



Short communication

Suitability of group-level oral fluid sampling in ruminant populations for lumpy skin disease virus detection



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ABSTRACT

The geographic expansion of Lumpy skin disease (LSD) from the near East into the European Union highlighted again the need for appropriate disease detection tools applicable to animal host populations where access to individual animals is difficult. This is of particular importance considering that the clinical manifestation of LSD is often mild making early disease detection challenging under the above-mentioned conditions. Building on positive experiences of group-level oral fluid sampling for pathogen detection as it is known to work for swine herds and wild boar, the concept was transferred to ruminants. Two groups of six cattle were infected experimentally with Lumpy skin disease virus (LSDV) under controlled conditions. Blood as well as oropharyngeal and nasal swab samples were collected at regular intervals. Group samples were obtained by placing cotton gauze around a salt lick block provided commonly as dietary supplement. Pieces of the gauze with visible signs of manipulation were tested in parallel to samples obtained from individual animals. Genome load analysis by qPCR technology revealed LSDV detection window starting from day 2 post infection until day 28 post infection, the end of the animal trial. At the individual level, detection periods varied between animals and type of sample and included intermittent detection. The accumulative character of the alternative sampling method makes it suitable to detect LSDV DNA at group-level even at times of the infection where a selective sampling of individuals from a group – as normally done in LSD surveillance – would have most likely failed in the detection.

1. Introduction

Lumpy skin disease (LSD) is an internationally notifiable disease in cattle, caused by the infection with lumpy skin disease virus (LSDV), a DNA virus of the genus *Capripoxvirus*. Its appearance remained confined to the African continent for many decades and its spread is believed to be propagated mainly through blood-feeding arthropods, yet many epidemiological features of the disease are still poorly understood (Tuppurainen and Oura, 2012). With the geographic expansion of the occurrence of LSD over the past decade (FAO, 2017), an increasing number of countries is faced with the need to establish appropriate surveillance methods for early disease detection in their cattle populations.

When addressing a disease like LSD, where passive surveillance based on clinical suspicion is expected to be insensitive and unspecific at the early stages of an epidemic due to a low number of animals with clinical disease manifestation, active surveillance using virological

methods is recommended (Tuppurainen et al., 2017). In populations where the access to the individual animal is not a particular challenge, the standard methods of obtaining blood or swab samples is considered suitable at different points of the infection (Babiuk et al., 2008). In populations however, where this access is not easily given, be it the extensive cattle rearing or potentially in wildlife populations, complementary methods are needed to complete the repertoire of sampling methods allowing resource efficient surveillance of epidemiological relevance. To overcome the shortage of timely information on the circulation of pathogens, oral fluid testing offers an opportunity to easily collect group-level pathogen data (Prickett and Zimmerman, 2010). Whilst the collection of oral fluid at group-level has been successfully tested for the detection of transboundary animal diseases (TAD) in domestic pigs (Dietze et al., 2017; Ramirez et al., 2012) and wild boar (Mouchantat et al., 2014), for ruminants this approach has not been explored.

In this study, we test the suitability of group-level, oral fluid testing

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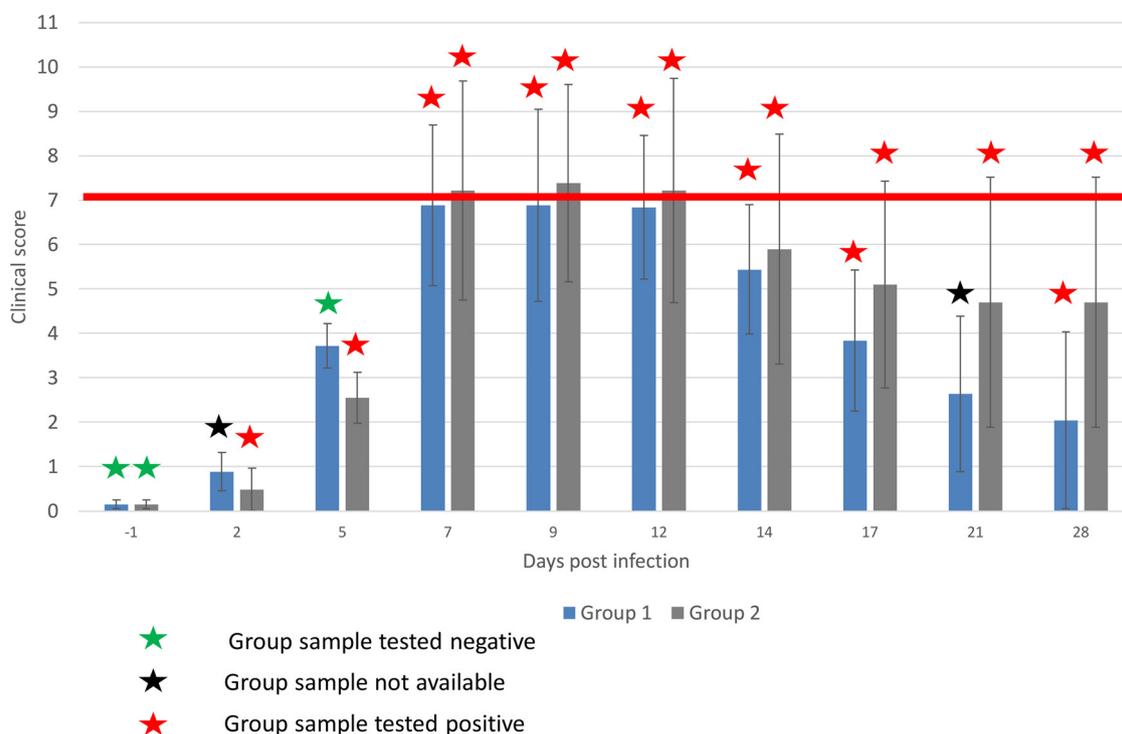


Fig. 1. Development of the clinical score at group-level through the course of the infection (mean values and standard deviation). The red line at the score level 7 indicates the threshold for scores of generalized disease characteristics. The stars depict the results of the capripoxvirus genome detection of the samples taken at group-level (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

for the detection of LSDV in ruminants through the course of an LSDV infection, comparing it with the detection of LSDV in samples obtained at individual animal level.

2. Materials and methods

2.1. Animal experiment

Twelve female Holstein cattle with a body weight of approximately 150 kg in an age of around six months were purchased from commercial breeders. They were separated in 2 groups of 6 animals each for management reasons and kept in a high containment isolation unit. Each animal was inoculated with 1 ml subcutaneous and 3 ml intravenous LSDV-Macedonia 2016 field strain isolated at the Friedrich-Loeffler-Institute, Germany, from skin nodules of infected animals (at 10^7 50% tissue culture infective dose per ml on Madin Darby Bovine Kidney (MDBK) -cells). Starting from day -1, all animals were examined daily until day 28 post infection (pi) following the clinical scoring scheme (Carn and Kitching, 1995). The scheme allowed a structured observation of the clinical course of the infection and to determine humane end points for the individual animal. Scores of seven and above indicate the generalized form of the disease, animals with clinical scores of 10 were euthanized. To comply with the biocontainment regulations of the research facility, all animals that survived until the end of the study were slaughtered by legitimated staff of the Friedrich-Loeffler-Institut.

2.2. Sampling

Sampling of the animals took place on days -1, 2, 5, 7, 9, 12, 14, 17, 21, and 28 pi. Blood samples were taken using blood collection tubes Kabevette G E772 (EDTA blood) and Kabevette G S792 (Serum) (Kaba Labortechnik) and stored at -70°C until further analysis. Oropharyngeal and nasal swab samples were taken in parallel to the blood samples while animals were restrained using Rayon®-swab (Copan Diagnostics). These were subsequently soaked in 4 ml of fetal

calf serum (FCS) - free cell culture medium (MEM: Minimum Essential Medium) and stored at -70°C until further analysis.

For the oral fluid sample collection at group-level, conventional salt lick blocks provided to the animals as part of the mineral supply were wrapped with one layer of cotton gauze (Cosmoplast® Fixierschlauch, Cosmoplast prime, Germany). The cotton gauze was removed for analysis at the day of sampling and replaced accordingly. The parts of cotton gauze that have been manipulated by the animals were soaked for 30 min in 5 ml of MEM. Two ml of the obtained medium-oral fluid solution were subsequently stored at -70°C until further analysis.

2.3. Sample analysis

Extraction of DNA from all obtained samples was performed using the NucleoMag Vet Kit (Macherey-Nagel GmbH) according to the manufacturer's instructions. Viral DNA, specific for capripox viruses, was detected through a quantitative real-time PCR following the protocol from Bowden et al. (2008) with the following modifications. The used pan-Capripox assay contains the same primers but a new TaqMan probe (Capri-P32-FAM-Taq: 5'-FAM-ATG GAT GGC TCA TAG ATT TCC TGA T-BHQ1-3'), instead of the original MGB probe. For the amplification, the PerfectA qPCR ToughMix (Quanta BioSciences) was used: 3 min at 95°C , 45 cycles (15 s at 95°C , 15 s at 60°C , 15 s at 72°C) on the CFX96 real-time PCR system (Bio-Rad Laboratories Inc.). The collection of the fluorescence data were performed in the annealing step. Cq values above 40 were regarded as negative.

Selected LSDV genome positive samples of the cotton gauze and oropharyngeal swabs were used in a virus isolation procedure. MDBK cells were seeded in T25 cell culture flasks (Corning®) using MEM with Earle's salts, Hanks' salts, nonessential amino acids and 10% FCS. The cells were incubated for 24 h at 37°C . Subsequently, the cell culture medium was removed, the cells were washed one time with 4 ml FCS-free medium and covered with further 4 ml FCS-free medium including antibiotics (penicillin and streptomycin). Afterwards 1 ml of the respective sample (cotton gauze or oropharyngeal swabs fluid) was added

to the cells. After the incubation of 1 h at 37 °C further 10 ml of medium with 10% FCS including antibiotics were added. After seven days, the cells were frozen at –80 °C and after thawing 1 ml of the cell culture was passaged. The passage procedure was repeated twice and a typical CPE was verified by qPCR.

3. Results

Each individual animal developed clinical signs of LSD but with differing degrees of severity. In each group, three animals developed a generalized form of LSD and one animal per group had to be euthanized before the scheduled end of the experiment. The course of the clinical signs summarized in the clinical score are summarized in Fig. 1.

In all animals, capripoxvirus-specific DNA was detected in blood samples as well as in nasal and oropharyngeal swab samples. The time window in which this detection was possible varied between the animals and for the different samples obtained. In blood samples, the earliest detection was possible at day 5 pi and the latest at day 21 pi. For the nasal and oropharyngeal swabs, the capripoxvirus genome was detected from day 2 pi until the end of the animal trial. The laboratory results for all the samples taken throughout the experiment per animal and at group-level are summarized in Table 1.

In both groups, the animals approached the salt lick block on a regular basis leaving visible signs of contact and manipulation on the cotton gauze (see Fig. 2). Observations by researchers and animal caretakers throughout the time of the study additionally confirmed that animals did not only had contact with the salt lick block through their tongue but also touched it with other facial parts when smelling and curiously examining it. For group one, on two sampling dates (days 2 and 21 pi) no samples were obtained since the animals managed to completely dismantle the cotton gauze from the salt lick block and no remains were found in the pen.

The cotton gauze samples that were obtained tested positive for DNA fragments specific for capripox viruses starting from day 2 pi throughout the experiment until day 28 pi except the sample from day 5 pi obtained from group 1. That particular sample was also missing clear visible signs of contact or manipulation.

For virus isolation, the earliest and strongest PCR-positive samples were chosen, to avoid interaction with potential subsequently developing neutralizing antibodies. From the original saliva sample, taken 7 days post challenge (Cq = 28.6), virus could be successfully propagated by passaging on MDBK cells. After the second passage, a strong cytopathic effect was visible and the successful replication of LSDV could be confirmed by qPCR (Cq = 24.3). In contrast, from the original LSDV genome positive cotton gauze sample, starting with a Cq-value of 25.8, virus isolation was not successful. The LSDV-DNA positive sample material was passaged three times on MDBK cells until no capripoxvirus specific DNA was detectable and no cytopathic effect was visible.

4. Discussion

With the ubiquitous application of modern and fast PCR-based pathogen detection methods for disease confirmation, early field detection of TADs remains one of the core bottlenecks in today's animal disease control. Surveillance options fit-for-purpose are therefore of eminent importance, and need to take into account the host population characteristics as well as the pathogen and disease specific pathogenesis.

Unlike for many other infectious diseases of international importance, the detection of LSDV genome in different matrices over the course of an infection is described with rather inconsistent time ranges (EFSA, 2015). Our study confirmed the described heterogeneous clinical course of the infection, viremic periods and shedding of virus (see Table 1). Combined with the moderate morbidity, the wide range of clinical appearances from asymptomatic to severely affected and the overall low mortality of LSD in the field, make early detection through the sampling of individual animals difficult. The success of risk based surveillance will depend on the selection of the right animals and sample specimen, a particular problem in populations where the access to selected individuals is challenging. Early detection of diseases in ruminants at herd or group-level is normally done either indirect using syndromic surveillance (Veldhuis et al., 2016) or often via bulk milk sampling (Velasova et al., 2017). Both approaches have proven to be suitable, but are limited to herds or animal groups where the underlying data-needs can be satisfied.

An approach taking parameters of low-access ruminant populations and LSD into account was therefore needed aiming to find suitable sampling methods for ruminants that do not require direct animal contact and ideally provide a pool sample. Nasal and oropharyngeal excretions have been described previously to provide a much wider time window for detection of LSDV genome than blood (EFSA, 2015) making them a preferred sample matrix. Salt lick blocks used for dietary supplementation of ruminants have earlier been described as potential source of infection for the individual animal due to the fact that all animals sharing the habitat have access to these blocks and can leave traces of infectious material on them (Kaneene et al., 2016). This study has turned this conclusion by actively using it for pathogen detection in oral and nasal excretions collected at salt lick blocks. The underlying idea for this study originated from preliminary results of field tests reported by Khomenko et al. (2013) and positive virological findings in an ad hoc sampling during the LSD epidemic in Bulgaria in 2016, where the described sampling technique was used in addition to the standard sampling approaches during the implementation of control measures (Alexandrov, personal communication).

The method for non-invasive sampling applied in this study served as prove of principle. Under controlled conditions capripoxvirus genome was detectable in the obtained group-level samples as early as through the conventional individual sampling methods and before any individual animal showed the generalized form of the disease (Fig. 1 and Table 1). The detectability lasted all the way through the

Table 1

Summary of qPCR results of all samples taken throughout the experiment. Results are listed as “number of qPCR positive samples / total number of samples” for the samples of individual animals. Colored boxes indicate sampling days and sample type where at least one sample was positive. DPI = days post infection; NS = nasal swab; OPS = Oropharyngeal swab.

DPI	Group 1					Group sample	Group 2				Group sample	
	Individual samples				Group sample		Individual samples					Group sample
	EDTA Blood	Serum	NS	OPS			EDTA Blood	Serum	NS	OPS		
-1	0/6	0/6	0/6	0/6	neg	0/6	0/6	0/6	0/6	neg		
2	0/6	0/6	2/6	1/6	no sample	0/6	0/6	0/6	1/6	pos		
5	3/6	3/6	0/6	0/6	neg	4/6	5/6	0/6	0/6	pos		
7	6/6	5/6	4/6	0/6	pos	5/6	6/6	2/6	4/6	pos		
9	6/6	5/6	3/6	4/6	pos	4/6	5/6	5/6	4/6	pos		
12	3/5	1/5	3/5	3/5	pos	4/6	4/6	6/6	6/6	pos		
14	2/5	1/5	5/5	5/5	pos	3/5	3/5	5/5	5/5	pos		
17	1/5	1/5	4/5	4/5	pos	3/5	1/5	5/5	5/5	pos		
21	0/5	1/5	5/5	5/5	no sample	1/5	1/5	4/5	4/5	pos		
28	0/5	0/5	3/5	3/5	pos	0/5	0/5	5/5	5/5	pos		

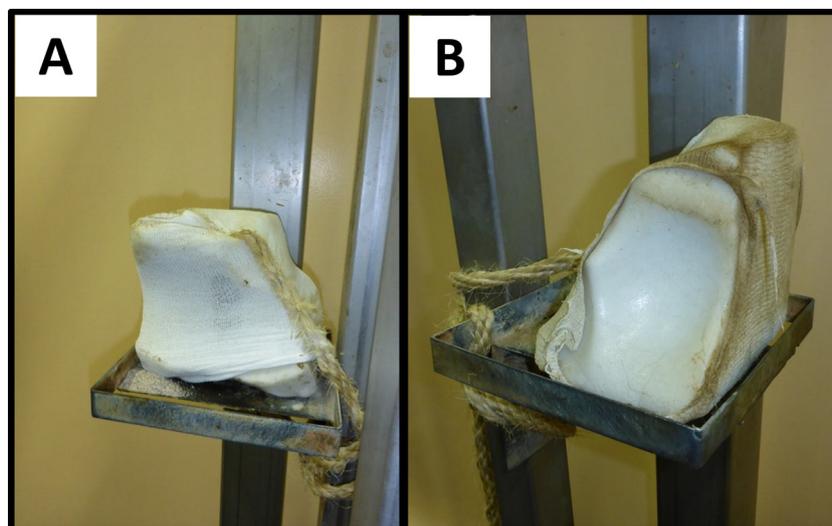


Fig. 2. Salt lick block covered with cotton gauze used to obtain group-level samples. Installation secured with a rope to avoid dismantling. A: before being approached by cattle. B: after being approached by cattle with visible signs of manipulation.

experiment. The observed behavior of the animals approaching the salt lick blocks stresses the fact that the obtained samples should not be considered purely oral fluid but can have components of nasal and ocular secretions or skin abrasions, hence are mixed matrix samples. Adding the fact that the obtained samples are cumulative samples over several days, the wide diagnostic time window that was achieved can be explained. In particular on day 5 pi, where no swab sample of individual animals tested positive, the weight of this characteristic leading to improved group sensitivity becomes evident. The same counts for the later days of the experiment (e.g. day 28 pi in group 1), where next to the group-level sample not all individual swab samples tested positive for LSDV genome. In how far the detectable capripoxvirus genome at these later stages of the experiment has been conserved in the salt lick block since earlier sampling dates was not verified, as it is of subordinated relevance in the context of a detection tool where a cumulative or conserving effect is considered beneficial.

A downside of the methodology, as shown in group one, is that it currently comes along with an uncertainty to obtain a sample. This can be optimized by better securing the cotton gauze and maybe adding an extra layer. Additional studies on the frequency these salt lick blocks are approached by the animals under field conditions must provide information on the timeframe the gauze must be in place in order to obtain a representative herd sample. In our preliminary steps aiming for virus isolation from a cotton-gauze sample, the potential to act as source of infection, as postulated by Kaneene et al. (2016) could not be confirmed within the applied setting. Whilst it was possible to isolate LSDV from PCR-positive samples obtained from individual animals, this was not successful in the sample obtained from a PCR-positive cotton-gauze piece. At this stage it can only be speculated, but most likely, the combination of a saturated salt solution that undergoes drying and re-moistening over the course of time before the sample is collected, is at least not favoring LSDV survival. A study specifically targeting the tenacity of LSDV under these particular conditions would be needed though to provide solid scientific data on this issue. The importance to develop fit-for-purpose tools for the surveillance of some of the major TADs that can affect ruminants became once more evident with the incursion of LSD in the European Union. This is underlined by the fact that initial LSD outbreaks in most European countries were detected in extensive grazing systems often in remote areas. For these host populations with limited access to the individual animal, sample acquisition will to some extent rely on non-invasive group-level testing.

5. Conclusions

It can be concluded that under experimental conditions, the described “salt lickblock sampling” technique has confirmed its suitability in principle to detect circulating LSDV. This can potentially lead to easier and cheaper field sample collection leading to overall more cost-effective surveillance in specific target populations and allow quicker response due to improved timeliness in disease detection after initial incursion.

Ethics and consent to participate

The infection experiment was approved by the competent authority, the State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Wester Pomerania, Rostock, Germany. The respective reference number was LALF M-V/TSD/7221.3-1-061/16.

Consent to publish

Not applicable

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Competing interests

The authors declare that they do not have competing interests

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