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Posted Date: 4 February 2024

doi: 10.20944/preprints202402.0183.v1

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 doi:10.20944/preprints202402.0183.v1

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Susceptibility of Mediterranean Buffalo after Experimental Infection with Lumpy Skin Disease Virus

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Abstract: Lumpy skin disease (LSD) is a viral disease of cattle and water buffalo characterized by cutaneous nodules, biphasic fever and lymphadenitis. LSD is endemic in Africa and Middle East but in recent years has spread in different Asian countries. Drop of animal productions, vaccination campaign, surveillance costs and trade restrictions have significant impact on the economy of affected countries. The disease is well characterized in cattle while little is known in buffaloes and no experimental studies have been conducted in this species. Six buffaloes and two cattle were inoculated with an Albanian LSD virus (LSDV) field strain. Over a period of 42 days animals were clinically monitored and sampled for serological and virological analysis. Only two buffaloes showed clinical sign, fever, skin nodules and lymphadenitis. All samples tested in real time PCR (blood, swabs, biopsies and organ) gave negative results. Three buffaloes seroconverted at day 39 and 42 post-inoculation, detected by ELISA, but all sera were negative in serum neutralization test. Cattle showed more severe clinical signs. Moreover, viraemia and virus shedding were proven by positive PCR results and antibodies were detected by both ELISA and serum neutralization test. These findings demonstrated the limited susceptibility of buffaloes compared to cattle once experimental infected with LSDV. Further studies are needed to investigate the epidemiological role of the buffaloes.

Keywords: lumpy skin disease; buffalo; ELISA; real time PCR; SN test

1. Introduction

Lumpy skin disease (LSD) is a viral disease of cattle and water buffalo characterized by multifocal cutaneous nodules, biphasic fever, oral and nasal discharge, accompanied by lymphadenitis. Clinical infections range from sub-clinical through mild to acute, and are often influenced by the age, breed, immune status and production period of animals [1]. LSD is associated with moderate-to-high morbidity and low mortality. Despite that, the disease has a high socio-economic impact, resulting from the decrease in milk production, decreased weight gain, permanent damage to hides, reduced reproduction due to increased infertility and abortion, costs related to surveillance activity and serious trade restrictions [2–4]. For these reasons, the Animal Health Law (Regulation (EU) 2016/429), classified the LSD in the list of diseases belonging to Category A. LSDV is subject to early notification and reporting throughout the European Union Countries, and its prevention and control measures are governed by the Regulation (EU) 2016/429 and supplemented by Delegated Regulation 2020/687 and Implementing Regulation 2021/1070.

 doi:10.20944/preprints202402.0183.v1

The disease is caused by the lumpy skin disease virus (LSDV) which belongs to the family *Poxviridae,* genus *Capripoxvirus,* together with sheeppox virus (SPPV) and goatpoxvirus (GTPV). Transmission of LSDV occurs mainly mechanically via blood-feeding insects and ticks. In addition, direct and indirect transmission via common use of feeders or drinking troughs by infected cattle [5] as well as seminal fluid [6] has been reported. LSD diagnosis is primarily based on the clinical diagnosis of LSD, confirmed by the PCR analysis of lesion crusts or biopsies of the nodules [7–9]. However, LSDV infection is not always apparent, and mild and subclinical disease occurs, also confirmed by cattle experimentally infected, when up to 50% of animals remain uninfected or sub-clinically infected [10,11]. The incubation period in field conditions varies from 2 to 4 weeks, while in experimental disease from 4 to 14 days [12,13]. The first skin lesions appear at the inoculation site after 4-20 days. In the acute form, animals develop a biphasic febrile reaction that may exceed 41°C and lasts from 4 to 14 days. This is accompanied by depression, reluctance to move, inappetence, and sialorrhea, nasal discharge from mucous to purulent and tearing. Lymph nodes are enlarged, especially pre-scapular and pre-crurarl [12–14].

LSD is endemic in most of the African continent and in recent years has spread throughout the Middle East, including Turkey. In 2015 it arrived in Europe, in particular in Greece where more than 100 outbreaks were reported [15]. In 2016, cases were also reported in Bulgaria, Serbia, the Former Yugoslav Republic of Macedonia (FYROM) and Albania [16]. Thanks to mass vaccination campaigns with homologous LSD vaccines in the infected countries of south-eastern Europe as well as in neighbouring countries (Bosnia and Herzegovina, Croatia), the spread of the disease was contained, and no LSD cases have been reported since 2017. LSD outbreaks were reported in Armenia in 2015, Georgia, and Kazakhstan in 2016, later in 2019 and 2020 in Israel, Russia, Saudi Arabia and Syria. Between July and August 2019 LSDV was introduced in Asia affecting Bangladesh, China and India and in 2020 it was reported in Bhutan, Hong Kong, Myanmar, Nepal, Sri Lanka, Taiwan and Vietnam [17]. To date, no LSDV outbreaks have been reported in Italy.

In Italy the number of buffalo breedings and animals are constantly increasing, resulting the country with the largest number of buffaloes reared in the EU. The buffalo breeding represents an important economic reality, with increasing potential. Due to the triple aptitude of the buffalo, for milk, meat and work, in the last 10 years its breeding increased almost around 50%. In particular in the central and south regions of Italy, such as Campania, Lazio and Molise, it is mainly raised for the production of milk, PDO mozzarella (EEC 2081/92) and ricotta. The demand for buffalo meat has also increased in recent years thanks to the increasingly appreciated organoleptic properties.

Under field condition, LSDV infection in water buffalo is a controversial matter. Some studies described isolation of LSDV from skin lesions in buffalo [18,19]. Previously, Davies (1991)[20] reported that African buffalo (*Syncerus caffer*) and Asian water buffalo (*Bubalus bubalis*) did not show lesions in the field during LSD outbreaks though they had seroconverted. A recent field study reported that blood and skin biopsy samples collected from buffaloes in outbreaks in Egypt were negative for the presence of LSDV [9].

To date, there are some descriptive articles on buffaloes that confirm their sensitivity to LSD [9,21,22] but, there is only a field study on buffaloes naturally infected by LSDV [23]. No experimental trials are reported. The susceptibility of water buffalo to lumpy skin disease virus (LSDV) and their role in spreading the disease are unclear, so further studies are needed to fill these gaps

The aims of this study were to determine the susceptibility of buffaloes to LSDV infection and to describe the clinical, virological and serological responses of water buffaloes after the LSDV infection.

2. Materials and Methods

2.1. Animals

A group of eight Mediterranean buffaloes (*Bubalus bubalis*), 5 males and 3 females, and two cattle used as positive control, were included in the study. All animals were between seven to eleven months old. The animals were consecutively numbered from 1 to 10 and maintained in the high-

 doi:10.20944/preprints202402.0183.v1

containment animal facilities (Insect proof-establishment) of the Institute Zooprofilattico Sperimentale G. Caporale, Teramo, Italy, and housed with a 12-hourly light-dark cycle, temperature between 10°C and 25°C, relative humidity of 40% to 70%. Animals were fed concentrated rations twice daily and given *ad libitum* access to hay and water. Environmental enrichment was provided, including rubber toys and a hollow ball stuffed with hay.

The animals, sourced from a commercial herd, were confirmed as negative for Bovine Viral Diarrhea Virus, Parainfluenza type 3 virus, Bovine Adenovirus, Bovine herpesvirus-4, Bovine herpesvirus-1, Bluetongue, *Chlamydia psittaci* and *Coxiella burnetii* prior to study commencement.

To detect any possible dipteran presence, indoor blood-feeding insect UV light traps and sticky traps were mounted at regular intervals on the walls of the high-containment animal facilities.

The respective experimental protocols were reviewed by the state ethics commission (OPBA) and approved by Italian Ministry of Health. The experimental procedures were conducted according to the Law decree n. 26, art. 31, 2014, and they were approved by the Minister of Health (n.722 of 13/07/2020).

2.2. Experimental infection

LSDV inoculation stock was obtained at "Istituto Zooprofilattico Sperimentaledell'Abruzzo e del Molise G. Caporale" by five consecutive passages on MDBK cells of the LSDV field strain named 7416/5, isolated from a symptomatic calf skin nodule, as described by Babiuk et al. (2008) [7]. The sample was collected during an outbreak occurred in Albania in 2017 and was kindly provided by Dr. LediPite and Dr. Liljana Cara working at the Food Safety and Veterinary Institute (FSVI) in Tirana. Six of the 8 buffaloes (nos 3 to 8) were randomly assigned to inoculated group, the remaining 2 buffaloes (nos 1-2) were used as control group, while the two cattle (nos 9-10) were used as positive control. The six buffaloes, from number 3 to 8, were inoculated intravenously into the jugular vein with 5 ml of LSDV field strain suspension with titre of $10^{5.8}$ $TCID_{50}$/ml, and with 1 ml injected intra dermally in 2 sites on each side of the neck (0.25 ml in each site). The same procedure and the same LSDV suspension were used to inoculate two calves used as positive control. The remaining two buffaloes were mock inoculated with the same amount of supernatant of LSDV negative cell culture using the same procedure. All animals were examined daily for clinical signs, in particular fever, anorexia, depression, lesions, including cutaneous nodules, and lymphadenopathy, for 42 days.

2.3. Clinical observation

Buffaloes and calves were clinically monitored daily during the entire trial. Body temperature was registered each day and scored as fever if ≥ 39.5°C for at least 2 days. Others observations were collected (Table 1) and used to calculate a cumulative clinical scores [11,24].

Table 1. Clinical scoring system. The animals were clinically evaluated daily and classified using the scoring system in this table (adapted from Aerts et al., 2021)[24].

General Health Status		Food intake		Nasal Discharge		Number of Nodules		Dissemination of Nodules		Lymphadenomegaly	
Normal	0	Normal	0	Normal	0	No Nodules	0	No Nodules	0	No Lymphadenomegaly	0
Mild Illness	1	Slightly Decreased	1	Mild	1	≤ 10	1	Localized	1	Localized	1
		Decreased	2	Marked	2	<20	2				

 doi:10.20944/preprints202402.0183.v1

				Mucous							
Severe Illness	2	Does Not eat	3	Purulent	3	≥20	3	Generalized	2	Generalized	2

2.4. Samples

For molecular analysis, EDTA blood samples were collected from all animals from day 2 post-inoculation (p.i.) to day 22 p.i., and oral, nasal, ocular swabs were collected daily from day 2 p.i.to day 14 p.i. and then every 3-4 days until the end of the experiment. For serological analysis, serum samples were collected every 3-4 days from day 3p.i. until the end of the study.

Skin biopsies were carried out on three buffaloes (nos 3, 6 and 8) and cattle n.10 at different time. On day 13 a skin biopsy was collected from buffalo 6 from a nodule at the inoculation site. On buffalo no. 3, six biopsies were taken on day 16, 22, 23, and 25 from nodules present in different parts of the body (neck, thorax, dewlap, inoculum site, and intermandibolar zone). On buffalo no. 8, skin biopsies were taken from one nodule at inoculation site on day 16 and another one at the tip of the right shoulder on day 30. From cattle no. 10, skin biopsy was collected from a nodule on the neck on day 7. Hair was removed from the biopsy sites with electric clippers and cleaned with skin wipes containing 2% chlorhexidine in 70% alcohol (Clinell, GAMA Healthcare); 2.5 ml of lignocaine (Lidocaine Hydrochloride injection 2%, Hameln Pharmaceuticals) was injected subcutaneously, and after 10 minutes, a 0.8 cm punch biopsy was taken using a disposable biopsy punch (Integra Miltex). Half of the biopsy tissue was placed into 10% sterile buffered formalin (Merck) for a minimum of 48 hours. The remaining tissue was tested in real time PCR [8]. Animals were sedated with guaifenesin 80 mg/Kg, rompum 50 mg/Kg (xylazine hydrochloride 23,32 mg/ml, Bayer AG 51368 Leverkusen, Germany), were anesthetized with pentothal sodium 7-13 mg/kg (tiopentalesodico MSD Animal Health S.r.l.) and then euthanized using Tanax T-61 (Mebezonium iodide 50,00 mg/ml, Embutramide 200,00 mg/ml, Tetracaine 5,00 mg/ml, MSD Animal Health S.r.l.). During necropsy, a panel of organs and tissues (spleen, liver, kidney, tonsils, skin, lymph nodes, heart, rumen, abomasum, ileum, testicles, ovaries, nasal mucosa, and tongue) was collected and analyzed using the pan-capripox real-time qPCR [8].

2.5. Serologicalexamination

Serological analysis was carried out by commercially available ID Screen Capripox Double Antigen ELISA (ID.vet, Montepellier, France) and SN test. The ID Screen Capripox ELISA was performed according to the manufacturer's instructions. Samples with an S/P% ratio of ≥ 30% were considered positive.

For the detection of neutralizing antibodies, an LSDV specific SN test based on modified protocol of WOAH was performed [25,26]. For this purpose, test serum samples were inactivated at 56°C for 30 minutes, and log2 dilution series in serum-free minimal essential medium from 1:5 to 1:640 were prepared in duplicate using a 96-well format (50µl of each serum dilution/well), in order to be titrated against a constant titre of 100 $TCID_{50}$ in 50µl of LSDV Neethling strain. Microtiter plates with serum-virus suspension were incubated for 2 hours at 37°C in 5% CO2 and after incubation 100µL of MDBK cell suspension was added to each well. Plates were observed daily using an inverted microscope (20-40X Leica DFC425 C, Leica Microsystem Ltd.) to evaluate the presence of virus–specific cytopathic effect (CPE), and after 4 days at 37°C in 5% CO_2 the titre was determined. Wells were scored as positive for neutralisation of virus if 100% of the cell monolayer is intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in half of the test wells is the 50% end-point titre of that serum. A titre of 1:10 or greater was considered to be positive.

 doi:10.20944/preprints202402.0183.v1

2.6. DNA extraction and molecular analysis

Organ samples and skin biopsies were homogenized in PBS plus antibiotics (10^6IU/L penicillin, 10 g/L streptomycin, 5 × 10^6 IU/L nystatin, and 125 mg/L gentamicin, IZSAM) using a TissueLyser II tissue homogenizer (QIAGEN, Hilden, Germany). Nasal, oral, ocular and rectal swabs were frozen at -80°C and thawed 3 times before being tested. DNA was extracted from homogenized organ and biopsy samples, EDTA blood and swab samples using BioSprint 96 One-For-All Vet Kit (Indical Bioscience, Leipzig, Germany) following the manufacturer's instruction. Subsequently, all samples were tested using the pan-capripox real-time qPCR [8].

3. Results

3.1. Clinical observation

Six water buffaloes and two cattle were inoculated with an LSDV field strain while two buffaloes were inoculated with placebo. All the animals were monitored for clinical signs (Table 2; Figure 1) and viraemia over 42 days. Two mock inoculated animals did not show any clinical signs. Instead, buffaloes nos 4, 5, 6 had enlarged prescapular and prefemoral lymph nodes from day 5 to day 8, but they did not developed any other clinical sign apart from nodules at inoculation sites. Buffaloes nos 3 and 8 developed fever on day 14 and on day 27, respectively, associated with a decrease of food intake, and generalized lymphadenomegaly. After 24 hours from inoculation, small nodules appeared on both sides of their neck and spread to the whole body (neck, legs, back and flanks) on the following days. Buffalo no. 3 developed well-circumscribed cutaneous nodules which reduced in size and disappeared by day 30 onward. Nodules in buffalo no. 8 were well-circumscribed, and some nodules start to reduce in size by day 37.

In the challenged control group, typical clinical signs of LSD were observed from day 4 p.i.: calves showed pyrexia, enlargement of superficial lymph nodes, appearance of skin nodules at inoculation site, inappetence (Figure 1; Table 2). Calve no. 9 developed fever on day 7 and, while calve no. 10 had a fever from day 4 (39.7 °C) to day 8 (39.5°C) and then increased again until day 10 (40.4°C) when the animal was humanely euthanized due to severe clinical signs and to avoid unnecessary suffering.

Table 2. Clinical findings and viremia in six water buffaloes and two cattle used as positive control group, after experimental inoculation with a field strain of LSDV.

Clinical finding	Inoculated animals							
	Buffalo						Cattle	
	3	4	5	6	7	8	9	10*
Fever	39.8-40.5°C for 6 days	-	-	-	-	39.5°C for 2 days	39.5°C for 2 days	39.5-40.4 for 7 days **
Decreased food intake	For 2 days	-	-	-	-	For 2 days	For 2 days	For 4 days
Nodules								
Number	>20	2	4	4	3	12	4	>20
Size (ø)	0.5-5.0 cm	0.2-0.5 cm	0.5-2.0 cm	0.5-2.5 cm	0.2-2.0 cm	0.5-3.5 cm	2.0-6.5 cm	2.0-7.0 cm
Location	Generalized	Inoculation sites	Inoculation sites	Inoculation sites	Inoculation sites	Generalized	Inoculation sites	Generalized
Lymphadenomegaly	Generalized	Prescapular and prefemoral	Prescapular and prefemoral	Prescapular and prefemoral	Not detected	Generalized	Generalized	Generalized
Oedema	ND	ND	ND	ND	ND	ND	ND	Dewlap
Nasal and ocular discharge	ND	ND	ND	ND	ND	ND	From mucopurulent to	From mucopurulent to

 doi:10.20944/preprints202402.0183.v1

Viremia	ND	ND	ND	ND	ND	ND	serous; bilateral From day 9 (Ct 28.54) to day 14 (ct 38.40)	serous; bilateral From day 5 (Ct 35.57) to day 9 (Ct 32.97)*

*Cattle was euthanized on day 10 p.i. **Fever had a biphasic course: on day 4 was 39.7 °C rose to 40.2 on day 7, decreased to 39.5 on day 8 and then increased again from day 9 to day 10, when it was 40.4°C.

Clinical score was recorded daily after the infection to measure the severity of LSDV infection (Figure 1). All the animals started from score 0 on the day of the inoculation. The difference in clinical scores between the two buffaloes with clinical signs and the other 4 animals without was evident from day 14 to day 30, when the score increased in buffaloes nos 3 and 8 up to 10 and then decreased. The other buffaloes had a clinical score ranging between 0 and 5, while calves of positive control group had a clinical score between 9 and 14 (from day 6 to day 9 and then start to decrease).

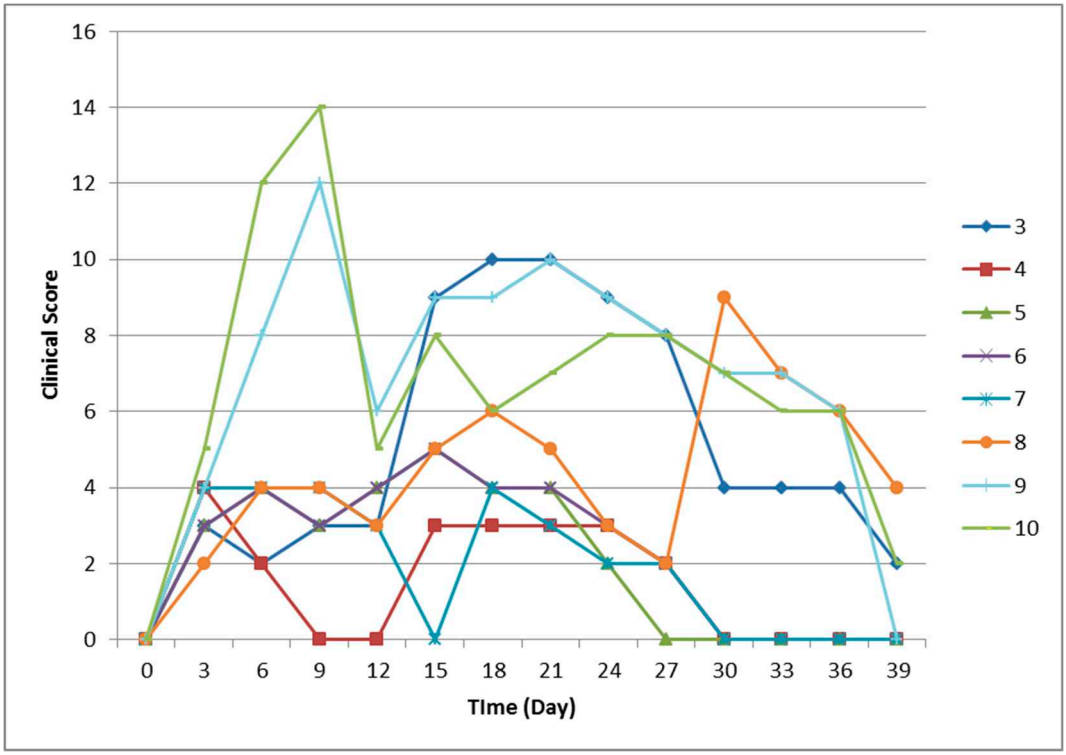

Figure 1. Clinical scores of six water buffaloes and two cattle infected with LSDV strain. Buffaloes naïve to the virus were infected on Day 0, and clinical scores were assessed every 3 days.

3.2. Serological analysis

Serological analysis were conducted using ELISA and the SN test to assess the seroconversion. On the day of the inoculation (D0), all animals were sero-negative. Buffaloes inoculated with placebo remained serologically negative during the 42 days of the experiment. The two buffaloes with characteristic clinical signs resulted positive in ELISA on day 39 (no. 3, S/P 38%), 25 days after the onset of fever, and on day 42 (no. 8, S/P 30%), 15 days after feverish rise, and remained positive to the end of the study. Buffalo no. 6 seroconverted on day 42 (S/P 32%). The other inoculated buffaloes did not show any serological response during the whole study. None of the infected buffaloes developed neutralizing antibodies against LSDV. Calf no.9, of the positive control group, seroconverted from day 31p.i., and remained ELISA positive until the end of the study. The calf developed neutralizing antibodies from day 14 (1:80) till the end of the trial. Neutralization titres peaked on day 22p.i.(1:320) and remained strongly positive until the time of euthanasia, confirming the development of neutralizing antibodies against LSDV.

 doi:10.20944/preprints202402.0183.v1

3.3. Molecular analysis

No viraemia was detectable in any buffalo, at any time during the trial, even in febrile animals. Viral DNA was not found in the skin biopsies, swabs and organs of the challenged animals (Table 3). On the contrary, viral DNA was found in blood sample of the cattle no. 9 from day 9 to day 14 with a Ct value between 28.54 and 38.4, while in cattle no. 10 viraemia was detected from day 5 (Ct 35.57) to day 9 (Ct 32.97). Real-time PCR were performed on nasal, oral and conjunctival swabs at different times (Table 4).After 42 days viral DNA was found in prescapular lymph nodes of calf no. 9 and in all organs and in the skin biopsy collected from calf no.10, that was euthanized on day 10, by real time PCR (Table 3).

Table 3. Detection of LSDV by real-time PCR in buffalo and cattle tissues and swabs, adapted from Babiuk et al., 2008 [7].

	Inoculated Animals							
	Buffalo						**Cattle**	
Tissue	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Skin	ND	ND	ND	ND	ND	ND	ND	Ct 19.63
Skin nodule	NDb	a	a	ND	a	NDb	a	Ct 20.39 Ct 19.39 and 18.47c
Spleen	ND	ND	ND	ND	ND	ND	ND	Ct 29.14
Kidney	ND	ND	ND	ND	ND	ND	ND	Ct 29.84
Lung	ND	ND	ND	ND	ND	ND	ND	Ct 29.02
Liver	ND	ND	ND	ND	ND	ND	ND	Ct 30.49
Bronchial LN	ND	ND	ND	ND	ND	ND	ND	Ct 29.58
Inguinal LN	ND	ND	ND	ND	ND	ND	ND	Ct 24.11
Mesenteric LN	ND	ND	ND	ND	ND	ND	ND	Ct 28.44
Right prescapolar LN	ND	ND	ND	ND	ND	ND	Ct 35.11	Ct 24.70
Left prescapolar LN	a	a	a	a	a	a	ND	Ct 25.70
Submandibolar LN	ND	ND	ND	ND	ND	ND	ND	Ct 23.63
Rumen	ND	ND	ND	ND	ND	ND	ND	Ct 28.43
Nasal mucosa	ND	ND	ND	ND	ND	ND	ND	Ct 18.73d

LN, lymph node; ND, not detected in real time PCR. [a]No sample was taken. [b]The animal had clinical signs of LSD and the sampled skin nodule was negative real-time PCR. [c]Nodule located on the back and on the scrotum respectively. [d]Nodule located on the nasal mucosa.

Table 4. Detection of LSDV in swab samples by real-time PCR in experimental infected buffaloes and cattle.

		Day post inoculation												
Sample	**Animal**	**2**	**3**	**4**	**5**	**7**	**9**	**14**	**17**	**21**	**24**	**31**	**35**	**38**
Nasal swab	Buffalo 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

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	Buffalo 4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Calf 9	ND	ND	ND	ND	ND	ND	ND	**37.12**	**37.42**	**39.44**	**35.92**	**37.16**	ND
	Calf 10	ND	ND	ND	ND	**34.04**	**31.97**							
Oral swab	Buffalo 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Calf 9	ND	ND	ND	ND	ND	**37.45**	ND	**38.45**	**36.83**	ND	**34.78**	ND	ND
	Calf 10	ND	ND	ND	ND	**37.37**	**37.97**							
Conj[b] swab	Buffalo 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Buffalo 7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

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Buffalo 8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Calf 9	ND	ND	ND	ND	ND	ND	ND	ND	**36.93**	ND	ND	**38.45**	ND
Calf 10	ND	ND	ND	ND	ND	**37.05**							

ND indicates the absence of detectable DNA. [a]Oneach sampling day, real-time PCR data are shown ct value was reported for each sample; [b]Conjunctival.

4. Discussion

There is little evidence of the susceptibility of water buffaloes to the LSDV infection as well as their epidemiological role in course of outbreaks. In order to fill these gaps 6 Mediterranean buffaloes were experimentally inoculated with LSDV isolated from an Albanian calf in 2016 to monitor the clinical, serological and virological response evoked by the infection.

As previously described in other experimentally infected cattle, the intravenous inoculation route was used because current data indicate this route to be the most effective to produce severe generalized disease [4,13]. In addition, intradermal inoculation was also selected in order to reproduce the natural route of infection.

The total clinical score of the LSD clinically diseased buffaloes reached 9-10, while the score for animals without clinical signs did not surpass 5, which is a clinical evolution comparable to the patterns described previously [42,43]. It is important to note that the same inoculum evoked an important clinical response when used to infect cattle [27].

The susceptibility of buffaloes to develop the typical clinical symptoms following LSDV infection is controversial. Ahmed and Dessouki (2013) [35] reported LSD in cattle while water buffaloes from the same affected area appeared clinically healthy. On the contrary, other papers reported the evidence of natural infection with LSDV in Egyptian buffaloes [36]. In recent years, anobservational study conducted on buffaloes during an outbreak in Egypt, confirm their low susceptibility to this virus [37]. Researchers hypothesized that the reason is that buffalo have thick skin, and the mouthparts of blood sucking insects such as mosquitoes, flies and ticks cannot easily pass through the skin of buffalo, so the transmission rate and susceptibility of this disease are low [23,38]. In the current experiment the virus was injected in the skin and blood of the buffaloes, nevertheless only two out of six animals developed a mild generalized disease with the appearance of small skin nodules resembling the “Neethling disease” often observed after the use of attenuated vaccines[39].

The diagnosis of LSD is mainly based on clinical surveillance, confirmed by laboratory test on the biopsies of nodules or blood samples[33,40]. Identification of infected subclinical animals remains difficult as nodules are absent and viraemia is short or intermittent making it difficult to identify the virus in the blood [41]. The assumption is particularly true in buffaloes which develops LSD mainly in the subclinical form [33] with mild or absent clinical signs as confirmed in our trial.

One of the most interesting outcomes of the trial is the lack of detection of LSDV in any sample collected from the infected buffaloes regardless the development of clinical signs. The presence of the virus in nasal and ocular discharges in infected animals and the amount of virus shed by infected animals is key to virus spread. Clinical signs and molecular results obtained in this trial, suggest that buffalo did not shed the virus making us assume they do not have a preminent role in the spread of the disease.

This data agrees with what was recently reported in a work by Elhaig et al. (2021)[28], during Egyptian outbreaks between 2016 and 2018, in which all collected blood and biopsy samples were negative in real time PCR, but the presence of antibodies confirmed LSDV infection in the animals. Such findings strengthen the hypothesis of the buffalo’s resistance to LSD and their role as an accidental non-adapted host [33,34].

 doi:10.20944/preprints202402.0183.v1

LSDV DNA was detected neither in the blood nor in the skin nodules collected from the buffalos with generalized lesions or in the animals with local skin reactions in the inoculation sites. It suggests the development of a weak viraemia and a scarce distribution of the virus in the peripheral sites once inoculated in this species. Whether the virus is promptly cleared from the blood circulation and/or not capable to replicate efficiently to evoke the severe generalized form of the disease is hard to say and deserve further studies to investigate LDSV pathogenicity in buffaloes.

The limited susceptibility of water buffalo against LSD was also confirmed by the different evolution of the infection in the challenged *vs* the positive control group. In the challenged group only two buffaloes showed mild clinical signs (fever, skin nodules) that have totally regressed at the end of the trial. On the contrary, the same inoculum evoked a severe disease leading to the euthanasia of cattle n.10 to avoid the suffering of the animal. Both animals developed a detectable viraemia, eliminated LSDV through nasal and ocular discharge and displayed a peripheral distribution of the virus in the skin nodules detected by PCR.

The cattle infected with the field LSDV strain developed severe clinical signs proving the efficacy of the experimental infection procedure.

Seroconversion was detected in 3 out of 6 challenged buffaloes between 39 and 42 days p.i. only by ELISA test. The short duration of the trial and the late development of the antibody response have probably prevented to record the seroconversion of the remaining animals. However, the immune response to LSDV infection is predominantly cell-mediated [35] and the scarcity of humoral immune response in buffaloes following capripoxvirus infection has been described also in field condition. A study conducted in Turkey [29] reported a low seroprevalence (7.6%) in buffaloes vaccinated against LSDV with heterologous sheep-goat pox vaccine, using a commercial ELISA.

Nevertheless, the ELISA test confirmed its sensitivity in the early detection of anti-capripoxvirus antibodies [7,9,13] compared to the neutralization assay. In fact, virus neutralization test is the most specific serological method, but lacks sensitivity [30–32].None of our buffaloes developed neutralizing antibodies during the experimental trial. Whether the lack of circulating neutralising antibodies is attributable to the short duration of the trial or to the immunological response of buffaloes to LSDV infection is difficult to say.

However, the poor neutralizing response to LSDV in this species is not a novelty being reported by several authors. Elhaig et al. (2017) [33] did not detect neutralizing antibodies in naturally infected buffaloes with clinical LSD lesions. In a previous work of Fagbo and colleagues conducted on buffalo sera collected (2014)[21] during an inter-epidemic period, the SN test results confirm only partially the results obtained by the ELISA and withlow neutralizing titres were limited to two buffaloes (1:20).

On the contrary, a different scenario occurred for the surviving bovine, with the detection of neutralizing antibodies from day 14 p.i. followed by positive ELISA results seventeen days later (day 31 p.i.).

We should probably consider that the sensitivity of the ELISA may differ between animal species, as already reported [7].In recent experimental study on bulls, the authors reported seroconvertion after 42 days [10], confirming what observed in our trial.

If Water buffaloes' limited susceptibility to LSD infection, could be ascribed to specific genetic variants[48] as for tuberculosis, mastitis or foot-and-mouth disease [44–47], is difficult to say but the hypothesis deserves to be further explained in future studies.

5. Conclusions

LSD is a cross-border disease characterised by severe economic losses that continues to spread worldwide. Real time PCR is the method of choice for a rapid routine diagnosis in cases of suspicion, however, in species not particularly susceptible to infection such as buffalo it may also be useful to combine genome detection with serological tests to detect any previous contact with the virus. The present study addresses the hypothesis that water buffaloes are less susceptible to the LSDV infection under experimental conditions compared to cattle and that diseased buffaloes may develop mild clinical signs of the disease.

Author Contributions: Conceptualization, F.M. and D.M.; methodology, F.M..; software, E.D.F., C.P.; validation, M.D.V., F.M. and E. R.; formal analysis, G.D.D..; investigation, E.D.F., C.P., F.I., A.T., A.G., E.M., G.D.T.; resources, F.M.; data curation, C.P., E.D.F., G.D.D.; writing—original draft preparation, E.D.F. G.D.D.; writing—review and editing, E.D.F., G.D.D., F.M., G.F.R, M.T.M.; visualization, E.D.F.; supervision, F.M., D.M.; project administration, D.M.; funding acquisition, F.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Ministry of Health, grant number IZSAM 07/15 RC.

Acknowledgments: The authors would like to thank to professor David Wallace (Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, P/Bag X4, Pretoria 0110, South Africa) for his support in the drafting of the experimental protocol and to all the technicians that with their work have allowed the realization of this experimental study taking care of the animals used.

Conflicts of Interest: The authors declare no conflict of interest.

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