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Molecular Diagnosis and Application of Combined Alternative Treatment in Lesions Developing in the Oral Region due to Orf Virus in Sheep and Goats

Y.S. Orta[1], G.B. Kaya[1], K. Atli[1], M. Kale[1], O. Ozmen[2], Y. Yildirim[1]

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ABSTRACT

Background: Orf virus (ORFV) is a zoonotic pathogen that infects sheep and goats, causing significant economic losses. The infection results in proliferative and self-limiting crustal lesions, commonly seen on the skin of the lips and around the nostrils of sheep and goats and occasionally on their feet and teats. ORFV infection is prevalent worldwide and endemic in regions where sheep and goats are raised. Vaccines are widely used to protect against ORFV-induced ecthyma in these animals. However, the disease has become increasingly prevalent throughout the year in small ruminants, necessitating the development of new approaches to treat it.

Methods: Crust samples were taken from ORFV lesions in the mouth (lips, gingiva) of 29 sheep and 29 goats aged two years or older from various barns in Burdur-Center and its districts. Conventional polymerase chain reaction was used to investigate the presence of the ORFV genome in the samples, using seven different types of primers (GIF/IL-2, PPP1-PPP4, Orf1-Orf2, VIR1-VIR2, vIL-10-3, vIL-10-4, B2L and Alpha tubulin). Additionally, samples from oral mucosa lesions were examined using histopathological and immunohistochemical methods. As an alternative treatment approach, PAPILEND™,®cream was applied and Ivermectin was administered subcutaneously twice every 15 days, along with 10 grams of Alquermold™ premix powder daily for 10 days to all animals with a detected ORFV viral genome.

Result: The presence of viral genomes was determined in 10 (34.48%) of 29 sheep and 14 (48.28%) of 29 goats using 7 different types of primers, which have been detected intensively in our region before. In sheep the GIF/IL-2, PPP1-PPP4 and B2L primers detected viral genome (10/29 positive) while in goats Orf1-Orf2 and B2L primers (14/29 positive). Histopathological examination showed epidermal hyperplasia, hyperkeratosis, parakeratosis and crust formations at the epidermal layer. Both epithelial and dermal cells expressed orf virus antigen throughout the immunohistochemical investigation. The alternative treatment of PAPILEND™,®cream + Ivermectin + Alquermold™ premix powder triple combination applied to sheep and goats with ORFV genome determined was successful in treating oral lesions caused by ORFV. PCR tests provide rapid and reliable results in the diagnosis of ORFV. The use of triple combined alternative treatments, in addition to ORFV preventive vaccinations in sheep and goats, was found to be successful in treating ORFV infections. The animals showed recovery or regression within a range of 5 to 21 days.

Key words: Combined alternative treatment, Goat, Orf virus, PCR, Sheep.

INTRODUCTION

Orf virus (ORFV) is a zoonotic pathogen that infects sheep and goats and is responsible for significant economic losses (Karki *et al.,* 2019). ORFV belongs to the Parapoxvirus genus, *Chordopoxvirinae* subfamily and *Poxviridae* family (Cargnelutti *et al.,* 2011). Bovine papularstomatitis virus (BPSV) and Pseudocowpox virus (PCPV) are the most important members of this family (Cargnelutti *et al.,* 2011; Khalafalla *et al.,* 2020). ORFV enters the live body through damaged tissues and replicates in epidermal cells (Atli, 2017; Bergqvist *et al.,* 2017). It usually spreads to healthy animals through contact with infected animals. ORFV infection in sheep and goats causes proliferative and self-limiting crustal lesions that can be seen frequently on the skin of the lips, around the nostrils and rarely on the feet and teats (Cargnelutti *et al.,* 2011; Fleming *et al.,* 2015). ORFV infection is widespread worldwide and endemic in countries where sheep and goats are raised. While ORFV infection in sheep and goats has low mortality, it has a mortality rate of 10% to 90% in lambs and kids due to their failure to suckle their mothers and secondary bacterial infections (Nandi *et al.,* 2011; Atli, 2017; Karki *et al.,* 2019). Vaccines (live or live attenuated) are commonly used for

[1]Department of Virology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey.
[2]Department of Pathology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey.

Corresponding Author: M. Kale, Departments of Virology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey. Email: drmkalex@yahoo.com

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the prevention of ecthyma disease caused by ORFV in sheep and goats (Karki *et al.,* 2019). The need for new approaches in the treatment of this disease has arisen due to the recent occurrence of the disease throughout the year, which was previously seen seasonally in animals. In this study, the aim was to determine the presence of viral genomes in lesions developed in the mouths (lips, gums) of sheep and goats due to ORFV using specific ORFV primers and to investigate the effectiveness of triple combined alternative treatment methods in these animals.

MATERIALS AND METHODS

Samples were collected from the lesions on the lips and gums of 29 sheep and 29 goats aged 2 years and older with ORFV infection from various sheepfolds in Burdur-Merkez and its districts. All samples were collected, all field tests were performed and all analyses were carried out in our laboratories (Departments of Virology and Pathology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University) between January and April 2023. DNA extraction was performed from the lesion crusts using the DNeasy Blood and Tissue Kit (Qiagen, Germany). The application was carried out according to the protocol provided by the manufacturer. The obtained DNA extract was stored at -20°C until PCR application. In PCR applications, typing of ORFV in ecthyma samples collected from sheep and goats in Burdur province was performed using 7 different types of primers from Metabion (Synthesis Report Metabion International AG, Germany). For this purpose, the primers used in this study (GIF/IL-2, PPP1-PPP4, Orf1-Orf2, VIR1-VIR2, vIL-10-3 vIL-10-4, B2L and Alpha tubulin) were applied by modifying the procedures developed by different researchers (Inoshima *et al.,* 2000; Kottaridi *et al.,* 2006a; Gu *et al.,* 2011; Alam *et al.,* 2012; Mwanandota *et al.,* 2016; Adedeji *et al.,* 2017). The amplified products were then run on 1.5% Tris Acetate EDTA (TAE) agarose gel with 10 µl safe view dye (ABM, Canada) using a thermal cycler device and visualized using an imaging system (BIO-RAD GelDoc Go Imaging System, USA). Samples from oral mucosa lesions were collected and fixed in 10% neutral formalin solution. The tissue samples were then processed using Leica ASP300S (Leica Biosystems, USA) fully automatic tissue processing equipment and embedded in paraffin wax. Subsequently, 5 µm thick sections were obtained from the paraffin blocks using a Leica RM2155 rotary microtome (Leica Biosystems, USA) after cooling. These sections were stained with hematoxylin-eosin (HE) and examined under a light microscope. The sections were placed on polylysine slides and stained with streptavidin biotin peroxidase for immunohistochemical analysis. For ORFV detection, sheep orf antibody (positive control) was used as the primary antibody [sheep orf virus disease antibody IgG (ORFV-IgG) ELISA Kit, SL00097Sp, Sunlong Biotech Co. Ltd., China]. DAB chromogen and the Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (ab64264) were used as secondary antibody kit. After counterstaining the prepared slides with Harris Hematoxylin, a cover slip was applied and the slides were then examined under a microscope. A triple combination therapy was tried in all animals in which the presence of ORFV viral genome was investigated and positively detected; the positive cases were treated daily with 2 to 5 grams of PAPILEND™,®cream (Almer Kimya İlaç San. Tic. Ltd.Şti., Ankara, Turkey) according to severity of lesions for 10 days, 2 doses of subcutaneous injection of 1 mL per 50 kg live weight of Ivermectin (Zolimectin™, Zoleant İlaç A.Ş. Şişli, İstanbul, Turkey) at 15-day intervals and daily 10 grams of Alquermold™ premix powder (Biovet, S.A., 25 Poligono Industrial, Tarragona, Spain) per animal for 10 days was added to animal feed.

RESULTS AND DISCUSSION

PCR Results

In the study, with the use of 7 different types of primers as previously detected intensively in our region (Kaya and Kale, 2022), the presence of viral genome was detected in 10 out of 29 sheep (34.48%) and 14 out of 29 goats (48.28%) (Table 1). The GIF/IL-2 (2), PPP1-PPP4 (2) and B2L (2) primers were detected the most in sheep, while Orf1-Orf2 (4) and B2L (2) primers were detected the most in goats (Table 1, Fig 1).

Histopathology and Immunohistochemistry

According to histopathological analyses, acanthosis was caused by epidermal hyperplasia, hyperkeratosis and parakeratosis, as well as pronounced thickening of the epidermis. In addition, there was degeneration and necrosis

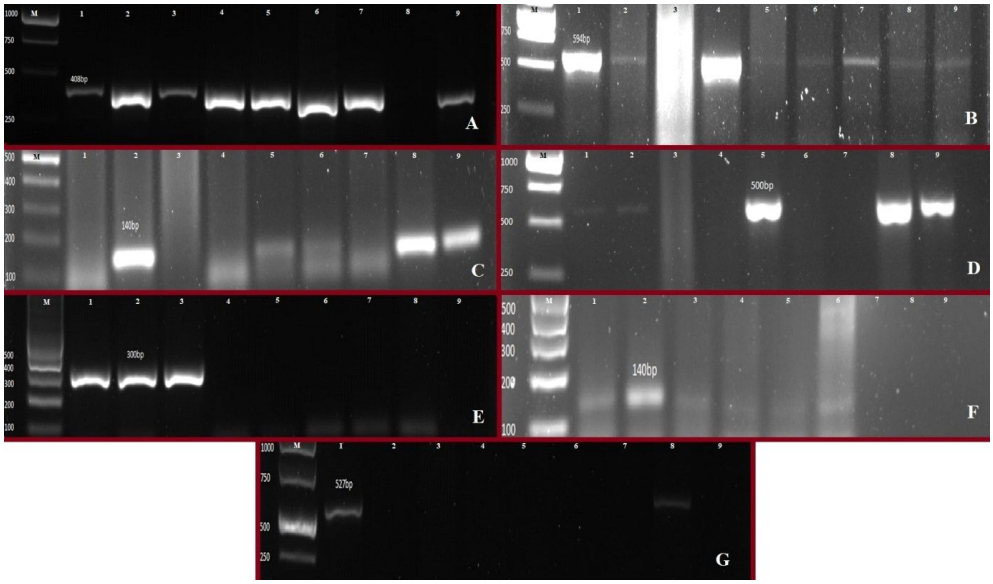
Table 1: PCR analysis results according to ORFV primers.

Sheep		Goats	
Primers	Positive	Primers	Positive
GIF/IL-2	2	GIF/IL-2	2
PPP1 ve PPP4	2	PPP1 ve PPP4	1
Orf 1 Orf 2	1	Orf 1 Orf 2	4
VIR1 VIR2	1	VIR1 VIR2	1
vIL-10-3 vIL-10-4	1	vIL-10-3 vIL-10-4	2
B2L	2	B2L	3
Alfa-tubulin	1	Alfa-tubulin	1
Total	10/29 (34.48%)	Total	14/29 (48.28%)

of the epithelial cells, along with the presence of multifocal or consolidating necrotic crust on the epidermal layer. The most common finding was the marked epidermal extensions of the epidermis into the dermis, which are called rete ridges. Increased mitotic activity was noticed in epidermal cells. Degenerative and necrotic changes in the stratum spinosum's spinous cells were also evident, with spongiosis, vacuolation, pyknotic and karyorrhectic nuclei. Micro abscess, scab development and peripheralized keratohyalin granules were common in the lesions. Keratinocytes exhibited typical intraepithelial ballooning degeneration and eosinophilic intracytoplasmic inclusion bodies. Inflammatory cell infiltration, intracellular edema and sporadic hemorrhages caused the superficial dermis to enlarge. At the immunohistochemical examination, positive reactions with ORFV were detected in both epidermal and dermal cells (Fig 2).

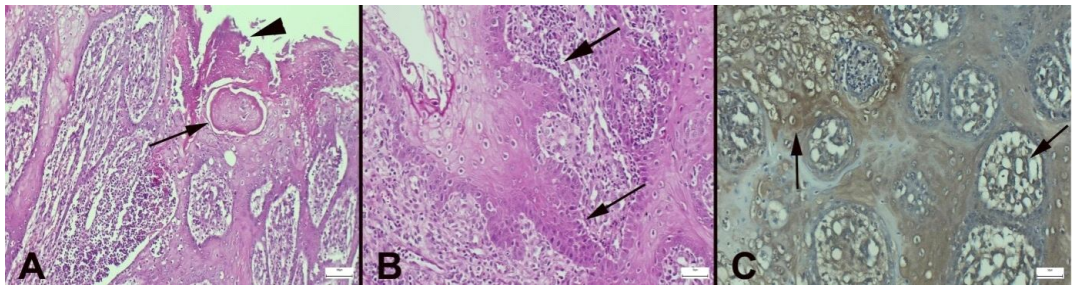
Results of alternative treatment application

Successful (complete healing or regression) results were obtained in the oral lesions developed as a result of the triple combination application in all animals with positive detection of ORFV viral genome (Fig 3). The complete healing or regression time in sheep and goats with positive



A) GIF/IL-2 [M: Marker, 1: Positive Control (PC), 8: Negative Control (NC), 3 and 9: Positive Samples]; B) PPP1-PPP4 (M: Marker, 1: PC, 5: NC, 2,4,6,7,8 and 9: Positive Samples); C) Orf 1-Orf 2 (M: Marker, 2: PC, 3: NC, 5 and 8: Positive Samples); D) VIR1-VIR2 (M: Marker, 5: PC, 7: NC, 1,2,8 and 9: Positive Samples); E) vIL-10-3 vIL-10-4 (M: Marker, 2: PC, 4:NC, 1 and 3: Positive Samples); F) B2L (M: Marker, 2: PC, 5: NC, 1 and 3: Positive Samples); G) Alfa-tubulin (M: Marker, 1: PC, 3: NC, 8: Positive Sample).

Fig 1: Gel electrophoresis images of ORFV primers.



(A) Representative histopathological microphotos of a goat orf lesion, crust (arrow head) and micro abscess formation (arrow), HE; Scale bar=100µm. (B) Rete ridge formations (thin arrow) and inflammatory cell infiltrations (thick arrow), HE; Scale bar=50µm. (C) Orf virus immunohistochemistry findings, positive cells (arrows), streptavidin biotin peroxidase method, Scale bar=50µm.

Fig 2: Microscopical appearance of the orf cases.

detection of ORFV viral genome and developing oral lesions occurred between 5-21 days as a result of the triple combination applications.

The most important way to achieve economic gain in sheep and goat herds is to obtain healthy offspring and increase the herd size. ORFV infection in sheep and goats commonly causes proliferative and self-limiting scab lesions on the lip skin, around the nostrils, rarely on the oral mucosa, feet and teats (Atli, 2017).

In the study, using 7 different types of primers that we previously detected intensively in our region (Kaya and Kale, 2022), viral genome presence was detected in 10 out of 29 sheep (34.48%) and 14 out of 29 goats (48.28%). Tedla *et al.* (2018) determined the ORFV prevalence in sheep as 15.5% and in goats as 8.5%, while Ifende *et al.* (2019) reported the ORFV prevalence in sheep as 4.2% and in goats as 3.6%. In contrast, Esmaeili *et al.* (2021) obtained a result of 44.6% positive for ORFV presence in sheep and 45.5% in goats.

In this study, sheep had the highest detection of GIF/IL-2, PPP1-PPP4 and B2L primers, whereas goats had the highest detection of Orf1-Orf2 and B2L primers. Alam *et al.* (2012) and Kaya and Kale (2022) have previously identified the presence of ORFV GIF/IL-2 primers in sheep and goats and in lambs and kids, respectively. PCR studies using PPP1-PPP4 primers in sheep and goats have been found positive in some studies (Kottaridi *et al.,* 2006b; Castro *et al.,* 2019; Şevik, 2019; Esmaeili *et al.,* 2021). In a study conducted in Nigeria, 100% positivity was found in 60 male goats using Orf1-Orf2 primers (Adedeji *et al.,* 2017). B2L gene primers were found in 34 adult local breed goats from Belbis and Sharkia cities in northern Egypt by Shehata *et al.* (2019). Zeedan *et al.* (2015) detected positivity in 3 out of 15 sheep and 4 out of 15 goats using B2L primers in a PCR analysis, while El-Tholoth *et al.* (2015) found 24 out of 30 PCR-positive results using B2L primers in a study conducted on a farm with 500 animals showing clinical signs of ORFV. Our study also identified positivity using VIR1 VIR2, vIL-10-3 vIL-10-4 and Alpha-tubulin primers. Indeed, the presence of VIR1 VIR2 (Maganga *et al.,* 2016), vIL-10-3 vIL-10-4 (Kottaridi *et al.,* 2006b) and Alpha-tubulin (Markoulatos *et al.,* 2000; Kottaridi *et al.,* 2006b) has been identified in sheep and goats by various researchers.

The infected animals had the typical multifocal to consolidating, ulcerated lesions on the epidermis of the gums, lips, mouth margin, muzzles, nose and udder. Additionally, considerable proliferative papules, pustules, crusting and brownish scabs appeared on infected animals. Within a few days, orf pustules appear, followed in a few weeks by ulcers and a thick scab with no formation of scar tissue (Abbas and Mughal, 2014). A superficial lesion's etiological agent may cause it to crust, scab over and

Fig 3: Gross appearance of the goats before and 5-days after treatment.

Case 1 (A-B) and Case 2 (C-D) [Gross appearance of the goats before and 5-days after treatment]. Case 3 (E-F) [Gross appearance of the goat before and 21-days after treatment].

eventually fall off. Additionally, opportunistic microbes could enter these wounds and spread disease (Kumar *et al.,* 2014). The hallmarks of microscopic lesions in the vesicular stage include marked epidermal hyperplasia, ballooning degeneration and eosinophilic intracytoplasmic inclusion bodies within keratinocytes (Tahir *et al.,* 2014; Vellucci *et al.,* 2020). Additionally, the stratum granulosum and spinosum are the main locations for keratohyalin granules. These granules, which are water-insoluble and support cell dehydration, are present in the cytoplasm. These granules go through cellular adjustments during keratinocyte development that lead to the conversion of keratin tonofilaments into a homogenous keratin matrix, a crucial step in cornification (Hermanns-Le *et al.,* 2004; Takahashi *et al.,* 2010). The macroscopic and histopathological lesions observed in this study were in consistent with ORFV infection in sheep and goats, according to earlier research (Ozmen and Dolu, 2018; Aneed and Al-Saad, 2019; Hussain *et al.,* 2022).

Various alternative methods have been applied for the treatment of contagious ecthyma disease in sheep and goats caused by ORFV (Geerlings and Wageningen, 2001; Liu *et al.,* 2006; Sunitha *et al.,* 2019; Wang *et al.,* 2019) and successful results have been achieved in these studies. In this study, successful healing / regression was observed in the oral lesions that developed as a result of the triple combination application in all animals with positive ORFV viral genome. It was determined that the complete healing or regression period occurred within a range of 5-21 days as a result of triple combination applications. In this study, topical PAPILEND™® cream was applied daily to the lesions that developed as a result of the triple combination application in all animals with positive ORFV viral genome presence. The PAPILEND™® cream product contains Glacial acetic acid, Salicylic acid, Garlic oil, Tea tree oil, Glycerol monostearate, Stearic acid, Cetyl stearyl alcohol, Hydrogenated castor oil, podophyllin and water. Some of the ingredients in PAPILEND™® cream show topical cytotoxic and antimitotic effects. It has been reported that some of these ingredients (*e.g*. Tea tree oil, podophyllin) stop mitosis in epithelial cells at the metaphase stage, are very effective on warts with less keratin deposition such as plantar warts compared to warts with intense keratin deposition, have lytic effects that do not extend beyond epidermal cells, remain intact in the basal layer, do not cause scar formation when topically applied, are absorbed systemically and when applied to bleeding or recently biopsied warts, the amount of absorption increases (Saltık *et al.,* 2022). In addition to these animals, 1 mL of ivermectin was administered subcutaneously every 15 days to animals weighing 50 kg, for 2 times. Ivermectin is a potent antiparasitic drug that is effective against many helminths and arthropods and belongs to the macrocyclic lactone class. Avermectin B is derived from *Streptomyces avermitis*, an actinomycete found in soil. While structurally similar to macrolides, it does not exhibit antibacterial activity (Omura, 2008).

Ivermectin stimulates both cellular and humoral immunity. The prevalence of papillomas in the body is linked to the immune system. Therefore, cellular immunity is more effective than humoral immunity in reducing papillomas. Ivermectin's anti-tumor activity has also been reported. In mice, ivermectin application has been shown to increase antibody production, serum specific antibody activity and responses from T lymphocytes and macrophages (Rao *et al.,* 1987; Blakley and Rousseaux, 1991; Uhlir, 1991; Drinyaev *et al.,* 2004; Korystov *et al.,* 2004; Börkü *et al.,* 2007). Additionally, in the study, infected animals were given 10 grams of Alquermold™ premix powder per day per animal for 10 days. Vitamin E, selenium, copper and zinc are important substances that strengthen the immune system against diseases in cattle (Spears and Weiss, 2008). Vitamin E is mainly obtained from feed (Baldi *et al.,* 2000). When prepared feed is stored for a long time or processed, the amount of vitamin E decreases. Additionally, when grazing, they may not receive enough vitamin E from plants (Baldi, 2005). Selenium may not be present in sufficient amounts in the soil or in plants (Sharma *et al.,* 1983). Selenium is an essential component of the organism's antioxidant defense system (Kieliszek and Błażejak, 2016). Selenium exhibits a synergistic effect with vitamin E (Willshire and Payne, 2011). Zinc plays an important role in keratin production (Tomlinson *et al.,* 2004). Copper and zinc are important in fighting microorganisms and strengthening the immune system (Spears, 2000).

Lyophilized live vaccines are widely used for protective purposes in Turkey and worldwide in cases of orf disease (Rziha *et al.,* 2000; Musser *et al.,* 2008; Hosamani *et al.,* 2009; Bimeda Biologicals, 2022; Colorado Serum Company, 2022; Dollvet, 2022; NOAH Compendium, 2022; Ministry of Agriculture and Forestry Istanbul Pendik Veterinary Control Institute, 2022; Vetal, 2022). Advances in the field of recombinant DNA vaccines are still ongoing for the development of ORFV vaccines through experimental studies (Orta *et al.,* 2022). Vaccination is the most basic protection method against orf disease, but short-term immunity and the need for annual revaccination are significant drawbacks. Therefore, alternative treatment methods can be used in the early stages of the disease to prevent its spread within the herd. In recent years, the use of alternative treatment products developed by various researchers for orf disease has gained importance. Therefore, it has been suggested that efforts should be made to develop new commercial products in this field as well (Orta *et al.,* 2022).

CONCLUSION

In conclusion, different ORFV primers and PCR test applications that are commonly used for detection can be appropriate and it is recommended to work with other different ORFV primers. Tests such as histopathology and immunohistochemistry can also be used for confirmation. In addition to ORFV preventive vaccinations in sheep and

goats, our study has shown that the use of triple combined alternative applications was successful for treating affected animals effectively.

Conflict of interest: None.

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