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Comparative study of three different routes of experimental inoculation of the orf virus

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ABSTRACT

Three different methods of inducing experimental infection of lambs with orf virus (ORFV), the cause of Contagious Ecthyma, were examined in nine animals; intradermal inoculation, subcutaneous injection and epithelial scarification. The objective was to identify the most appropriate experimental method to reproduce the disease with lesions of similar severity in all infected animals. Subcutaneous inoculation failed to reproduce orf lesions in two of the three infected animals, whereas both the groups that were inoculated by intradermal and scarification routes, respectively, displayed a significantly higher number of lesions at 12 dpi than the group inoculated subcutaneously. However, the lesions following scarification spread from the inoculation site with no ORFV-associated lesions found in other areas of the mucous membrane or skin. Finally, following intradermal inoculation, ORFV-associated lesions developed homogeneously in all infected animals, with lesions progressing from the point of inoculation in different areas of the skin of the lips, yet also spreading to the interior of the mouth, gums, palate and tongue, as occurs in natural infections. Thus, it was concluded that for studies investigating the efficacy of new approaches to treatment and vaccination for improved welfare of affected animals and control of ORFV transmission, the most appropriate route for experimental ORFV infection is intradermal inoculation.

1. Introduction

Contagious ecthyma, also known as Scabby Mouth or orf, is a contagious disease of worldwide distribution caused by the DNA orf virus (ORFV) belonging to the Parapox-virus genus of the Poxviridae family (Nandi et al., 2011). Orf mainly affects domestic and wild ruminants of any age, although the disease is mostly observed in young animals on affected farms, with older animals eventually immunised by exposure to ORFV (Spyrou and Valiakos, 2015). Morbidity may be high as 100%, although mortality is usually low and less than 5% (Haig et al., 1997; Hosamani et al., 2009). The repetition of outbreaks in each season, when susceptible young animals are present, is explained by the contamination of the environment with infectious scabs and close contact with infected animals. Of importance is that orf is a significant zoonotic disease affecting mainly vets and farmers. Lesions in humans are usually located in contact sites, primarily the hands, where they are often painful and itchy. As in ORFV infections in animals, lesions in humans may persist for several weeks (Nandi et al., 2011; Spyrou and Valiakos, 2015).

ORFV replicates exclusively in the cytoplasm of regenerating keratinocytes of the basal layer of the epidermis (McKeever et al., 1988; Haig and McInnes, 2002). Injuries from oral epithelial trauma, including the eruption of milk teeth in neonates, facilitate virus entry through the mucosa, establishing infection in the entire the oral cavity. As ORFV encodes several immunomodulatory proteins, it is able to evade the host immune response (Lloyd et al., 2000; Haig, 2006; Rohde et al., 2012).

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These include: viral chemokine binding protein (Lateef et al., 2010); viral endothelial growth factor (Martins et al., 2021); viral Interleuken-10 (Fleming et al., 1997); ORFV interferon resistance factor (Haig et al., 1998); and granulocyte-macrophage colony-stimulating factor (Deane et al., 2000). Although ORFV generally causes a self-limiting disease with the immunological mechanisms involved in viral neutralisation unknown, infection and replication uniquely in keratinocytes and evasion of the immune system through immunomodulatory proteins may explain the ability of ORFV to reinfect sheep and goats.

Clinical signs in sheep and goats are usually sufficient to establish the diagnosis, with the disease characterised as multifocal proliferative and pustular dermatitis affecting mainly the skin of the lips, muzzle, nose, and sometimes the eyelids and ears. Infections of the oral cavity may cause erosive to ulcerative stomatitis, affecting the gums, palate, and tongue. Although less frequent, infections can also involve the genitalia, udders and limbs of animals (Nandi et al., 2011; Spyrou and Valiakos, 2015). Lesions establish and progress through several stages, from macula to papule, vesicle, pustule, and crusts (Fleming et al., 2015), usually resolving in approximately 6–8 weeks. As painful scabs on the mouth and lips often prevent infected animals from feeding adequately, severe loss in body weight may occur (Lovatt, 2013). Orf outbreaks may cause both significant financial losses and animal welfare concerns in livestock production, particularly in association with intensive sheep and goat husbandry (Windsor et al., 2017). Orf is also associated with indirect losses from weight loss and increased susceptibility to other diseases due to immunosuppression, compromising production and profit margins from increasing costs associated with the provision of feeds, medicines, and veterinary services (Lovatt, 2013).

Clinical diagnosis must be differentiated from other relevant diseases that present with similar gross lesions, such as sheeppox, foot-and-mouth disease, bluetongue or peste des petits ruminants Nandi et al., 2011; Windsor et al., 2017). Histopathology provides a reliable diagnostic method, with microscopic features of the ecthyma lesions, including vacuolation and swelling of keratinocytes in the stratum spinosum, reticular degeneration, epidermal hyperplasia, mainly acanthosis, intraepidermal microabscesses and scab formation (Windsor et al., 2017). Eosinophilic inclusion bodies in the cytoplasm of keratinocytes can be seen but not in all phases of infection. Usually, these epidermal changes are accompanied by a mononuclear superficial dermatitis variable in severity. Lesions complicated by secondary infections can obscure the main microscopic changes by a severe and acute infiltration of neutrophils (Jenkinson et al., 1990). Molecular diagnostic methods, including quantitative polymerase chain reaction (qPCR) test or indirect ELISA, are increasingly widely used. ORFV qPCR is based on the detection of the B2L gene and is capable of detecting viral DNA in animal tissues and fluids before seroconversion (Wang et al., 2017; Kottaridi et al., 2006). The in-house indirect ELISA, based on recombinant protein ORFV 109, can detect anti-ORFV antibodies in sheep (unpublished results).

At present, no effective treatment against ORFV is available. However, since it is a self-limiting disease, symptomatic treatment with dressings and local antiseptics can be helpful. Topical and systemic antibiotics have been repeatedly recommended and promoted to reduce secondary bacterial contamination of ORFV-associated lesions. However, with the emergence of antimicrobial stewardship in response to the priority one health issue of managing antimicrobial resistance (AMR) risk, there is a need for treatment protocols that provide more effective topical treatment of orf without the use of antibiotics (Lacasta et al., 2021). Control of ORFV by vaccination has been used effectively in some countries (e.g. Australia). Currently, there are no orf vaccines commercialised in many European countries (Lacasta et al., 2015), and those registered are purified scab-based vaccines (Bukar et al., 2021) and cell culture-based live-attenuated vaccines (Bukar et al., 2021; Zhu et al., 2022) that present the risk of reversion to virulence. For this reason, prototypes of DNA and subunit vaccines based on proteins ORFV B2L (ORFV011 gene) and ORFV F1L (ORFV059 gene) are being studied as vaccines against ORFV infections (Zhao et al., 2011; Zhu et al., 2022; Wang et al., 2022).

Despite widespread distribution among sheep and goat populations globally and causing significant economic losses and welfare concerns, contagious ecthyma has not been extensively studied. Research on new treatments to aid more rapid recovery following the onset of clinical signs, methods that prevent the spread of the virus, and the development of new safe and effective vaccines, is required. To assess the efficacy of new preventative and therapeutic approaches, it is necessary to establish an experimental infection methodology that ensures similar lesion development in all inoculated animals. This will reduce both the number of animals required for clinical trials and heterogeneity occurring amongst cohorts in trials. Several studies have successfully reproduced ORFV infection using scarification, involving the scratching of the lips or buccal mucosa of a healthy animal with an emulsion of scabs from an infected case (Yirrell et al., 1989; Cargnelutti et al., 2011; Tryland et al., 2013). However, this technique precludes measurement of the viral concentration used and fails to produce a homogeneous progression of lesion development in infected animals.

Prior to the commencement of new studies on treatments and vaccines for contagious ecthyma, a study was designed to identify the preferred route of ORFV inoculation in establishing a similar rate and severity of lesion development and ensuring a homogeneous viral load in all infected animals, reducing both the heterogeneity between the treated and control groups and the number of animals needed in each group. Three different methods of experimental infection with the ORFV were proposed: epithelial scarification, subcutaneous injection, and intradermal inoculation.

2. Materials and Methods

To investigate the preferred route of inoculation of ORFV in establishing experimental infections, the three different methods of epithelial scarification, subcutaneous injection and intradermal inoculation were examined in nine newborn lambs obtained from a commercial sheep farm without orf outbreaks occurring during the last three years. Serum samples were obtained from their mothers to confirm the absence of anti-ORFV antibodies by an in-house ELISA test.

All the procedures were supervised and approved by the Ethics Advisory Commission for Animal Experimentation (nº PI33/21), the Biosafety Committee and the Occupational Risk Prevention Unit of the University of Zaragoza, in accordance with current regulations regarding these procedures. aspects (R.D. 53/2013, Law 31/1995, R.D. 664/1997, R.D. 1299/2006).

2.1. Studied lambs

Once the negative results of the ewes were confirmed, nine 15-day-old lambs were transported to the Veterinary Faculty of Zaragoza facilities and tagged with individual identification (85, 91, 93, 95, 109, 111, 112, 113 and 117). After spending a week in quarantine, they were transferred to an isolated facility to avoid contagion to other animals during the experiment. The lambs were fed with artificial milk, commercial feed, and straw ad libitum during their stay.

The nine lambs were randomly distributed into three experimental groups (3 per group), kept in separate boxes and under controlled conditions throughout the experiment to avoid transmission and contamination between groups. In each handling and management of the lambs, gloves, plastic boots and overalls were changed between groups and biosecurity measures were strictly followed, and each animal had its own bottle for feeding that was disinfected after use.

2.2. Experimental infection with ORFV

The experimental infection of the lambs was conducted with isolated

wildtype ORFV, and three routes of administration were evaluated: intradermal (group 1), subcutaneous (group 2) and scarification (group 3).

2.3. Wildtype ORFV isolation

Wildtype ORFV used for the experimental infection (T0) was isolated by standard methods (Zwartouw et al., 1962). Briefly, scabs collected from skin lesions of Rasa Aragonesa lambs affected during a naturally occurring outbreak of ORFV in Navarra (Spain) were macerated with liquid nitrogen. The resulting powder (viral isolate) was diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (fetal bovine serum), 1% L-glutamine, and 1% antibiotics/antimycotics mix (Sigma Aldrich, St. Louis, Missouri, USA). Subsequently, dilutions were centrifuged and filtered for the first purification step. Purified ORFV was added to bovine oesophagus cells (KOP-R) cultures, and the cytopathic effect was monitored for 24 h. Different freezing (-80 ºC) and thawing cycles were performed. By the third cycle, the cytopathic effect was not observed until 48–96 h. Then, the supernatant was collected, and the presence of the virus was confirmed using the commercial qPCR kit EXOone Contagious Ecthyma (Exopol, Spain), targeting the BL2 gene. Titration was performed on KOP-R cells using the Reed–Müench method (Reed and Muench, 1938). Finally, ORFV was distributed in 1 ml aliquots containing 5 ×104 TCDI50. One aliquot per lamb was used for the experimental infection (T0).

2.4. Experimental infection by intradermal inoculation (ID)

The lambs included in group 1 (ear tags 91, 93 and 113) were inoculated using the intradermal route (ID). ID delivery was achieved via jet injection, a needle-free method that creates a fine stream of pressurised liquid that penetrates the skin. The used gun was regulated to deliver 0.05 ml in each shot, and 1 ml/animal was inoculated in 20 shots distributed in different lips and gum areas. Four inoculations were applied to the left upper lip, 4 to the left lower lip, 4 to the right upper lip, 4 to the right lower lip, 2 to the central interior of the upper lip and 2 to the central interior of the lower lip.

2.5. Experimental infection by subcutaneous inoculation

Each of the lambs included in group 2 (tags 85, 95 and 112) was experimentally infected subcutaneously using a syringe with a 23Gx25mm fine needle. The inoculum volume was distributed in 5 punctures in the labial commissure with 0.2 ml at each point: left upper lip, left lower lip, right upper lip, right lower lip, and front area of the lower lip.

2.6. Experimental infection by scarification

As previously described, lambs from group 3 (109, 111 and 117) were infected by scarification in the labial commissure (Yirrell et al., 1989; Cargnelutti et al., 2011; Tryland et al., 2013). One cm x 3 cm strips of 80-grain sand-paper were previously autoclaved, and the upper and lower lips on the left and right sides were scraped, leaving intense irritation and micro-wounds. Next, half the volume of inoculum (0.5 ml) was applied with sterile pipettes throughout the injured area in the left lip and 0.5 ml in the right lip.

2.7. Clinical examination

After the experimental infection procedures, the lambs were kept for a period of 20 days in isolated facilities, with clinical examinations and images taken daily, involving the front and both sides of the face and including oral cavities in all animals. Subsequently, the images were grouped by lamb based on the numbering of the ear tags. For the statistical study, the images were analysed individually, and the lesions were numerically coded based on the anatomical location and severity.

In addition, a more detailed clinical examination was carried out on days 1 (T1), 5 (T2), 12 (T3) and 18 (T4) post-infection (dpi). Parameters including body condition, temperature, heart and respiratory rates were recorded.

2.8. Haematological analysis

Whole blood samples were collected from the jugular vein into EDTA anticoagulant tubes for subsequent haematological analysis. Samples were taken just before the experimental infection (T0) and at the end of the experiment (T5).

Haematology was performed with an IDEXX ProcyteDx automatic haematology counter (IDEXX laboratories, Westbrook, ME, USA). Measured parameters included leukocytes (K/ml), erythrocytes (M/µl), haemoglobin (g/dl), haematocrit (%), platelets (K/µl), VCM (Mean Corpuscular Volume; fl), HCM (Corpuscular Hemoglobin Mean; pg), MCHC (Mean Corpuscular Hemoglobin Concentration; g/dl) and reticulocytes (K/µl). White series blood cells were also evaluated by counting neutrophils (K/µl), lymphocytes (K/µl), monocytes (K/µl), basophils (K/µl), and eosinophils (K/µl).

2.9. Biomolecular analysis

For detection of ORFV viral load in infected skin and mucous membranes, samples were collected on T0, T1, T2, T3, T4 and T5 (20 dpi; necropsy) using sterile swabs. Swabs were passed superficially through two or three lesions per animal. When there were no lesions, the swab was passed to the lips where the experimental infection had been carried out. A simple smooth pass was performed in order to not produce high bacterial contamination that interferes with the qPCR. Nucleic acid was extracted using the MagMAX™ Pathogen RNA/DNA commercial kit (Thermo Fisher Scientific) and the KingFisher Flex System automated magnetic particle processor (Thermo Fisher Scientific), following manufacturer's instructions. Finally, qPCR was performed on a FAST 7500 cycler (Applied Biosystems) using the commercial qPCR kit EXOone Contagious Ecthyma (Exopol, Spain), targeting the BL2 gene.

Serum samples were taken on T0, T1, T2, T3, T4 and T5, with levels of ORFV-specific antibodies measured by in-house indirect ELISA. The initial fraction of ORFV envelope protein 109 (109rec), and three other peptides from the C-terminal part of the protein, also called P1, P2 and P3 were synthesised. To amplify the sequence that encodes the ORFV envelope protein 109, primers and PCR conditions described by Peralta et al. (2015), were used. Three combinations in different ELISA plaques were made: 109rec+P1, 109rec+P1+P2 and 109rec+P1+P2+P3.

2.10. Post-mortem study

Twenty days after the experimental infection (T5), animals were euthanised, and a detailed necropsy was conducted, with the number of ORFV-associated lesions, anatomical location, and severity recorded. Tissue samples from the skin, lip, tongue, palate, oesophagus, and rumen were systematically taken, although not all of these presented with macroscopic lesions. These samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and 4-µm thick sections were stained with haematoxylin and eosin (HE) and observed under a light microscope. The severity of macroscopic and microscopic lesions was scored from 0 to 3 independently: 0, absence; 1, mild; 2, moderate; 3, severe (Fig. 1).

2.11. Statistical analysis

All the data collected were integrated into a statistical matrix of the SPSS STATISTICS 26.0 program (IBM Corp., Chicago, IL, USA), with statistical tests applied and interpreted according to Petrie and Watson (2013). The comparison between groups for type and severity of injury

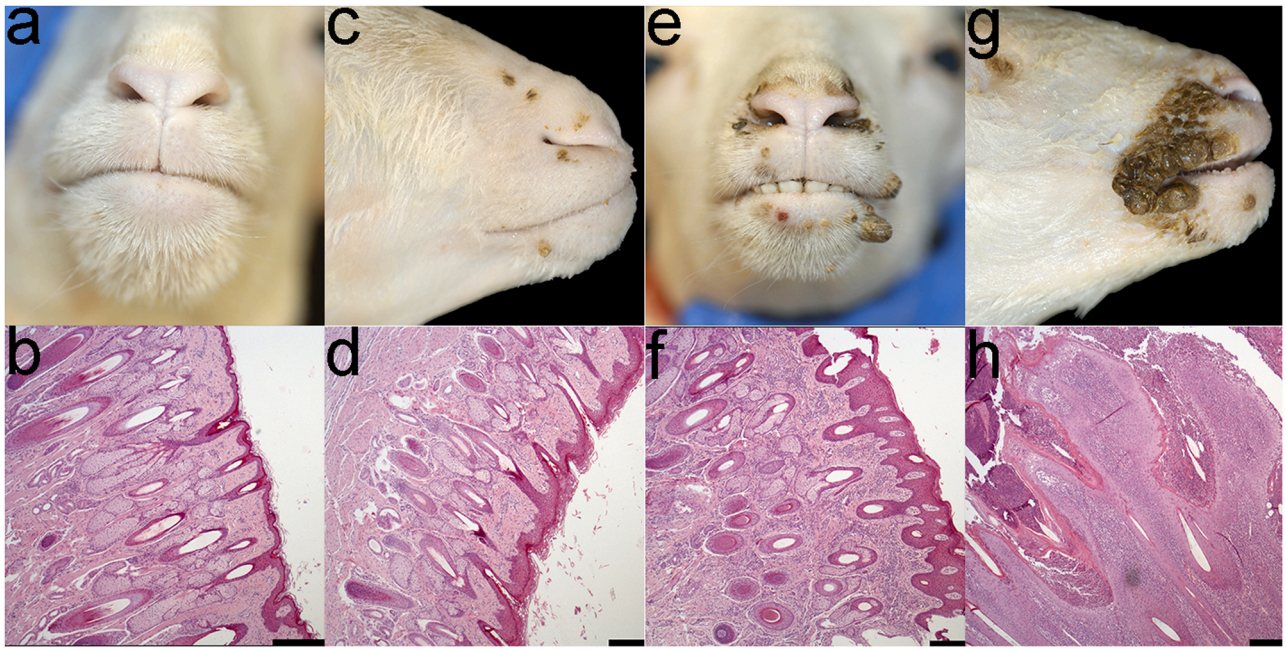

Fig. 1. Severity grade of gross and microscopic ORFV-associated lesions. Grade 0 is classified as the absence of macroscopic (a) and microscopic (b) lesions. Grossly, grade 1 shows papules, vesicles and pustules distributed around the muzzle (c). Histologically, grade 1 is composed of mild multifocal lymphoplasmacytic dermatitis admixed with acanthosis, orthokeratosis, hyperkeratosis and multifocal swelling and ballooning of the epidermis (d). Grade 2 is composed of multifocal scab formations with less number pustules (e). Microscopically, grade 2 is characterised by severe acanthotic proliferative dermatitis (f). Grade 3 shows multifocal to coalescence scabby proliferative and necrotising dermatitis (g), histologically observed as a total loss of skin architecture, replaced by lymphoplasmacytic, neutrophilic infiltrate and pericellular crusts (h).

(qualitative and ordinal variables, respectively) was made using Pearson's chi-square test. For the comparison between groups of the number of lesions per individual, the Kruskall–Wallis test (non-parametric) was used. The study of the time until the appearance of the first lesion was conducted using survival analysis (Kaplan-Meier method), and the comparison between groups was performed using the Breslow test. The relationship between quantitative variables (number of lesions per individual and number of PCR cycles) was analysed using the Rho se Spearman coefficient. The ANOVA (analysis of variance) was used for the comparison between groups of the number of PCR cycles (quantitative variable). In the case of multiple comparisons, the Bonferroni correction was applied. In all the statistical tests with which the association between variables was determined, values of $p<0.05$ were considered significant.

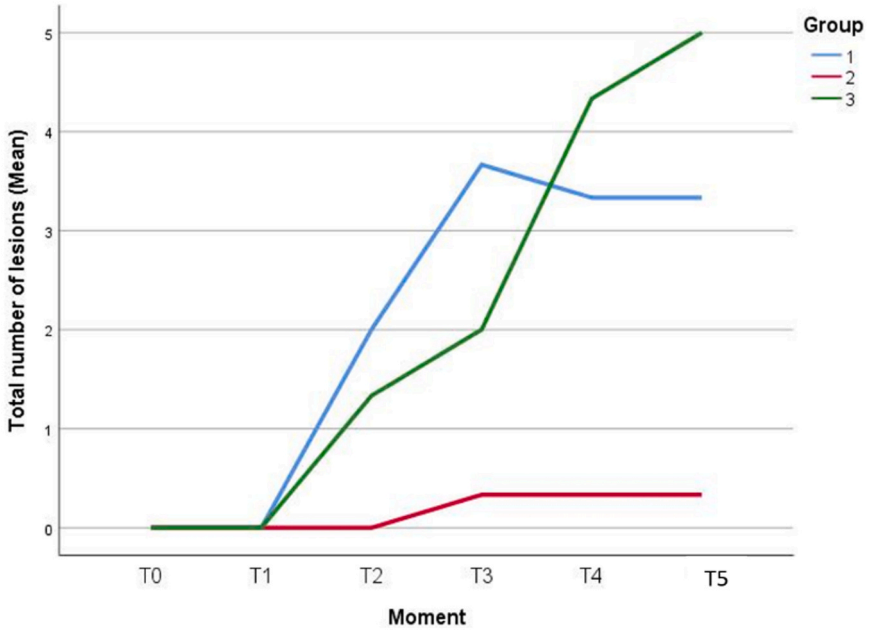

Fig. 2. Temporal evolution of the total number of ORFV-associated lesions observed in the clinical examination in the different inoculation groups: intradermal (group 1), subcutaneous (group 2) and scarification (group 3).

3. Results

3.1. Clinical examination

The lambs of groups 1 and 3 commenced the presentation of ORFV-associated lesions on 5 dpi (T2) (Fig. 2). The lambs of group 2 did not show lesions throughout the experiment, with the exception of lamb 85, commencing the development of mild lesions on the right muzzle on 7 dpi. Groups 1 and 3 showed a significantly higher number of lesions than Group 2 at T3 ($p = 0.041$) and T5 ($p = 0.03$), respectively.

Development of the ecthyma lesions in the three groups throughout the 20 days of the experiment is displayed in Fig. 3 (intradermal), Fig. 4 (subcutaneous) and Fig. 5 (scarification).

Vesicles and papules took between 3 and 7 dpi to become crusts, depending on the extent and location of the lesions. Lesions in Group 1 spread to other anatomical locations, including the oral mucosa, affecting the palate, tongue and/or gums (Fig. 3) with an exudate observed, suspected as indicative of a possible secondary bacterial infection. In Group 3, proliferative tissue spread only from the inoculation site (commissures), progressing as a locally extensive, raised, circumscribed inflammatory mass (Fig. 5) and no lesions were observed in locations far from the point of inoculation.

Classification of lesions was conducted into four categories: no visible lesions; vesicles/pustules only; both vesicles/pustules and scabs; and scabs only (Fig. 6). At T2, vesicles or papules were already detected in all lambs of Groups 1 and 3, whereas lesions were absent in Group 2 ($p = 0.011$). Similarly, at T3, scabs had already appeared in Groups 1 and 3 only ($p = 0.036$), although the distribution between lesions categories was different between groups ($p=0.036$). Significant differences were only detected in the category "both lesions" ($p<0.050$), with all lambs of Group 1 present (Fig. 6). However, at T4 and T5, no significant differences were detected between the groups.

In the detailed clinical examination, all the animals presented tachycardia, although this observation was attributed to the stress of handling and management. Some lambs also displayed occasional bouts of mild diarrhoea that were attributed to dietary changes or imbalance (milk/feed intake).

3.2. Haematological analysis

The haematological parameters were within the normal ranges in all groups at the two samplings of T0 and T5, with no significant differences found between the groups.

3.3. Biomolecular and serological analysis

qPCR detected the presence of ORFV in skin swab samples of all inoculated animals (Fig. 7). The first positive qPCR results appeared at T1. For T1, T2, T3 and T5, no significant differences were detected between groups. However, at T4, Group 1 had a significantly higher ORFV viral load than Group 2 ($p < 0.050$). In addition, a higher number of lesions corresponded with a higher viral load (Spearman's rho $= -0.490$ and $p = 0.002$). Throughout the study, anti-ORFV antibodies were not detected in any of the lambs.

3.4. Port mortem findings

On gross examination, Group 1 displayed severe ORFV-associated lesions, distributed on the lips and muzzle. In addition, lamb 91 and 113 presented with lesions on the tongue, palate and gums (Fig. 3). Two of the lambs in Group 2 did not develop lesions, whereas lamb 85 presented with a focal mild lesion on the right muzzle (Fig. 4). Group 3 showed severe lesions, uniquely in the location of ORFV inoculation (Fig. 5). As histopathological examination revealed that mild and/or moderate lesions were present in tissues where macroscopic lesions were not observed, the statistical evaluation of the lesion severity incorporated findings from the microscopic examinations (Table 1). The severity of lesions between Groups 1 and 3 was similar, with 100% of the skin lesions classified as severe and both higher than in Group 2 ($p = 0.061$). Palate and tongue lesions were only observed in Group 1 and categorised as mild-moderate and severe, respectively. Lip lesions were categorised as moderate in Group 1 and severe in Group 3 ($p = 0.006$).

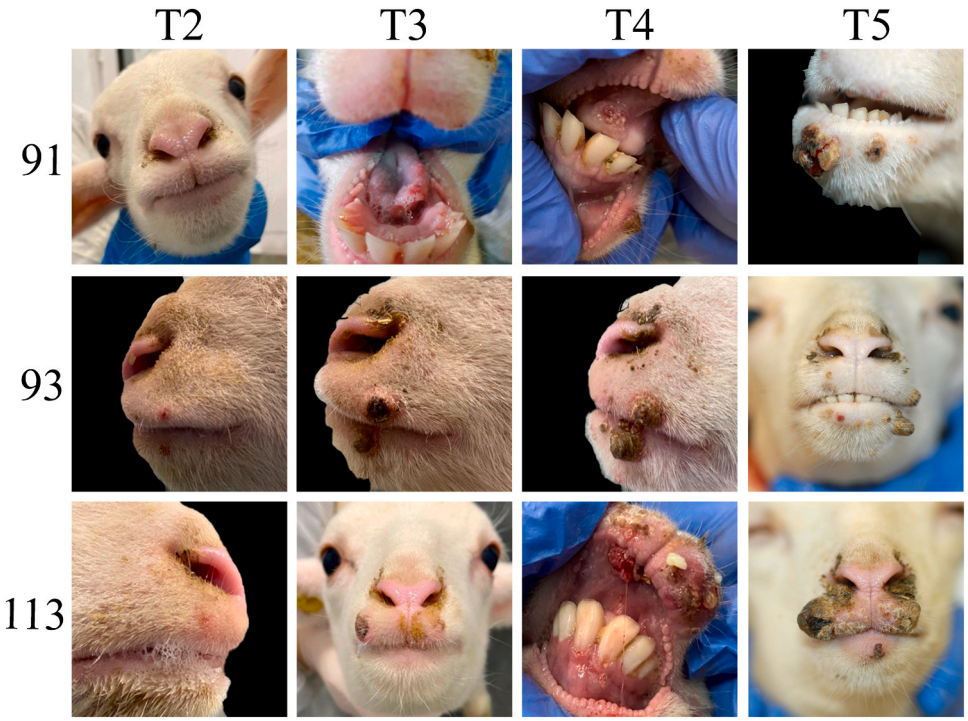

Fig. 3. Images of the ORFV-associated lesions in the lambs inoculated intradermally (Group 1) in T2 (5 dpi), T3 (12 dpi), T4 (18 dpi) and T5 (20 dpi).

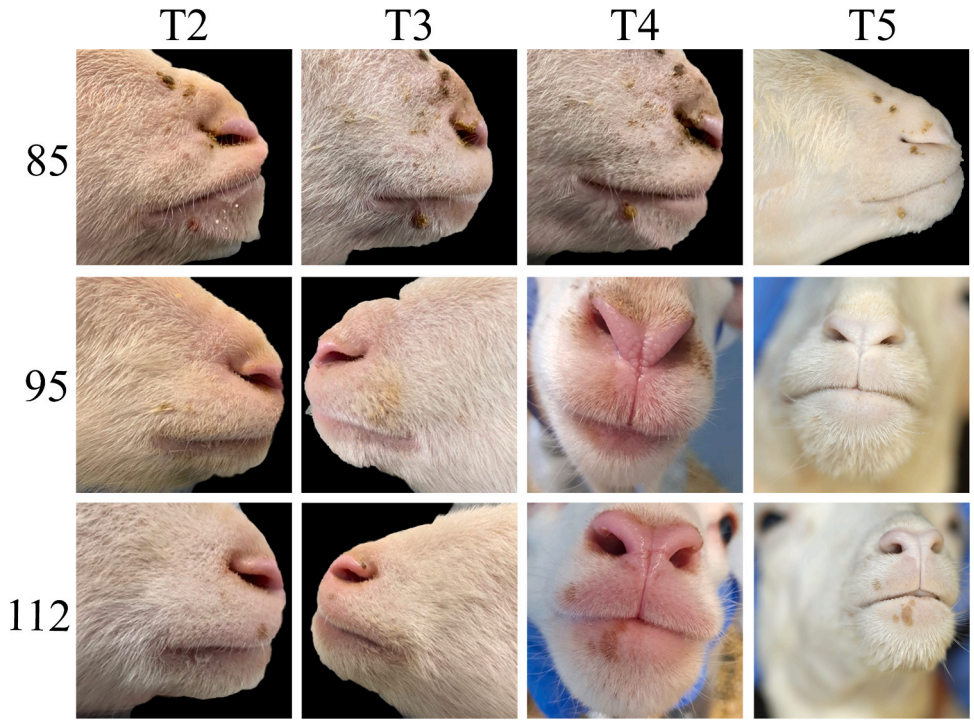

Fig. 4. Images of the ORFV-associated lesions in the lambs inoculated subcutaneously (Group 2) in T2 (5 dpi), T3 (12 dpi), T4 (18 dpi) and T5 (20 dpi). (Note: Animal 112 presented areas of hyperpigmentation on the muzzle. They should not be confused with ORFV-associated lesions.).

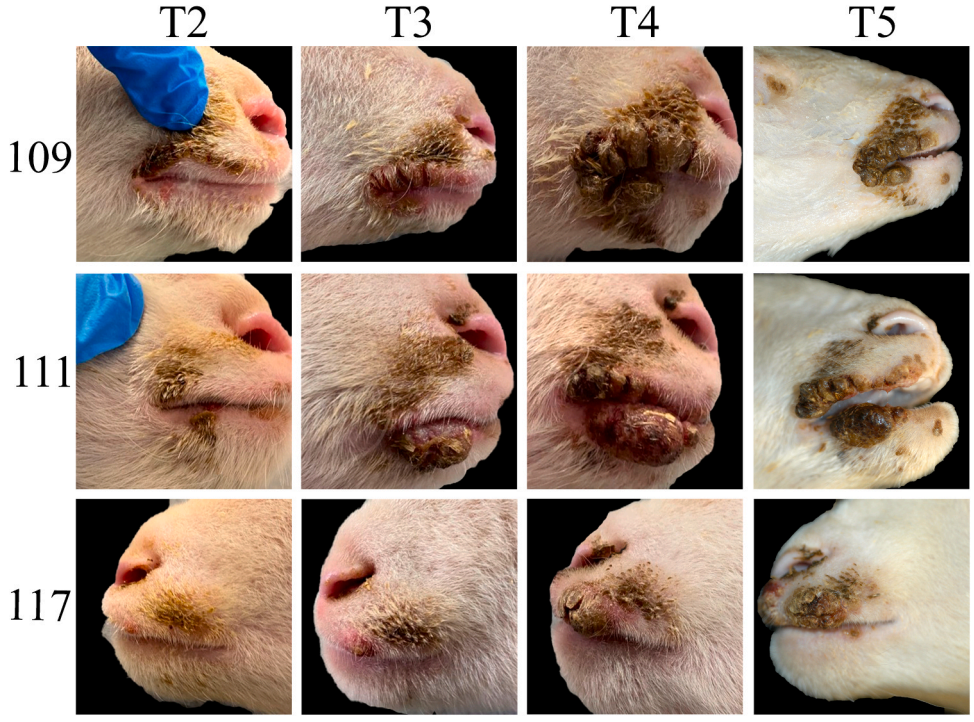

Fig. 5. Images of the ORFV-associated lesions in the lambs inoculated by scarification (Group 3) in T2 (5 dpi), T3 (12 dpi), T4 (18 dpi) and T5 (20 dpi).

4. Discussion

Contagious ecthyma is a zoonotic viral disease with a worldwide distribution that causes significant economic losses in the sheep and goat industries (Nandi et al., 2011), although few studies of ORFV infection have been reported in recent decades, particularly regarding new approaches to therapy and control. On affected farms, usual treatments involve the use of antibiotics to control potential secondary infections, despite the increasingly recognised risk that this may pose to the development of antimicrobial resistance. Further, vaccines are unavailable in many countries, and those that do exist are purified scab-based vaccines (Lacasta et al., 2015; Bukar et al., 2021) and cell culture-based live-attenuated vaccines (Bukar et al., 2021; Zhu et al., 2022), risking reversion to virulence. New studies on therapeutic and vaccination

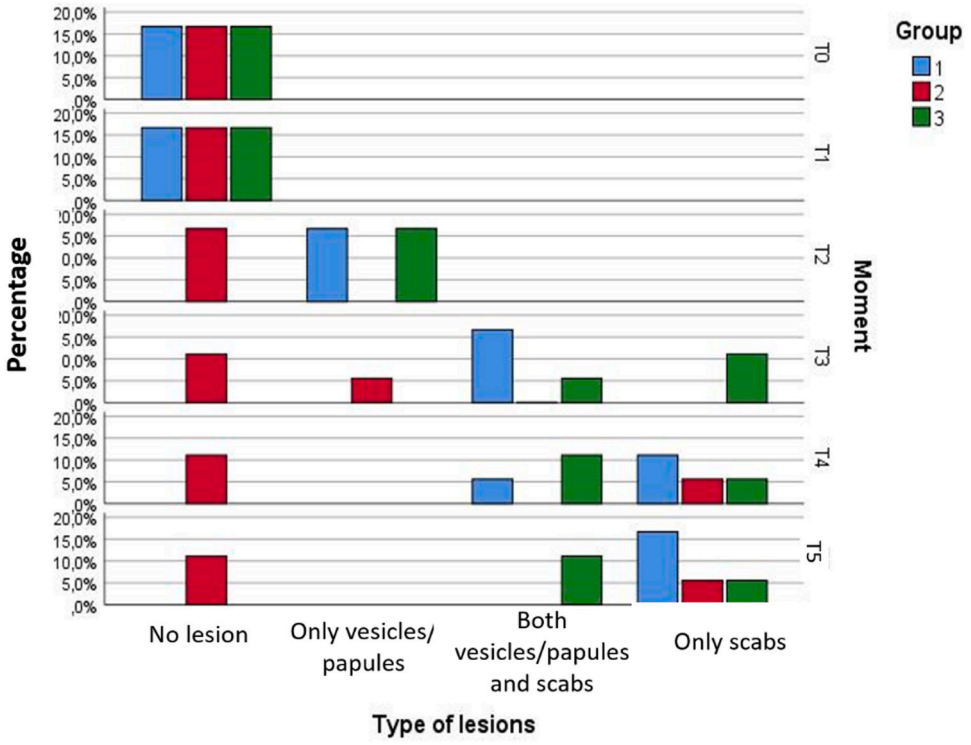

Fig. 6. Temporal evaluation of ORFV-associated lesion types (No lesion /only vesicle-papules/ vesicle-papules and scabs/ only scabs) present in the different inoculation groups: intradermal (group 1), subcutaneous (group 2) and scarification (group 3) in T0, T1 (1 dpi), T2 (5 dpi), T3 (12 dpi), T4 (18 dpi) and T5 (20 dpi).

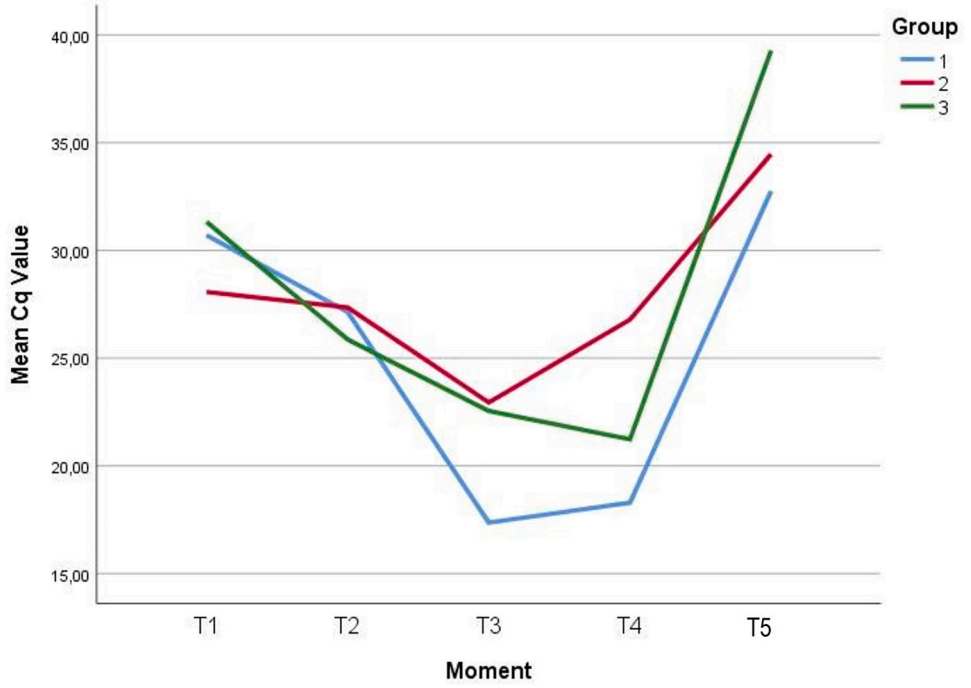

Fig. 7. Progression of the ORFV viral load in the skin of the different inoculated groups: intradermal (group 1), subcutaneous (group 2) and scarification (group 3) in T1 (1 dpi), T2 (5 dpi), T3 (12 dpi), T4 (18 dpi) and T5 (20 dpi). (Note: Lower Cq values indicate higher ORFV viral load.).

approaches are required to achieve control of this disease on small ruminant farms.

Prior to these further studies, a trial was conducted with the objective of determining the most appropriate route of experimental infection with ORFV. The aim was to achieve a reliable and homogenous progression of the lesions whilst being able to accurately determine the viral load inoculated. This is the first report comparing three different methods of administration of ORFV in lambs; intradermal inoculation, scarification and subcutaneous injection.

Scarification has been reported in the literature as the only route of inoculating ORFV in experimental infections in sheep, goats, reindeer, rabbits and mice (Yirrel et al., 1989; Cargnelutti et al., 2011; Tryland

Table 1
Severity of histopathological lesions at T5, categorised from 0 to 3: 0, absence; 1, mild; 2, moderate; 3, severe.

Severity of the lesions (Absence: 0; Mild: 1; Moderate: 2; Severe: 3	Group 1			Group 2			Group 3		
Affected organs	**91**	**93**	**113**	**85**	**95**	**112**	**111**	**117**	**109**
Skin	3	3	3	1	0	0	3	3	3
Tongue	3	0	3	0	0	0	0	0	0
Palate	2	1	1	1	0	0	0	0	0
Lips	2	2	2	1	0	0	3	3	3
Oesophagus	0	0	0	0	0	0	0	0	0
Rumen	0	0	0	0	0	0	0	0	0
Limbs skin	0	0	0	0	0	0	0	0	0

et al., 2013; Nashiruddullah et al., 2022). However, this route poses several challenges. Knowledge of the inoculated viral concentration is not possible as following skin abrasion, application of the inoculum over the injured skin results in most being lost. Further, lesions are limited to the injured area and the surrounding skin. In the present study, in concordance with previous reports (Yirrell et al., 1989; Cargnelutti et al., 2011; Tryland et al., 2013; Nashiruddullah et al., 2022), the inoculation by scarification was successful, with the disease reproduced in all 3 animals. However, the lesions following scarification spread from the inoculation site (commissures), progressing as a locally extensive, raised, circumscribed inflammatory mass, with no ORFV-associated lesions found in other areas of the mucous membrane or skin as happens in natural infections. Further, the process of both intradermal and subcutaneous administration enabled knowledge of the exact viral concentration inoculated. Interestingly, subcutaneous inoculation failed to reproduce orf lesions in two of the three infected animals, with only a single mild lesion observed in lamb 85, whereas both the groups that were inoculated by intradermal and scarification routes respectively, displayed a significantly higher number of lesions at 12 dpi than the group inoculated subcutaneously. Finally, following intradermal inoculation, ORFV-associated lesions developed homogeneously in all infected animals, with lesions progressing from the point of inoculation in different areas of the skin of the lips, yet also spreading to the interior of the mouth, gums, palate and tongue, as occurs in natural infections (Hosamani et al., 2009; Nandi et al., 2011; Windsor et al., 2017).

ORFV is epitheliotropic and replicates in regenerating epidermal keratinocytes, evading the defences of the host (Fleming et al., 2015). In lambs or kids, after an incubation period of approximately a week, an initial rise in temperature is accompanied by the development of skin lesions in the area of mouth, lips and nose (Spyrou and Valiakos, 2015). All the lambs from the intradermal inoculation and scarification groups developed ORFV-associated lesions by 5 dpi. In previous reports, experimentally infected lambs developed the first ORFV-associated lesions at 2 dpi (Yirrel et al., 1989) or 3 dpi (Cargnelutti et al., 2011), with first lesions observed in rabbits at 3 dpi, mice at 5 dpi, and reindeer at 5 dpi (Tryland et al., 2013), as in our results.

The general clinical progression of contagious ecthyma is from localised erythema, macula, vesicle, papule, pustule and then scab formation (Nandi et al., 2011; Spyrou and Valiakos, 2015). The gross and histopathological changes of the oral lesions of the lambs in this experiment were consistent with ORFV-associated lesions previously described (Jenkinson et al., 1990). Vesicles or papules were the first lesions to appear in groups inoculated intradermally and by scarification at 5 dpi, whereas lesions were not observed then in the group inoculated subcutaneously. Vesicles and papules progressed between 3 and 7 dpi to become scabs, whereas the first scabs were reported at 9 dpi previously (Yirrell et al., 1989). In our research, when the lambs were euthanised at 20 dpi for post-mortem study, no significant differences were found between the groups.

Contagious ecthyma usually lasts for 3–4 weeks, with lesions resolving in 1–2 months following the shedding of scabs without leaving a scar (Nandi et al., 2011). The final recovery reported in experimental infections varies from 20 to 28 dpi (Yirrel et al., 1989; Cargnelutti et al., 2011; Tryland et al., 2013; Nashiruddullah et al., 2022). In our experiment, at necropsy, 20 dpi, affected lambs only displayed scabs and proliferative tissue, with no vesicles or papules observed.

Infectious ORFV virions are detected 12 hours after infection, and the maximum viral titre has been reported to be between 24 and 72 hours post-infection (Lear, 1995). In our study, the first positive qPCR results appeared at 1 dpi, and all the infected animals remained positive throughout the experiment. However, at 18 dpi, Group 1, inoculated intradermally, had a significantly higher viral load than the group inoculated subcutaneously. In addition, a higher number of lesions corresponded with a higher viral load. A previous report found that virus isolation from the lesions in lambs was achieved between 2 and 19 dpi in lambs and between 2 and 14 dpi in rabbits, with most lesions yielding virus from 3 and 11 dpi (Cargnelutti et al., 2011). Similarly, viral nucleic acid in the skin could be detected by qPCR in goats at 8 dpi but not at 28 dpi when the animals were completely recovered (Nashiruddullah et al., 2022).

The immunity that develops against the ORFV is relatively short-lived, with a clear understanding of the mechanism of protective immunity yet to be achieved. Many studies have suggested that humoral immunity does not play a major role in protection (Buddle et al., 1984), with antibody titres merely indicating a previous infection in adult-infected animals. However, other studies suggest that a specific isotype (IgG2) has an important role in infection (Haig et al., 1998), although few studies report when experimentally infected animals begin to show specific ORFV antibodies. In our study, none of the lambs showed anti-ORFV antibodies throughout the experiment. However, the lambs were euthanised 20 dpi and in a subsequent study carried out by our group, the detection of anti-ORFV antibodies by the in-house ELISA was possible 30 days after infection (Lacasta et al., 2023). This is in accord with previous results in infected lambs that found antibodies at 28 dpi (Yirrell et al., 1989; Cargnelutti et al., 2011); it is of interest that infected reindeers displayed increased antibody levels by day 20 dpi (Tryland et al., 2013).

5. Conclusions

To the knowledge of the authors, previously reported experimental ORFV infections used the inaccurate method of scarification, with no reported analysis of different routes of inoculation. Our study indicates that the intradermal inoculation of ORFV offers a better approach, enabling knowledge of the precise concentration of virus inoculated, with lesions most resembling those occurring in natural infection, including spread from the area of infected skin to the tongue, gums and palate. In conclusion, the intradermal route of inoculation should be recommended as the preferred method for future experimental infections with ORFV.

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CRediT authorship contribution statement

Pablo Quilez: Investigation. **María Cuadra:** Investigation. **Alex Gómez:** Investigation, Methodology, Writing – review & editing. **Ramsés Reina:** Investigation. **Teresa Navarro:** Investigation. **Delia Lacasta Lozano:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft. **Héctor Ruiz:** Investigation. **Maite Verde:** Investigation. **Sergio**

Villanueva-Saz: Investigation, Writing – review & editing. **María Teresa Tejedor:** Data curation, Formal analysis, Writing – review & editing. **Marta Ruiz de Arcaute:** Investigation, Methodology, Resources, Writing – review & editing. **Juan Ramos:** Investigation, Project administration. **Ana Rodríguez-Largo:** Investigation. **Peter Windsor:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Aurora Ortín:** Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors of the article entitle: "Comparative study of three different routes of experimental inoculation of the orf virus" declare no conflict of interest.

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