

Identification and characterization of Orf viruses isolated from sheep and goats in Southern Italy

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Summary

Orf virus (ORFV; Family: Poxviridae) is the causative agent of contagious ecthyma, or Orf disease in sheep, goats and other domestic or wild ruminants with a worldwide distribution. The disease is endemic in Italy, but few data are available about its distribution and epidemiology. In the present study we analysed 32 clinical samples, obtained from crusted scab lesions of 5 goats and 27 sheep, from 19 suspected outbreaks of contagious ecthyma in Apulia and Basilicata regions between 2012 and 2014. Negative staining electron microscopy (EM) and polymerase chain reaction (PCR) targeting the late transcription factor gene (VLTF-1) were used to identify the virus. Isolation was also attempted on BHK-21 cell line. PCR was proved to be more sensitive than EM, as it detected the virus in 28 out of 32 samples, whereas the EM detected it only in 26 out of the 32 samples. The majority of isolated strains forms a monophyletic group; these isolates, according to the VLTF-1 sequencing, are highly related to ORFV strains previously shown to circulate in Southern Italy.

Identificazione ed analisi filogenetica di ORF virus isolati in focolai di ectima contagioso in pecore e capre in Italia

Parole chiave

Analisi filogenetica,
Colture cellulari,
Gene VLTF-1,
Microscopia elettronica,
ORF virus,
PCR.

Riassunto

L'ORF virus (ORFV; Family: Poxviridae) è l'agente causale dell'ectima contagioso, malattia infettiva contagiosa degli ovicaprini e di altri ruminanti domestici o selvatici, con distribuzione mondiale. La malattia è endemica in Italia, ma in letteratura scientifica sono disponibili pochi dati sulla sua distribuzione ed epidemiologia. In questo studio sono stati analizzati 32 campioni clinici (croste cutanee) prelevati da 27 ovini e 5 caprini provenienti da 19 focolai sospetti di ectima contagioso occorsi in Puglia e Basilicata tra il 2012 e il 2014. Per identificare il virus sono state effettuate sia l'analisi morfologica mediante microscopia elettronica (ME), sia una PCR mirata al rilevamento del gene del fattore di trascrizione tardiva (VLTF-1). I ceppi virali sono stati isolati anche su linea cellulare BHK-21. La PCR è risultata essere la metodica più sensibile, evidenziando la presenza di ORF virus in 28 dei 32 campioni analizzati, mentre la microscopia elettronica ha permesso di rilevare il virus solo in 26 dei 32 campioni. La maggior parte degli isolati forma un gruppo monofiletico; essi, sulla base delle sequenze del gene VLTF-1, risultano essere altamente correlati a ceppi di ORF virus già circolanti in Sud Italia in passato.

Introduction

Orf virus (ORFV) is an epitheliotropic virus of the genus *Parapoxvirus* (PPV), family *Poxviridae*, which also includes the bovine papular stomatitis virus (BPSV), Pseudocowpox virus (PCPV), causing Milker's nodule in man, PPV of the New Zealand red deer (PVNZ), and other tentative species, such as the camel PPV (CCEV), or seal PPV. Parapoxviruses may cause disease in a wide range of wild animal species (camels, red deer, reindeer, squirrels, seals, and grey seals) (Robinson and Mercer 1995, Scagliarini et al. 2011, Tryland et al. 2005, Thomas et al. 2003) as well as in humans, especially if immunocompromised (Ara et al. 2008, Mercer et al. 2014). At risk humans are those living in close contact with infected animals such as farmers, abattoir workers, veterinarians, and sheep shearers. In humans, the disease usually causes painful nodules on hands and arms and, more infrequently, on the face (Kuhl et al. 2003). In zootechny, ORFV has to be considered a problem for sheep industry, as it causes decrease of productions, but it also poses a risk for workers' health (Zhang et al. 2014).

Generally, the virus enters the host through skin lesions, and initiates a localized virus replication in the epidermal cells and keratinocytes, producing the typical clinical signs of contagious ecthyma. In sheep and goats, the disease is usually circumscribed to the skin of the lips, around the nostrils, and to the oral mucosa. Sometimes, it also affects the gum and the tongue, especially in young lambs (Hosamani et al. 2009). The disease is active for 3-4 weeks and lesions heal completely in 1-2 months. Despite contagious ecthyma is generally characterised by high morbidity, mortality rates are usually low. However, mortality up to 93% has been reported in younger animals (Mazur and Machado 1989, Mazur and Machado 1990).

This study aimed at identifying the ORFV as causative agent of the disease in 19 suspected outbreaks of contagious ecthyma occurred in Apulia and Basilicata regions, Southern Italy, between 2012 and 2014. Furthermore, we compared the reliability and suitability of negative staining electron microscopy (EM) propagation in cell cultures, and a polymerase chain reaction (PCR) assay for the direct identification of ORFV DNA from skin biopsies. Finally, we characterised the strains by analysing the 045 gene, encoding the late transcription factor VLTF-1, which is already used to detect genetic variations among PPVs (Mahmoud et al. 2010, Davari et al. 2013).

Materials and methods

Disease outbreaks and sample collection

Between 2012 and 2014, 19 outbreaks of suspected contagious ecthyma in sheep and goats were

reported in different areas of Apulia and Basilicata regions, in Southern Italy. In most cases, clinical signs were limited to scabs on the lips, and the disease spontaneously resolved in 3-4 weeks. Conversely, 2 outbreaks were characterised by high fever, oedemas of the face, and of the submandibular region, scabs on lips, tongue and teats, which led to anorexia (Figure 1). In these 2 outbreaks, about the 6% of the animals died. One to 5 clinical samples (listed in Table I) per outbreaks were collected from crusted scab lesions and analysed.

Electron microscopy

The first diagnosis was achieved by EM. All the crusty scab samples were mechanically homogenised in a sterile mortar with distilled water. After staining with 2% NaPt acid, pH 6.8 (Doane and Anderson 1987), the grids were observed using an electron microscope (EM Philips 208S, FEI, Eindhoven, Netherland) operating at 80 kV.

Propagation in cell cultures

The cell line used for the propagation of the virus was the baby hamster kidney (BHK-21), previously proved to be reliable for the cultivation of the ORFV (Kottaridi et al. 2006a). Homogenised samples were centrifuged at 3,000 x g for 5 minutes, and the supernatant was filtered through a 0.45 µM filter (Merck Millipore, Milan, Italy) and treated with antibiotics and antimycotic (namely, penicillin 100 U/ml, streptomycin 100 µg/ml, neomycin 50 µg/ml, and amphotericin B 2.5 µg/ml). The mixture was inoculated in the cell suspension, cultivated with Dulbecco's modified Eagles medium and supplemented with 10% bovine foetal serum. Cell cultures were incubated at 37 °C for 96 hours, daily observed for the presence of cytopathic effects (CPEs), and passaged up to 4 times.

Polymerase chain reaction

Total DNA was extracted from all clinical samples by using the DNeasy Blood and Tissue kit (Qiagen, Milan, Italy); DNA from tissues and supernatants from cell cultures were used as a template for PCR targeting the gene 045, which encodes the late transcription factor VLTF-1 of PPV (Delhon et al. 2004). PCR was performed as previously described (Kottaridi et al. 2006a) by using the 045F and 045R primers. The reactions were carried out in a volume of 50 µl. The reaction mixture consisted of 25 µl PCR master mix (REDTaq ReadyMix PCR, Sigma-Aldrich, Milan, Italy), 1 µM of each primer, and 5 µl of the extracted DNA. Cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 15 seconds, 57.7 °C for 30



Figure 1. Clinical signs of ORF virus infection in sheep in the two most severe outbreaks of the disease. Nodular lesions, evolving in scabs are well evident around nostrils, on the lips (**A, B**), in the oral mucosa, on the tongue (**C**) and in some cases also around the teats (**D**). The oedema of the head and the hypersalivation are evident (**A, B, C**).

Table I. Clinical samples analyzed in this study. —cont'd

Farm relative number	Clinical samples	Place and date of collection	Host	Cell culture	Electron microscopy	PCR 045 gene
A	5467 FG	Foggia, Puglia 07-2012	Sheep	+	+	+
B	3472 FG	Foggia, Puglia 08-2012	Sheep	+	+	+
C	12821 FG	Foggia, Puglia 08-2014	Sheep	+	+	+
D	13281 FG	Foggia, Puglia 09-2014	Sheep	+	-	+
E	868 FG	Foggia, Puglia 12-2014	Sheep	+	-	+
F	16232/1 BA	Bari, Puglia, 09-2013	Sheep	+	+	+
F	16232/2 BA	Bari, Puglia, 09-2013	Sheep	+	+	+
G	17205/1 BA	Bari, Puglia, 10-2013	Sheep	+	+	+
G	17205/2 BA	Bari, Puglia, 10-2013	Sheep	+	+	+
H	801 BA	Bari, Puglia, 12-2014	Sheep	+	+	+
I	3869 LE	Lecce, Puglia, 09-2012	Sheep	+	+	+
J	10278 PZ	Potenza, Basilicata, 06-2013	Sheep	+	+	+
J	10279 PZ	Potenza, Basilicata, 06-2013	Sheep	+	+	+
K	8598 PZ	Potenza, Basilicata, 06-2013	Sheep	+	+	+
L	9726/19 PZ	Potenza, Basilicata, 09-2013	Sheep	+	+	+
M	5174/1	Matera, Basilicata, 07-2013	Goat	+	+	+
M	5174/2	Matera, Basilicata, 07-2013	Goat	+	+	+
M	5174/3	Matera, Basilicata, 07-2013	Goat	+	+	+

continued

Table I. Clinical samples analyzed in this study. —cont'd

Farm relative number	Clinical samples	Place and date of collection	Host	Cell culture	Electron microscopy	PCR 045 gene
M	5174/4	Matera, Basilicata, 07-2013	Goat	+	+	+
N	5181/1	Matera, Basilicata, 07-2013	Sheep	+	+	+
N	5181/2	Matera, Basilicata, 07-2013	Sheep	+	+	+
N	5181/3	Matera, Basilicata, 07-2013	Sheep	+	+	+
N	5181/4	Matera, Basilicata, 07-2013	Sheep	+	+	+
N	5181/5	Matera, Basilicata, 07-2013	Sheep	+	+	+
O	6173/1	Matera, Basilicata, 09-2013	Sheep	+	+	+
O	6173/2	Matera, Basilicata, 09-2013	Sheep	+	+	+
O	6173/3	Matera, Basilicata, 09-2013	Sheep	+	+	+
O	6173/4	Matera, Basilicata, 09-2013	Sheep	+	+	+
P	1179	Brindisi, Puglia, 05-2013	Goat	-	-	-
Q	3559	Taranto, Puglia, 11-2013	Sheep	-	-	-
R	145	Foggia, Puglia, 01-2014	Sheep	-	-	-
S	9301	Potenza, Basilicata, 09-2014	Sheep	-	-	-

seconds and 72 °C for 50 seconds; final extension of 72 °C for 10 minutes.

Amplification products were run on 2% agarose gel stained with Sybr® Safe (Life Technologies, Milan, Italy) and were visualised by the mean of a the Molecular Imager Gel DocTM XR+ (Bio Rad, Milan, Italy).

Sequencing and phylogenetic analysis

Amplification products obtained from DNA purified from cell supernatants were purified by the QIAquick PCR purification kit (Qiagen, Milan, Italy) and both strands were sequenced by the Sanger method (Big Dye Terminator Kit, Life Technologies, Milan, Italy) using external facilities (Eurofins Genomics Italy, Milan, Italy). The high quality regions were assembled by Cap3¹. Nucleotide sequences were submitted to the NCBI GenBank under accession numbers from KR812113 to KR812140.

Sequences were compared with those present in GenBank by Blast to confirm ORFV identification. After the identification, our sequences and other ORF virus sequences submitted to the GenBank, were aligned by ClustalW algorithm implemented in MEGA 6.0 (Tamura et al. 2013) (Table II). Phylogeny was inferred by Maximum Likelihood estimation using the PhyML software (Guindon and Guascl 2003), with 1,000 non-parametric bootstrap replicates. The best fitting evolutionary model (GTR+G, alpha = 0.48) was determined by using

the Modeltest script implemented for web at FindModel².

Results

Negative staining electron microscopy

Typical PPV virions were observed in 26 of the 32 samples analysed by EM. The particles appeared as enveloped, ovoid virions, 220-300 nm long, and 140-170 nm wide. The surface membrane displayed regularly arranged surface tubules. In most of the analysed samples, several viral particles were visible. Mature viral particles of PPV were also observed from all the suspensions of the cell cultures.

Cell cultures and PCR detection

Twenty-eight of the 32 cell cultures showed CPE (ballooning, wounding, degeneration of cells up to the complete destruction of the monolayer) starting from passage 2. For 2 samples only (13281 FG and 868 FG), CPE was evident at passage 4. VLTF-1 was successfully amplified from DNA purified from all 28 ORFV-positive cell cultures.

VLTF-1 was also amplified from the corresponding infected biological samples while the other 4, shown to be negative by virus isolation, resulted negative (Table I).

¹ <http://doua.prabi.fr/software/cap3> (latest accessed on March 4, 2016).

² <http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>, latest accessed March 4, 2016.

Table II. List of strains from GenBank enrolled for phylogenetic analysis.

Strain	Species	Host	Isolation place (year)	GenBank Accession number
BV-AR02	Bovine papular stomatitis virus	Calf	USA (N/A)	AY386265
BV-TX09c1	Bovine papular stomatitis virus	Cattle	USA (2009)	KM875472
BV-TX09c5	Bovine papular stomatitis virus	Cattle	USA (2009)	KM875471
BV-TX09c15	Bovine papular stomatitis virus	Cattle	USA (2009)	KM875470
MyV	Myxoma virus	Brush rabbit	USA (1950)	KF148065
F00.120R	Pseudocowpox virus	Reindeer	Finland (2000)	GQ329669
PCPV776	Pseudocowpox virus	Human	Austria (2010)	HM589037
VR634	Pseudocowpox virus	Human	N/A (N/A)	GQ329670
B029	Orf virus	Human	Germany (1996)	KF837136
D1701	Orf virus	Sheep	Germany (N/A)	HM133903
Egypt	Orf virus	Sheep	Egypt (2006)	EU826136
NA1/11	Orf virus	Sheep	China (2011)	KF234407
NZ2	Orf virus	Sheep	New Zealand (N/A)	DQ184476
OV-IA82	Orf virus	Sheep	USA (1982)	AY386263
OV-SA00	Orf virus	Human	USA (N/A)	AY386264
Shiraz1	Orf virus	Sheep	Iran (2012)	KC534486
Shiraz3	Orf virus	Sheep	Iran (2012)	KC534488
Shiraz4	Orf virus	Sheep	Iran (2012)	KC534489
Shiraz5	Orf virus	Sheep	Iran (2012)	KC534490
HL953	Red deer parapoxvirus	Red deer	Germany (2013)	KM502564
SqPV	Squirrelpox virus	Red squirrel	UK (N/A)	HE601899

N/A = Information not available in GenBank.

Phylogeny

When compared to those present in GenBank, the nucleotide sequences from VLTF-1 gene were 99% identical to the correspondent sequences of previously submitted ORFV, thus confirming the detection of this virus in the analysed samples.

Phylogenetic analysis showed that all but 3 sequences were identical among themselves, and that they grouped together in cluster A (Figure 2). This cluster also included strains OV-IA82 isolated in Iowa (US) from nasal secretion of a lamb (Delhon *et al.* 2004), strain NZ2, isolated in New Zealand from sheep scab material (Mercer *et al.* 2006), and strain B029, isolated in Germany from humans (Friederichs *et al.* 2014).

Out of the 3 sequences not included in the cluster A, 2 (10278 and 10279) originated from sheep of the same farm (J, Table I), located in Potenza, Basilicata. They were 100% identical, while they differed for 3 nucleotides from sequences belonging to cluster A.

Finally, the most divergent sequence was obtained from isolate 3472, isolated from a sheep sample collected in Foggia in 2012. It differed

for 14 nucleotides from the other sequences of cluster A. However, no differences were shown at amino acid level. This latter sequence grouped in a distinct branch of the phylogenetic tree, with strong bootstrap support.

Discussion

The molecular approach was confirmed to be the most sensitive for the diagnosis of ORFV, and probably of other PPVs, from clinical samples. In fact, we found that PCR was able to detect ORFV DNA in 28 samples, while EM evidenced the virus in 26 only. Furthermore, ORFV isolation from PCR-positive samples tissue confirmed the presence of live viral particles. Reasonably this method requires more time and high-skilled personnel. As reported in other studies, the reduced sensitivity of EM might be due to a low viral load in the samples, as it happens when tissues are collected from animals during the healing phase of infection (Doane and Anderson 1987).

However, negative staining EM by the drop-to-drop method has proved to be very fast, and it can be

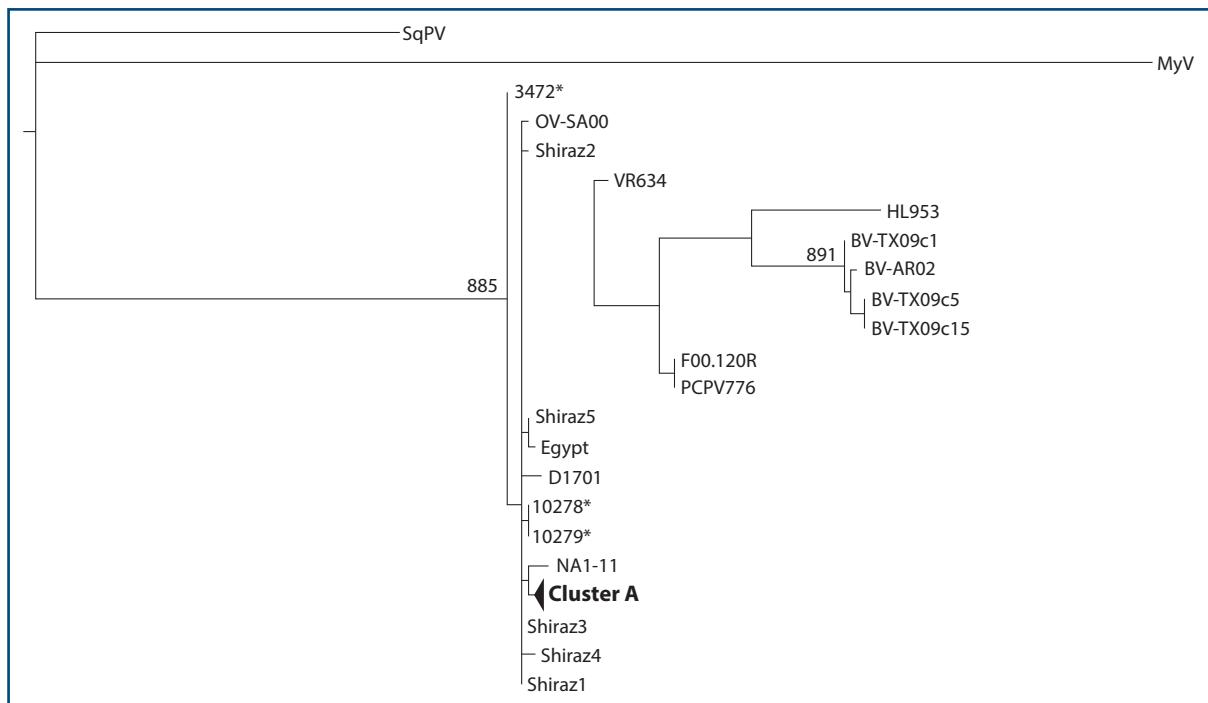


Figure 2. Maximum likelihood phylogeny of the ORF virus strains analysed in this study, based on the nucleotide sequences of the VLT-1 gene. Significant (> 850/1000) bootstraps are reported at nodes. Cluster A includes all the analysed strains, with the exception of 10278 PZ, 10279 PZ and 3472 FG.

considered a reliable option for the quick diagnosis of contagious ecthyma, especially during active outbreaks. In fact, by means of such technique, results can be achieved in 30 minutes.

VLT-1 gene is also useful for phylogenetic analysis. In the present study the gene was sequenced to check whether significant genetic differences could be found in the strains isolated in the outbreaks. While highly conserved, we found a limited variability due to point mutations, in agreement with a previous report (Mahmoud et al. 2010).

Contagious ecthyma is a very common disease, diffused all over the world. In Italy, the disease is present and it may be considered endemic in Southern Italy, where sheep and goat breeding is widespread. However, to the best of our knowledge, scientific literature upon ORFV circulation in Italy is limited (Kottaridi et al. 2006b). The present study reveals that the majority of ORFV strains, which have been circulating among sheep farms of Apulia and Basilicata during 2012-2014, is enclosed within a monophyletic group. Furthermore, they were highly related to well characterised strains, isolated 3 decades ago. In particular, the isolated strains belonged to the same group, which includes the strains OV-IA82 and NZ2. Importantly, several

isolates from all over the world are related to these two strains (Lojkic et al. 2010). NZ2-related strains were known to circulate in central Italy since the first decade of 2000's (Cardeti et al. 2005) and our study highlights its persistence.

However, we also evidenced the presence, in the regions involved in the present study, of other strains, which were more distantly related to the predominant group. In particular, the VLT-1 gene of the isolate 3472 appears well distinct from the others. Interestingly, we did not observe any correlation between phylogenetic clustering and disease severity. The strains causing the most virulent outbreaks were included in the cluster A, and their VLT-1 did not differ from the others. Such finding confirms the hypothesis that the clinical features of the disease may be due to host-related factors rather than to specific pathogenic traits of the virus (Gallina et al. 2008).

Overall, these data strongly suggest that most of the cases of contagious ecthyma of Apulia and Basilicata are caused by a strain characterized by the same VLT-1 gene that circulates in Southern Italy. Other strains could be present, probably imported from other countries as result of animal trade.

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