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Dermal Response to Experimental *Orfvirus* (*ORFV*) Infection in Goats, Mice and Rabbit

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ABSTRACT

Background: During a study on the outbreak of orf in goats, it was intended to study the disease transmissibility in different hosts from field samples and ascertain the infective potential of the agent in laboratory animals compared to goats.

Methods: Cutaneous clinical materials from orf virus (ORFV) infected goats was used to experimentally infect naive goats, rabbits and mice and ascertain its infective potential and transmissibility in different hosts. The processed inoculum was applied topically to mimic a natural transmission through injured skin. Regular skin biopsies were taken that revealed characteristic macroscopic and microscopic lesions typical of orf.

Result: Virus inoculum applied on abraded skin in goats successfully established the lesions of orf. A parallel inoculation in rabbit and mice could not successfully reproduce the disease in these unnatural hosts beyond a subtle vesicular stage on 3 dpi with subsequent healing by 7 dpi. The lesions in goats regressed spontaneously by 28 days post-infection (dpi). Intracytoplasmic inclusions were associated only in the vesicular stage. Immunopathological progression was observed by immunoperoxidase staining of $CD4^+$ and $CD8^+$ T-cells which were found to appear by day 5 in the dermis and became more abundant and distributed by day 8, but subsequently reduced in number by 15 dpi. $CD4^+$ cells were found to be more numerous and widespread. Viral antigen in tissues could be demonstrated by 4 dpi by immunohistological methods that increased in signal intensity progressively and disappear by 28 dpi. Similarly, viral nucleic acid in the skin could be detected on day 8 dpi but not on 28 dpi by PCR. The present experiment depicts the ease of disease transmissibility through traumatized skin in the primary hosts, but establishment in unnatural hosts may not be readily achieved. The infection was self-limiting with possibly no virus latency as indicated by immunofluorescence and PCR studies.

Key words: Goat, Mice, ORFV, Pathology, Rabbit.

INTRODUCTION

Orf is an exanthematous disease of sheep and goats, caused by a virus of the family Poxviridae and subfamily *Chordopoxvirinae*. Orf virus (ORFV) is the type species belonging to the genus *Parapoxvirus* (PPV). ORFV is an epitheliotropic virus, with no systemic spread; neither there is any evidence of virus latency (Haig *et al.*, 1996); while the virus is shed with the scab and forms a source of contamination in the environment.

Although the disease primarily affects sheep and goats, but can also infect humans and many other domesticated and wild animals.

After the disease was recorded in goats from Assam (Nashiruddullah, 2014), isolated in cell culture (Nashiruddullah *et al.*, 2016), confirmed by PCR and visualized by electron microscopy (Nashiruddullah *et al.*, 2018), it was intriguing to study the disease transmissibility in different hosts from field samples and ascertain the infective potential of the agent. In view of the above, a trial was designed to infect mice, rabbits and goats with a field isolate of the virus from clinical scab material to mimic a natural transmission through injured skin.

MATERIALS AND METHODS

Animals for experimental infection

Two apparently healthy female Assam hill goats of

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approximately 6-8 months of age, weighing 8 and 10 kg each, procured from a local source with no known history or visible lesion of orf infection. The animals were clinically examined for illness, ecto- and endo-parasites, de-wormed and acclimatized for two weeks before infecting them with clinical isolates of ORFV. They were housed and reared in the animal shed of Pathology department and allowed to graze during the day in a confined isolated enclosure with access to *ad libitum* concentrate feed, forage and water.

Experimental animals also included four adult healthy mice and two rabbits that were similarly inoculated with ORFV isolates. The proposal for animal experimentation was approved by the Institutional Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), approval no. 770/ac/CPCSEA/FVSc/AAU/IAEC/12-13/181, dated 13/09/2013.

Preparation of inoculum

Scab samples taken from natural orf infection in a goat (sample BAG17) was used as a source of orf virus (ORFV). The pathogenic *Parapoxvirus* isolate was verified by PCR and electron microscopy (Nashiruddullah *et al.*, 2018). Inoculum was prepared according to Nashiruddullah *et al*. (2016).

Experimental infection in goats

On three occasions on every alternate day, about 4 mg (1 ml) dexamethasone (Dexasone, Cadila Pharmaceuticals Ltd., India) was injected intramuscularly to make them immunologically compromised. On the 5th day (*i.e.* last day of injection), the animals were treated with an herbal shampoo and cleaned around the cheeks and lips. The next day, the area around the cheeks and lip commissures were shaved. The cheek area was gently rubbed with sterilized sandpaper and marked with three linear scratches about 5 cm long and 1-2 cm apart with a sterile needle. The 20% scab derived and processed inoculum was swabbed over the injured skin.

Incisional skin biopsies were collected from the animals on 3, 4, 5, 8, 15, 21 and 28 days post inoculation (dpi) from the cheek with a surgical blade under 2% lidocaine local anaesthetic infiltration (Xylocaine 2%, Astra Zeneca, India) for adequate nerve block. Materials were immediately collected in 10% buffered formalin for histopathological and immunoperoxidase histological preparations.

Experimental infection in rabbit and mice

On three occasions on every alternate day, about 0.2 mg (0.5 ml) and 0.4 mg (0.1 ml) dexamethasone was injected intramuscularly to both rabbits (two animals) and mice (four animals) respectively to make them immunocompromised. On the 5th day (*i.e.* last day of injection), the inner pinna of one ear was swabbed clean with sterile PBS and traumatized with three linear scratches about 5 cm long and 1 cm apart with a sterile needle. The 20% scab derived and processed inoculum was swabbed over the abraded and injured skin. The traumatized skin was observed regularly for developing lesions.

Immunohistochemical detection

For rapid detection of ORFV antigen in tissues, cryosections were prepared and immunohistochemically stained.

A commercially available mouse monoclonal Orf Virus (2E5) (Santa Cruz Biotechnology Inc., sc101589) was used for detection of viral antigen (Lear *et al*., 1996; Lloyd *et al.*, 2000) in tissue cryosections. Indirect fluorescent immuno-histochemistry was done by using a goat anti-mouse IgG conjugated with FITC (Santa Cruz Biotechnology, #sc2010).

Mouse anti-sheep CD4 monoclonal antibody (AbD Serotec, #MCA2213GA) and Mouse anti-bovine CD8 monoclonal antibody (AbD Serotec, #MCA837GA) cross reactive to goat was used in immunochemical labelling of $CD4^+$ and $CD8^+$ cells in formalin fixed paraffin embedded goat tissue respectively. Indirect immunoperoxidase histochemistry was done by using goat anti-mouse IgG Peroxidase Conjugate (Sigma, #A4416).

PCR confirmation

Full-length *B2L* gene was amplified from goat skin tissue by PCR on the basis of published sequences of primers (Hosamani *et al.*, 2006).

RESULTS AND DISCUSSION

Experimental infection with ORFV in goats

Erythema appeared soon after inoculation. Faint, darkened, macular patches developed by 2 day post-inoculation (dpi). Oedema of cheeks was visible with appearance of papules along the streak borders on 3 dpi. Tiny, guttate vesicles appeared in one animal by day 4. Papules coalesced to form larger plaques by day 7 and scabs by day 8. The scabs matured and were found to shed spontaneously by day 10. Once scabs shed, they left a healed epidermis with none or little sign of scarring. The skin appeared completely healed with regrowth of hair by 26 dpi. There was no permanent skin lesion after 28 dpi.

Histopathological progression of lesions

Within the first 24 hours, skin biopsy showed a traumatized and abraded epidermis, along with haemorrhages in the underlying dermis.

By about the end of 48 hours (3 dpi), various microscopic changes include swelling of the otherwise flattened nucleated stratum granulosum keratinocytes make them appear rounded. Ballooning degeneration and eosinophilic aggregates (Fig 1a) were seen in the cytoplasm. Oedema, hyperemia and inflammatory cell infiltration is seen in the dermis. Some cells showed progressive vacuolation of the cytoplasm filling up the entire cell. The nuclei became pyknotic and pushed to the periphery. Vacuolation of cells progress deeper towards the spinous cells (Fig 1b) of the epidermis and also along the external hair root sheaths situated deep in the dermis. The underlying dermis showed oedema and hyperemia and infiltration of polymorphonuclear cells.

By 4 dpi, the histopathological changes noticed included extremely swollen and vacuolated stratum granulosum keratinocytes. The spinosum cells became progressively affected and disintegrated (Fig 1c), often showing nuclear pyknosis and karyorrhexis. Some spinous cells contained eosinophilic inclusion bodies in the cytoplasm. Early acanthotic changes of spinosum cells occurred with marked and progressive thickening of the Malpighian epidermis. The corneum disintegrated in places and was pushed outward, shaped into a dome (Fig 1d), while inclusions are demonstrable in spinous cells. This probably reflected macroscopically as tiny vesicles on the skin surface. Infiltration of polymorphonuclear inflammatory cells in the dermis was increasingly evident. Intradermal microabscesses were also seen and might reflect secondary infection of the scarified/abraded skin.

On 5 dpi, histopathological changes that were noticed included hyperkeratosis, acanthosis and marked vacuolation of the Malpighian cells (Fig 1e). Infiltration by polymorphonuclear cells in the dermis with some mononuclear cells was also noticed. The injured area over the scarification was locally infiltrated by a massive population of polymorphonuclear cells mostly neutrophils, amidst the necrosed tissue, cellular debris and probable contaminating bacteria (Fig 1f). Vacuolation of spinous cells were seen along the external sheath, deep in the dermis. Mononuclear cells including macrophages infiltrated into the dermis. There was also extensive necrosis of the affected areas and the commencement of the downward progression of rete ridges between the dermal papillae.

On 8 dpi, microscopic changes included marked hyperkeratosis, parakeratosis, acanthosis and deepening of the rete ridges (Fig 2a). The rete pegs invaded into the dermis much like in squamous cell carcinoma and result in islands of massive infiltrating cells of the dermis between the keratinocytes. The inflammatory cells still were predominantly polymorphonuclear. Neutrophils were loosely dispersed in oedematous fluid filled matrix. Mononuclear cell infiltration was also found to increase in numbers in the dermis. The epidermal layer in affected areas might undergo widespread degeneration and hyalinization. The milieu of cellular debris resulting from degeneration and necrosis in the superficial layers, together with invading pathogens and exudations of the inflammatory cells made up an external scab (Fig 2b). The scab was sharply demarcated from the underlying regenerating epidermis and was shed by the intense inflammatory reaction in the delimiting zone. At one time, the entire overlying necrotic mass was eventually replaced by the underlying tissue.

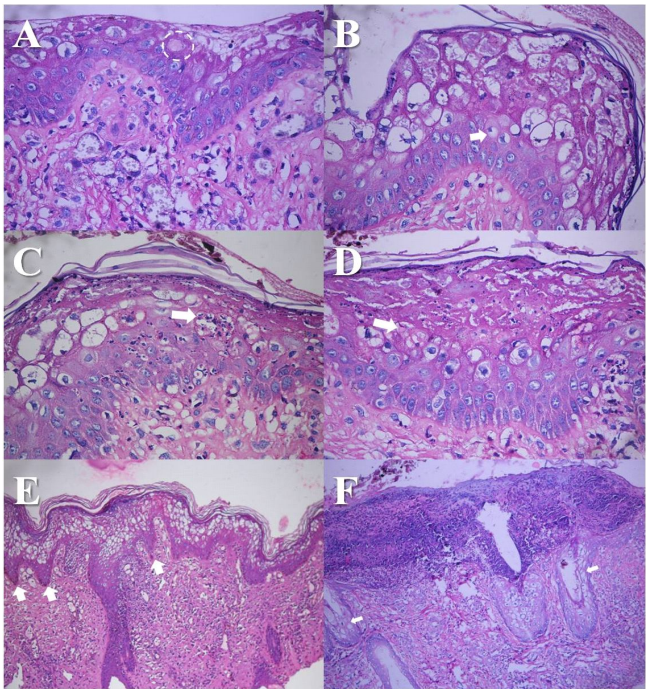

Fig 1: Histopathological changes in the skin of experimentally infected goats with orf virus (ORFV) at 3, 4 and 5 days post infection (dpi). (A) Skin biopsy at 3 dpi showing granulosum cells undergoing hydropic degeneration and eosinophilic cytoplasmic aggregates (circle). HandE X 400. (B) Skin biopsy at 3 dpi showing spinous cells with nuclear pyknosis and cytoplasmic vacuolation (arrow) like the granulosum cells above. H & E X 400. (C) The spinous cells at 4 dpi are swollen, vacuolated and most cells disintegrate (arrow). H & E X 400. (D) The corneal cells at 4 dpi disintegrate and inclusions appear in spinous cells. H & E X 400; (E) Hyperkeratosis, acanthosis and downward progression of the rete ridges (arrows) appear at 5 dpi, together with massive infiltration of inflammatory cells in the dermis. HandE X 100; (F) Vacuolation is seen in the spinous cells of the epidermis along the external hair sheaths at 5 dpi. Bacteria and polymorphonuclear cell infiltration seen in the epidermis of the scarified area. H & E X 100.

By 15 dpi, most of the acute histopathological changes subsided and the scab was shed in most cases. In certain areas there was papillary outgrowth of the epidermis with deep rete pegs. There is oedema in the dermis and infiltration by inflammatory mononuclear cells (Fig 2c) which persisted even on 21 dpi sections.

Biopsy tissue of 28 dpi showed complete healing and the epidermis was back to normal. The inflammation subsided and number of inflammatory cells in the dermis has reduced (Fig 2d). A slight parakeratotic change was evident (Fig 2e). Few inflammatory cells in the dermis persisted.

Immunopathological progression

No immunoreactive cells were noticed on 3 dpi when stained for $CD4^+$ and $CD8^+$ T-cells by immunoperoxidase staining. On 5 dpi, only isolated $CD4^+$ and $CD8^+$ positive cells could be noticed scattered in the dermis. The unstained lymphocytic cells probably include B-cells and other T-cell subsets. By 8 dpi, few immunopositive $CD4^+$ (Fig 3a) and $CD8^+$ (Fig 3b) T-cells could be demonstrated in the dermis amongst the other mononuclear cells. $CD4^+$ cells were relatively more abundant and some were found within the scab amongst the polymorphonuclear cells. On 15 dpi, both

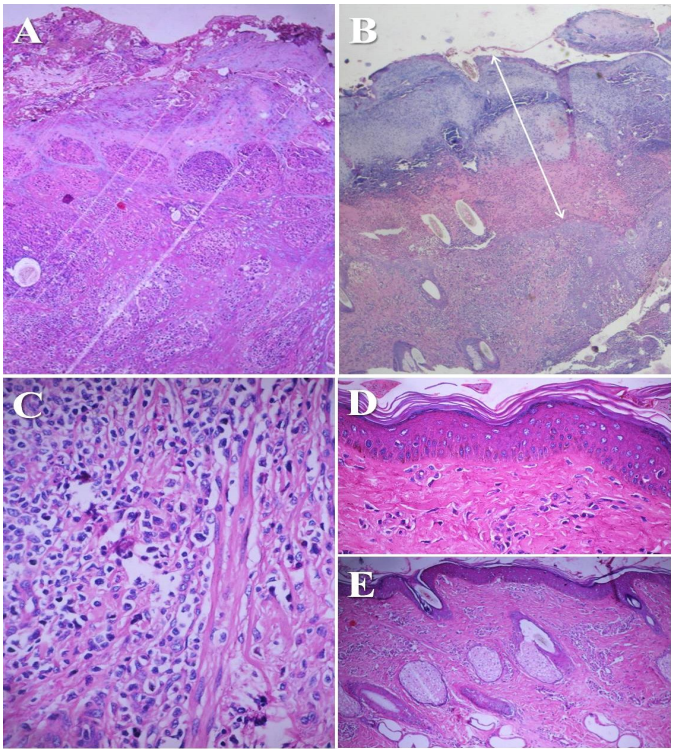

Fig 2: Histopathological changes in the skin of experimentally infected goats with orf virus (ORFV) at 8, 15 and 28 days post infection (dpi). (A) Marked hyperkeratosis, acanthosis and deepening of the rete ridges. HandE X 100; (B) Superficial scab formation (arrow) by necrotic debris and infiltrating cells, underlined by a reactive epithelium. HandE x 40. (C) The dermis at 15 dpi appears oedematous, with infiltration by predominantly mononuclear cells. HandE x 400; (D) The epidermis at 28 dpi in some areas has completely healed and inflammatory cells in the dermis have reduced. HandE X 100; (E) The epidermis at 28 dpi in some areas has healed completely, but with slight parakeratosis. HandE x 400.

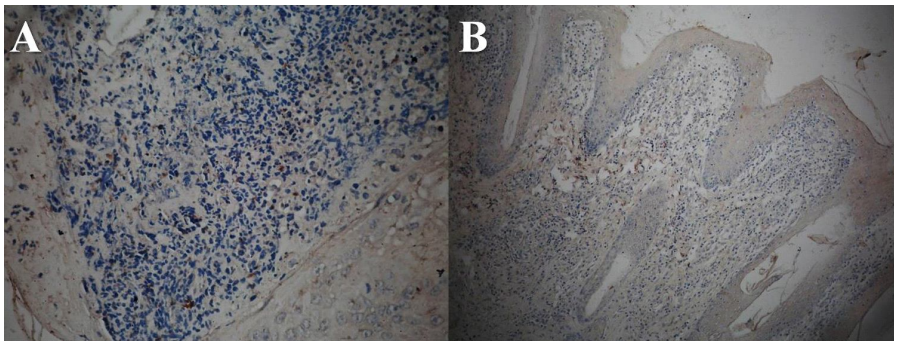

Fig 3: Immunoperoxidase detection of (A) $CD4^+$ and (B) $CD8^+$ T-cells in the skin of experimentally infected goats with ORFV at 8 dpi.

populations of $CD4^+$ and $CD8^+$ cells were slightly reduced but still present in the dermis and beneath the epidermal layer. By 28 dpi, only isolated $CD4^+$ and $CD8^+$ cells were found.

Detection of viral antigen

On 4 dpi, viral antigen with strong reactivity could be demonstrated in tissue cryosections by immunofluorescence technique in the keratinocytes of the epidermis and external root sheaths of the hair follicles. By 5 dpi, viral antigen with strong reactivity could be demonstrated in the affected cells of the epidermis and along the rete pegs (Fig 4a). Moderate to strong signal was also seen in the spinous cells around the root sheaths (Fig 4b). By 8 dpi, there was a very strong positive reaction to viral antigen within the epidermis and loosely adherent scab tissue. No virus antigen could be demonstrated with fluorophores on 28 dpi.

Detection of viral nucleic acid

DNA from affected goat skin tissue at day 8 was positive for ORFV, while they were negative from skin at 28 dpi. In rabbits and mice, *Orfvirus* DNA was negative on 8 dpi.

Experimental infection with ORFV in rabbit and mice

Two adult male rabbits and four mice were inoculated with clinical scab derived tissue lysate by scarification on the right inner pinna. The progressive lesions are depicted in Fig 5. Infection in the rabbits and mice could not be established beyond the erythema and vesicle stage even after the animals had been immunologically compromised by administration of steroids. Lesions in the scarified pinna of both the rabbits and mice were identical, which healed rapidly soon after, without progressing to clinical disease except for a transient vesicular stage. No histopathology or PCR was done on skin tissue.

The gross lesions were very similar to those that appeared in the literature (Abu-Elzein and Housawi, 2009) and conformed to the typified orf lesion. Yet, a distinct vesicular stage was not clearly evident grossly, except for tiny, guttate vesicles during the intervening 3-4 dpi.

The histopathological description of the lesions corresponds with the findings in experimentally infected sheep by Couch (1983).

The present findings indicates that immunoreactive $CD4^+$ and $CD8^+$ cells appear late and were demonstrable after 5 dpi, that increased progressively after 8 dpi, only to decline by 15 dpi. The findings in experimentally induced orf infection in goats by Garrido-Fariña *et al.* (2008) also corroborates $CD4^+$ and $CD8^+$ cell progression in the hypodermis from the macular stage onwards, through

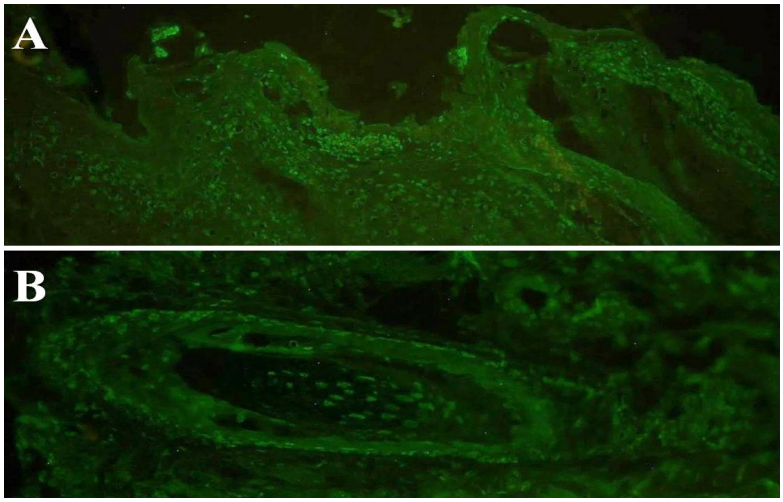

Fig 4: Fluorescent Immunohistochemical detection of orf virus (ORFV) antigen in the skin of experimentally infected goats at 5 dpi. (A) Strong fluorescence seen in epidermal cells including those of the stratum Malpighi. Spinous cells along the rete ridges are also positive. FITC X 100; (B) Moderate to strong fluorescence in the spinous cells of the external root sheath situated deep in the dermis. FITC X 400.

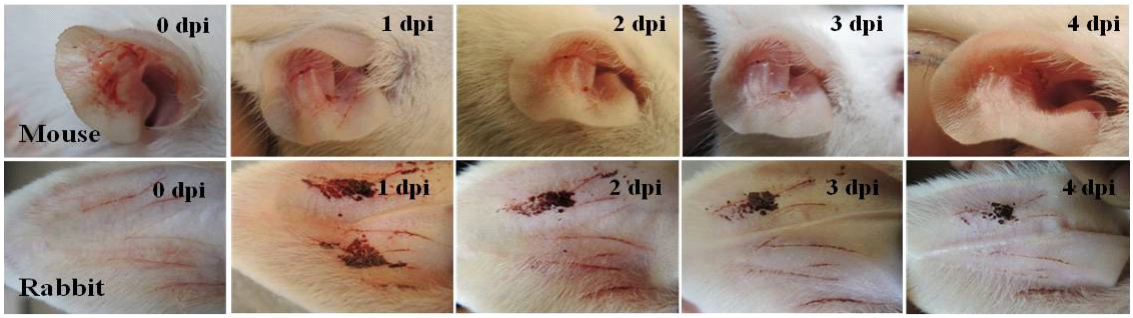

Fig 5: Progression of lesions in experimentally attempted orf virus (ORFV) infection on scarified inner pinna of mouse and rabbit.

papular and vesicular stage, thereafter diminishing during healing. Many workers have observed an initial and predominant population of neutrophils from the earliest establishment of the lesion, followed by a gradual accumulation of dendritic cells, $CD4^+$ T-cells, $CD8^+$ T-cells and B-cells around the virus infected epidermal cells (Jenkinson *et al.*, 1990; Jenkinson *et al.*, 1992; Lear *et al*., 1996; Lloyd *et al.*, 2000). The predominance of $CD4^+$ cells was also observed by Haig *et al.* (1996) who believed that $CD4^+$ T-cells and IFN-γ and to a lesser extent $CD8^+$ T-cells were important for partial protection against infection. The significance of $CD4^+$ cells, but not $CD8^+$ cells in conferring host immunity to ORFV have been highlighted by Lloyd *et al.* (2000) when studying CD4 depleted lambs which were unable to clear or raise humoral response against virus challenge, but on the other hand the virus was demonstrable in CD8 depleted lambs.

Demonstrable viral antigen in the histological lesions was reportedly absent during the first 24 hours of infection (Couch, 1983). Although a frequent cryohistological observation was not conducted in the present study, the first fluorescent signal of viral antigen was detected only by 4 dpi. In an earlier study involving infected lamb testes cell lines (OA3.Ts), the same viral antigen (BAG17) was demonstrable by 12 hrs of infection, with increasing signal intensity at 24 and 48 hrs and progressively at 72 hrs (3 dpi), vivid inclusions were detected in the cytoplasm (Nashiruddullah *et al*., 2016). It is speculated that host factors are probably responsible for delay in establishment of viral infection and their demonstration *in-vivo*. In an analogous experiment, Cargnelutti *et al.* (2011) reported successful isolation of virus from scarified lesions of lambs between 2-19 dpi, spontaneous regression of lesions by 19 dpi and absence of neutralizing serum antibodies at 28 dpi. Likewise, since no fluorescent signals could be detected at 28 dpi in the present study it may be indicative of virus clearance from the cutaneous tissue. Viral nucleic acid from curtaneous tissue similarly could not be demonstrated at 28 dpi, indicating absence of viral latency in the skin.

Although it was tempting to induce infection in a non-natural animal model, establishment of orf infection in mice and rabbits have received mixed success. On the other hand, transient lesions have been reported by Cargnelutti *et al.* (2011) with less consistent and conspicuous focal erythema, macules, papules, or a few vesicles and small scabs observed progressively between 5 and 12 dpi in scarified mice. In our study, it may be emphasized that the same antigen was used to successfully induce orf lesions in the primary host (goats). Speculations on the successful reproduction of the disease in experimental animals have been made by many workers, thereby suggesting various dependent factors such as the choice of viral strain, the virus titre in the inoculum, the procedure/site of inoculation, the genetic background and age of experimental animals (Cargnelutti *et al.*, 2011).

CONCLUSION

The present study shows the ease of disease transmissibility through traumatized skin, especially when the host is immunosuppressed or under stress. It would however be interesting to observe disease transmission without aided immunosuppressants or possibly through intact skin as an analogy to natural infection. The exact reason for variability in establishing successful infection in rabbits and mice is open for speculation. The present experiment also indicates to the absence of viral latency in host tissue as verified by negative immunofluorescence and PCR in healed skin. Infection therefore with ORFV may be regarded as a self-limiting cutaneous infection.

Conflict of interest

The authors declare no conflict of interest.

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