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RESEARCH ARTICLE

Detection and Phylogenetic Analysis of B2L Gene of *ORF virus* from Clinical Cases of Sheep in Serbia

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ABSTRACT

Infection of sheep by the ORF virus (ORFV) is very common in Serbia. ORFV is an economically important viral disease, distributed worldwide. Phylogenetic analysis based on the B2L gene of Serbian ORFV strains from two outbreaks that occurred in Serbia in 2016 is presented in this paper. Crust formation around the lips, nostrils, and udder was noted in all animals from the first outbreak, whilst in the second outbreak, all animals showed swollen and cyanotic lips and muzzle, with no visible crusts. Virus isolation was conducted using Vero cells. Cytopathic effects were evident on the third passage. However, all examined samples were positive using PCR. Phylogenetic analysis of the partial gene sequences (terminal gene regions were not included) encoding B2L gene of Serbian ORFV isolates showed 97.33-100.00% nucleotide and 92.86-100.00% amino acid similarity between each other. However, the viruses were divided into two clusters within the previously recognized Group 2, together with viruses from Croatia, Greece, Finland, China, South Korea and North America. This study is the first report of phylogenetic analysis of ORFV from Serbia and contributes to the data available in the GenBank database. The results of our investigation showed genetic diversity between ORFV strains in Serbia.

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INTRODUCTION

Contagious ecthyma (ORF) represents one of the most common viral infection of sheep caused by orf virus (ORFV). ORFV belongs to the genus *Parapoxvirus* (PPV), family *Poxviridae* together with bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), parapoxvirus of red deer in New Zealand (PVNZ), *Squirrel parapoxvirus* (SPPV), ORFV and sealpox virus (Robinson and Mercer, 1995; Haig and Mercer, 1998; Mercer and Haig, 1999; Becher *et al.*, 2002). Characteristics which distinguish PPV from other members of family *Poxviridae* is relatively small size, ovoid shape and crisscross surface pattern, together with high percentage of G+C content in the genome (Delhon *et al*., 2004; Mercer *et al*., 2006). As a member of family *Poxviridae*, ORFV possesses 138 kbp long linear double strand deoxyribonucleic acid (DNA) which encodes 132 putative genes (Mercer *et al*., 2006). Using the novel molecular techniques B2L gene (1137 bp) shows to be most suitable for detection, characterization and phylogenetic analysis of ORFV due to high conservation among ORFV isolates (Sullivan *et al*., 1994; Zhang *et al*., 2014a).

Sheep and goats are most susceptible for ORFV infection, but the other ruminants such as musk ox, camels and reindeer can be infected (Mombeni *et al*., 2013; Tryland *et al*., 2018). ORFV has also zoonotic potential affecting people who work with animals (Zhang *et al*., 2014b; Bergqvist *et al*., 2017). In Serbia, ORFV infection in humans and ruminants other than sheep and goats has never been reported. Proliferative crust lesions as pathognomonic clinical signs of ORFV infection could be found in the area of lips, nostrils, mouth, udder, and foot (Maganga *et al*., 2016; Peralta *et al*., 2018). There are many other diseases causing similar proliferative lesions, including Foot and mouth disease (FMD) (FitzGerald *et al*., 2015), Bluetongue (Backx *et al*., 2007), Peste des petits ruminants (OIE, 2013) and Sheep pox (OIE, 2017).

However, those diseases cause greater economic losses than contagious ecthyma due to international trade restrictions. Serbia is a country free from FMD, Peste des petits ruminants and Sheep pox while Bluetongue occurred in 2016 with 416 reported outbreaks and 767 affected animals (http://www.vet.minpolj.gov.rs/images/godisnji_izvestaji/2016god.pdf). As a highly contagious disease, ORFV can be transmitted through direct or indirect contact (Tedla *et al*., 2018). Based on crust location, infected animals are usually disabled to graze and walk, which leads to weight and production loss. Also, secondary bacterial infections additionally worsen the lesions (Gelaye *et al*., 2016). The mortality rate in general is very high comparing to mortality rate which is generally low. However, in young animals mortality rate can be high (Kumar *et al*., 2015).

ORFV infection of sheep is very common in Serbia. However, there are no available reports of molecular characterization and phylogenetic analysis of Serbian ORFV strains. For this reason, the aim of our investigation was to provide information on the sequences and phylogenetic analysis of B2L gene of ORFV strains from two outbreaks in Serbia which occurred during 2016.

MATERIALS AND METHODS

Sampling of ORFV from sheep: In total, 31 samples (17 samples from an outbreak in Tutin and 14 samples from an outbreak in Gornji Milanovac) of scabs from lips, nostrils, udder and vagina were taken from 14 sheep and 17 lambs during two ORF outbreaks in 2016. The outbreaks occurred during May in the municipality of Tutin and during August in the municipality of Gornji Milanovac. In both outbreaks, autochthonous breed sjenicka sheep and mixed breed sheep were affected. Sampling was performed using sterile forceps individually for each animal. The samples were immediately immersed into minimum essential medium (DMEM; Gibco, USA) supplemented with 1% antibiotics (Penicillin 1000 IU – Streptomycin 10 mg; Sigma, Germany) and 1% antimycotic (Amphotericin B; Sigma, Germany). The samples were chilled on ice during the transport to the laboratory and were stored at -80 °C until examination.

Virus isolation: All samples were subjected to virus isolation in Vero cells (ATCC CCL-81, IZSBS, Brescia, Italy). Before inoculation, samples were frozen and thawed three times and centrifuged at 600 *g* for 15 min. Subsequently, the supernatants were filtered using sterile 0.22 µm syringe filters (Merck, USA). Volumes (300 µL) of filtered supernatants were inoculated into 24 h old, 90% confluent monolayers of Vero cells grown in 12-well cell culture plates. The plates were incubated for 1 h at 37°C in an environment with 5% CO_2. After that, 1 ml of DMEM supplemented with 2% fetal calf serum (FBS-12A, Capricorn Scientific, Germany) was added. The plates were incubated and observed daily for the appearance of cytopathic effects (CPEs). If CPEs were not visible after 7 days, the plates with inoculated virus in the cell cultures were frozen and thawed three times and then passaged twice more. If no CPEs were visible after the third passage, the sample was considered as negative for the presence of virus. One well with Vero cells in each plate remained uninoculated as a tissue culture control (Fig. 2A).

Polymerase chain reaction (PCR): DNA from 31 filtered scab samples was isolated using QIAamp MiniElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The viral genome was detected by polymerase chain reaction (PCR) based on B2L gene amplification (1206 bp) using previously published primers (Hosamani *et al*., 2006). The PCR reaction was carried out in a total volume of 50 µL using HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) and 0.4 µM concentration of primers. The thermal profile for the PCR included the initial denaturation at 94°C for 3 min followed by 29 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min and elongation at 72°C for 1 min. A final extension step was performed at 72°C for 7 min. The PCR products were analysed by electrophoresis through 1.5% agarose gel stained with 0.5 mg/ml ethidium bromide and visualised under UV light.

Sequencing and phylogenetic analysis: In order to perform phylogenetic analysis of ORFV strains, the PCR products were purified using QIA quick PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions. The amplicons were sequenced at the Macrogen Europe Laboratory, Amsterdam, Netherlands. The sequences were analysed using Geneious 10.1.3 programme. The sequences were aligned and compared with the sequences available from the GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). All sequences used for phylogenetic analysis are listed in Table 1. Evolutionary analyses were conducted using MEGA 7 software. A phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates, and group formation according to Kumar *et al.* (2014).

RESULTS

Case presentation of ORFV infection in sheep: All infected animals manifested inappetence, fever, hypersalivation and mucopurulent nasal discharge. The tissue of lips, muzzle, and nostrils of affected sheep was swollen and cyanotic, with characteristic ORFV papules, pustules and crusts, which were noticeable on gums, hard palate and tongue (Fig. 1A). Crust formation around the lips, nasal cavity and udder (only nursing animals) was noted in all sampled animals from the first outbreak (Fig. 1C; Fig. 1D). Mild bleeding appeared after crust removal. In the second outbreak, all animals showed swollen and cyanotic tissue of lips and muzzle with papule and pustule formation on lips, gums and tongue, without the appearance of crusts (Fig. 1B). High morbidity rates, from 40% to 80%, were observed in both outbreaks. However, all infected animals recovered within 5-6 weeks after the appearance of the symptoms.

Molecular detection of ORFV from swab samples in sheep: ORF virus was successfully isolated from 15 out of 31 samples. The CPEs, characterized by the cell rounding, ballooning and degeneration (Fig. 2B; Fig. 2C), appeared on day 5 of the third passage. Due to fungal contamination, virus isolation from the other 16 samples was unable to be completed. All ORF isolates were confirmed by PCR.

Fig. 1: Clinical signs of ORFV infection in sheep. (A) Sheep with crust like proliferative lesions on the lips and muzzle; (B) Proliferative lesions on lips, gums and tongue with hyperemia and edema; (C and D) Crust formation on udder and vulva of nursing sheep.

Table 1: Details of ORFV sequences used for phylogenetic analysis

No.	Country	Year	Accession number	Host species
1.	Croatia	2010	HQ215588	Goat
2.	Croatia	2009	HQ215589	Sheep
3.	Greece	2004	JN368483	Sheep
4.	Greece	2003	JN368482	Sheep
5.	China	2010	JX968990	Goat
6.	South Korea	2011	JX968991	Sheep
7.	Finland	2011	JF773702	Sheep
8.	Finland	2011	JF773703	Sheep
9.	China	2010	JQ904794	Sheep
10.	North America	2004	AY424970	Sheep
11.	China	2010	JQ904799	Sheep
12.	India	2012	KC992325	Sheep
13.	India	2004	DQ263305	Sheep
14.	Turkey	2007	KC491191	Sheep
15.	India	2009	GQ390365	Camel
16.	India	2009	GU460370	Camel
17.	Finland	2011	JF773692	Reindeer
18.	Finland	2011	JF773694	Bovine
19.	Potreb	2016	MH883304	Sheep
20.	Potreb	2016	MH883305	Sheep
21.	Točilovo	2016	MH883306	Sheep
22.	Točilovo	2016	MH883307	Sheep
23.	Točilovo	2016	MH883308	Sheep
24.	Dobrinja	2016	MH883309	Sheep
25.	Dobrinja	2016	MH883310	Sheep
26.	Dobrinja	2016	MH883311	Sheep
27.	Majdan	2016	MH883290	Sheep
28.	Majdan	2016	MH883291	Sheep
29.	Majdan	2016	MH883292	Sheep
30.	Majdan	2016	MH883293	Sheep
31.	Majdan	2016	MH883294	Sheep
32.	Rudnik 1	2016	MH883295	Sheep
33.	Rudnik 1	2016	MH883296	Sheep
34.	Rudnik 1	2016	MH883297	Sheep
35.	Rudnik 1	2016	MH883298	Sheep
36.	Rudnik 1	2016	MH883299	Sheep
37.	Rudnik 2	2016	MH883300	Sheep
38.	Rudnik 2	2016	MH883301	Sheep
39.	Rudnik 2	2016	MH883302	Sheep
40.	Rudnik 2	2016	MH883303	Sheep

Since gene specific primers were used to amplify 1206 bp of the B2L gene, the appearance of this specific band was considered as a positive result. All the examined samples (31 in total) were positive using PCR. Further confirmation of the virus was performed by genome sequencing.

Sequence analysis of the PCR products showed high G+C content (64.1-66.0%) which is characteristic of *Parapoxviruses*. The partial gene sequences (terminal gene regions were not included) from Serbia used in this study were for the gene encoding the B2L protein of ORFV. All Serbian ORFV strains examined fell into the previously recognized phylogenetic Group 2 (Fig. 3). Within Group 2**,** all Serbian ORFV strains clustered into two main clusters with 97.33-100.00% nucleotide and 92.86-100.00% amino acid similarity between them. All ORFV sequences from Gornji Milanovac and ORFV sequences from Potreb village in Tutin municipality clustered together with 97.78-100.00% nucleotide and 92.86-100.00% amino acid similarity. ORFV sequences from the two other villages in Tutin municipality were grouped into a separate cluster with 99.56-100.00% nucleotide and 98.57-100.00% amino acid similarity between them. Detailed analysis of nucleotide and amino acid sequence similarities can be found in Tables 2 and 3.

DISCUSSION

ORFV infection of sheep is very common in Serbia, causing significant economic losses, and mainly occurring in spring and autumn, during the lambing seasons. During 2016, 6 ORFV outbreaks were reported in Serbia with 184 infected animals from which 20 cases reported as lethal[1]. The diagnosis is often based on clinical signs and characteristic tissue alterations in affected animals. The clinical features found in infected animals vary from multiple lesions around the lips, muzzle, nostrils, teats and oral mucosa, with occasional spread to the buccal cavity, oesophagus, stomach, intestine or the respiratory tract (Nandi *et al*., 2011; Maganga *et al*., 2016; Tryland *et al*., 2018). Despite very developed characteristic clinical symptoms, and in order to exclude other contagious diseases, two suspect ORF infections that were reported in 2016 were confirmed in the laboratory by the virus isolation, PCR, and genome sequencing. According to the veterinarian in charge of Gornji Milanovac municipality no contagious ecthyma had been recorded in the previous few years, but the veterinarian in Tutin municipality said that contagious ecthyma is recorded each year. All sheep in this study were housed in extensive farming conditions, and consequently, had direct contact with other animals from different flocks, increasing the possibility of the spread of ORFV. This type of epizootiological scenario could lead to countrywide spread of ORFV. Since those two municipalities are separated by more than 200 km, there is no other possibility of the virus spreading between them in the field expect by means of stock movement via traded animals. This conclusion could be concurred with recent study which showed that the virus can be introduced into a herd with new asymptomatic infected animals (Peralta *et al*., 2018). For isolation of ORFV, primary lamb testes cell culture is most commonly used because of its high sensitivity (Kottaridi *et al*., 2006).

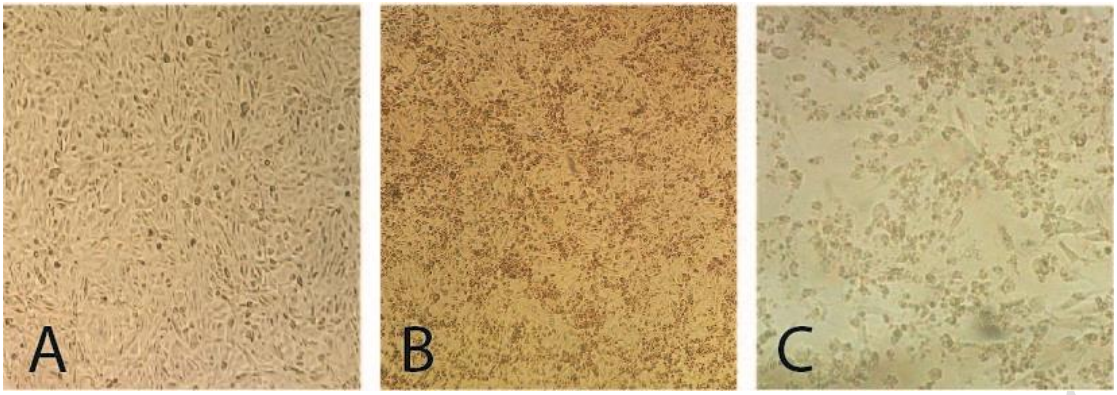

Fig. 2: Cytopathic effects (CPEs) of ORFV in Vero cell line. (A) uninfected Vero cells – negative control; (B) CPE 5 days post infection at 10x magnification; (C) CPE 5 days post infection at 100x magnification.

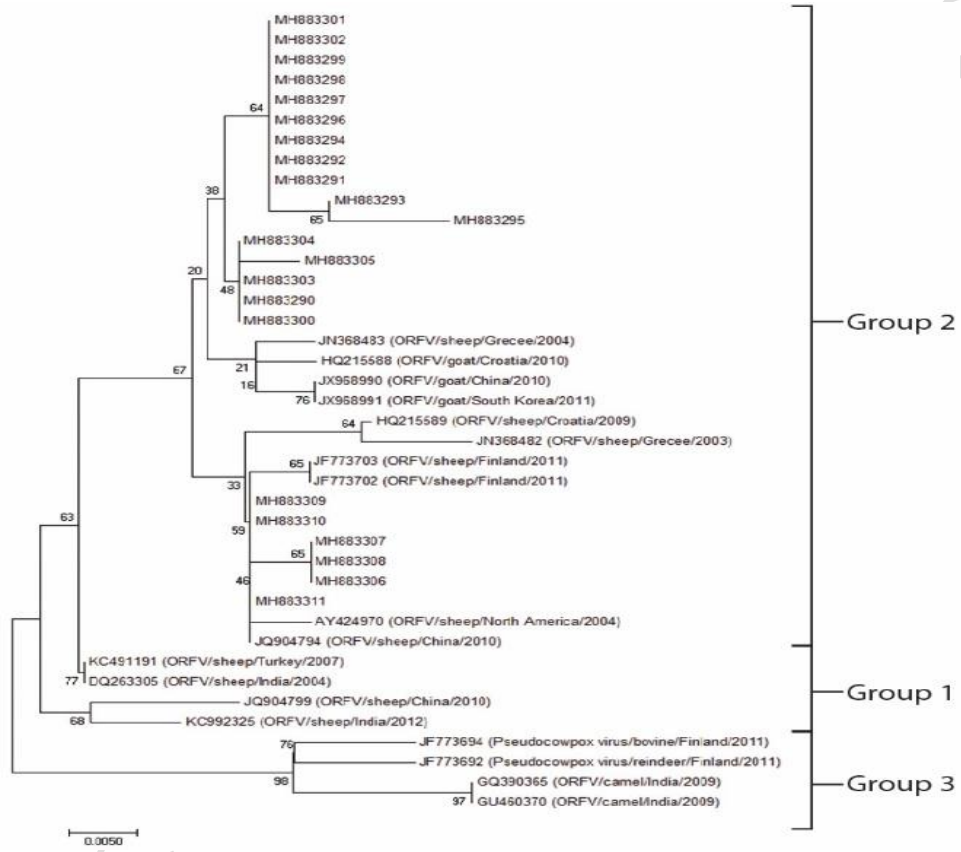

Fig. 3: Neighbor-Joining tree for B2L genes of Serbian and foreign ORFV strains. The phylogenetic relationship was calculated using MEGA 7.

Table 2: Percentage of nucleotide sequence similarity between ORFV sequences examined

	Tutin I	Tutin 2 and 3	Gornji Milanovac	Greece	Croatia	Group I	Group 2	Group 3
Tutin I	99.56	98.22-99.11	97.78-100.00	96.89-99.11	96.89-99.11	96.44-98.67	98.22-99.11	94.22-95.11
Tutin 2 and 3	98.22-99.11	99.56-100.00	97.33-99.11	97.78-98.22	97.78-99.11	96.44-98.67	97.78-100.00	94.22-95.11
Gornji Milanovac	97.78-100.00	97.33-99.11	98.22-100.00	96.00-99.11	96.89-99.11	95.56-98.67	97.33-99.11	93.33-95.11

Table 3: Percentage of amino acid sequence similarity between ORFV sequences examined

	Tutin I	Tutin 2 and 3	Gornji Milanovac	Greece	Croatia	Group I	Group 2	Group 3
Tutin I	98.57	95.71-98.57	92.86-100.00	92.86-97.34	95.71-97.14	94.29-97.14	97.14-98.57	88.57-90.00
Tutin 2 and 3	95.71-98.57	98.57-100.00	92.86-98.57	94.29-95.71	94.29-98.57	94.29-98.57	94.29-100.00	98.57-91.43
Gornji Milanovac	92.86-100.00	92.86-98.57	94.29-100.00	90.00-97.34	91.43-97.14	90.00-97.14	91.43-98.57	85.71-90.00

However, Vero cell line has also been successfully used for ORFV isolation, and CPEs of ORFV were observed on days 3 and 5 after the first or second passage (Gelaye *et al*., 2016). In our study using the Vero cell line for virus isolation, CPEs were observed on the third passage, 5 days after inoculation.

All sequences used for phylogenetic analysis in this study were separated into three groups: Group 1 comprises ORFV sequences from Asia (India, China, and Turkey), Group 2 comprises ORFV sequences from various countries (North America, Finland, South Korea, and China), and Group 3 comprises ORFV sequences

from camel (India) and PCPV (Finland) (Kumar *et al*., 2014). On the basis of results from our phylogenetic analyses, all ORFV strains from this study fell into the Group 2 and separated into two clusters together with sequences from Greece and Croatia. One cluster contains sequences from the municipality of Gornji Milanovac and sequences from Potreb village in Tutin municipality, while the second cluster contains only sequences from the other two villages in Tutin municipality. A possible explanation for the clustering together of sequences from Gornji Milanovac with sequences from Potreb village in Tutin could be infection in the field by the mixing with sheep from other herds. Since the sequences from the two other villages in Tutin municipality are separated in their own cluster, this suggests that some close neighbors brought sheep from Gornji Milanovac to Potreb. The clustering observed, together with the high nucleotide and amino acid similarity of ORFV sequences from this study with ORFV sequences from neighboring countries, Greece (96.00-99.11% and 90.00-97.34% respectively) and Croatia (96.89-99.11% and 91.43-98.57% respectively), implies that molecularly closely related ORFV are present in the region. ORFV sequences from this study were grouped into Group 2, together with sequences that are distributed worldwide, showing the highest nucleotide and amino acid identity with these sequences 97.33-100.00% and 91.43-100.00% respectively. This supports the findings of Kumar *et al.* (2014), who reported that Group 1 comprises only ORFV sequences from Asia.

Conclusions: The results of this study showed that ORFV strains in Serbia are genetically diverse, but closely related to ORFV strains from neighboring countries (Greece and Croatia). This study reports the first phylogenetic analysis of ORFV from Serbia, revealing that two different clusters within the same phylogenetic group circulate in Serbia. Considering that ORF is not a notifiable disease in Serbia, we assume many cases remain unreported, meaning that the actual diversity is likely even higher. Therefore, there is a need for further investigation of the distribution and biological properties of Serbian ORFV strains.

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Authors contribution: MM, VM and SR planed and designed the study. MM collected the samples and together with VM and NS conducted the virological study. MM, NS and VM carried out the molecular genetic studies; JN assisted in molecular genetic studies. MM and VM interpreted results and drafted manuscript. MV, NS, JN and SR assisted in drafting the manuscript and critically revised the manuscript. All authors have read the manuscript and approved submission of the manuscript.

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