were detected in EDTA blood, serum, and nasal and oral swabs as well as in all analyzed organs. In contrast, the attenuated LSDV vaccine strain caused only mild side-effects, especially local reactions at the inoculation site, and most samples were negative for the viral genome. Our newly developed independent duplex real-time qPCR assays based on TaqMan probes for the specific distinction between virulent and vaccine strains of LSDV display high analytical sensitivity and specificity and therefore provide rapid and reliable methods for the discrimination of field and vaccine strains of LSDV. Furthermore, the ID Screen Capripox DA ELISA from ID.vet, showing a sensitivity and specificity comparable to the SNT, represents a high-throughput alternative to the current gold-standard method for serological examination.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Statement on welfare of animals Each of the experimental protocols was reviewed by the state ethics commission and approved by the corresponding competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Vorpommern, Rostock, Germany; Ref. No. LALLF M-V/TSD/7221.3-2.1-022/10).

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