

## Western blot analysis of virus-specific antibody responses for capripox and contagious pustular dermatitis viral infections in sheep

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### SUMMARY

This paper reports the development and evaluation of serological tests for the differentiation of antibodies in animals infected with capripox and parapox viruses. Agar-gel immunodiffusion tests using sera from sheep with naturally-acquired infections and from sheep experimentally inoculated with orf or capripox viruses showed cross reactions. Virus-specific antibody responses to structural proteins of the viruses were analysed by Western-blot analysis. This analysis readily differentiated the infections as either capripox or contagious pustular dermatitis. The antibody responses to the 32 kDa and 26 kDa proteins of capripoxvirus provided a firm basis for differentiation.

### INTRODUCTION

Capripoxvirus (CPV) and contagious pustular dermatitis or orf virus (CPDV), are closely related serologically [1–4]. Both viruses cause very similar diseases in sheep and goats, and thus it is difficult to differentiate them clinically [5]. Virus-specific antibodies in sera are routinely used to indicate exposure to virus and it is therefore important to have a serological assay for the differentiation of CPV and CPDV infections. The viruses can easily be differentiated from each other by electron microscopy [6], and genomic comparison [7]. However, a major common precipitating antigen shown to be present in strains of CPV also reacts with CPDV positive serum [8]. Many serodiagnostic assays such as agar-gel immunodiffusion, complement-fixation, counter-immunoelectrophoresis, or ELISA, because they detect total antibody response including that from cross-reactive antibodies, are not reliable for differentiation between these infections.

In countries or areas free from CPV it is important to be able to distinguish CPV serologically from CPDV which is enzootic throughout the world [9]. In countries where CPV and CPDV coexist serological testing may be the only method by which their distribution and the success of control measures can be assessed. The purpose of this study was to determine whether CPV and CPDV infections of

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sheep could be differentiated from each other by the use of Western-blot analysis using serum from sheep with naturally-acquired infections and sheep inoculated with either CPV or CPDV. The present paper describes a system for the differential diagnosis of capripox and orf.

## MATERIALS AND METHODS

### *Viruses*

The CPV used for the study was a Kenya sheep and goat pox isolate (KS-1) that had been developed for a vaccine [8]. The CPDV was obtained from an infected sheep as a dried scab ground in sterile sand supplied by Dr G. R. Scott of the Centre for Tropical Veterinary Medicine, Edinburgh, UK.

### *Purification of Viruses*

The KS-1 isolate of CPV was grown on secondary lamb testis cell monolayers in 175 cm<sup>2</sup> flasks and purified as described by Kitching and colleagues [8]. CPDV was purified from scabs obtained from an experimentally inoculated sheep. Scabs from the groin region were collected 10–14 days after inoculation and processed [10, 11]. Approximately 3–4 g of tissue was mixed with carborundum (Turnpike Road, Cressex Industrial State, High Wycombe HP12 3NR, U.K.) in 15 ml TE buffer solution (10 mM Tris-HCl, pH 7.5 and 0.1 mM EDTA) and ground with a pestle and mortar. The resulting material was centrifuged at 1500 g for 30 min at 4 °C. The supernatant was layered onto a 2.5 ml 36% sucrose (w/v) cushion in an 18 ml tube and centrifuged in a Beckman SW-28.1 rotor at 45000 g for 30 min. The resultant pellet was suspended in 1 ml of TE buffer and carefully layered onto a composite gradient [25–50% sodium diatrizoate (Sigma Chemical Company, St Louis, MO, USA) gradient overlaid with 10% dextran T-10 (Pharmacia LKB, Uppsala, Sweden) (10)], and re-centrifuged at 45000 × g for 18 h (Beckman SW-28.1 rotor). The visible virus band located at about two-thirds from the top of the tube was aspirated and diluted 1/6 in TE buffer solution. The virus was recovered by centrifuging the diluted virus through a 36% sucrose cushion. The protein content of both viral preparations was calculated [12].

### *Serum samples*

Two batches of sheep serum (38 and 43 samples) routinely submitted by the G.R.M. International Pty. Ltd., Australia were used for CPV antibody screening. These were stored at –20 °C prior to analysis.

Sera were also obtained from two sheep experimentally infected with CPV and two with CPDV. Sera were obtained prior to inoculation and then weekly for 6 weeks.

### *Hyperimmune antisera*

Hyperimmune antisera were prepared against both of the viruses. Sheep inoculated with CPDV were given additional intramuscular inoculations (2.5 ml of 20% autogenous CPD-scab) 16 and 18 weeks after the first inoculation. The serum obtained 10 days after the final booster inoculation was used as hyperimmune serum (HIS). Hyperimmune serum against the KS-1 isolate of CPV was prepared in monozygotic sheep. The first animal was inoculated with KS-1 vaccine at 4 sites

in the flank region. The sheep was euthanized on the eighth day post inoculation. Skin biopsy samples from all 4 sites were collected and used to vaccinate the second monozygotic sheep (20% suspension of tissue in phosphate buffered saline solution [PBS]). Inoculations were then administered monthly for 10 months. Serum taken 10 days after the last inoculation was used as hyperimmune serum. The hyperimmune sera were stored at  $-20^{\circ}\text{C}$ .

#### *Monospecific antiserum*

Polyclonal monospecific antiserum to the 26 kDa and 32 kDa proteins of CPV was prepared in rabbits. The CPV proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then detected by staining with 0.3 M cupric chloride solution [13]. Protein bands were cut out and processed for inoculation into rabbits [14]. Rabbits were given two inoculations with an interval of 4 weeks. Serum from the rabbits was collected 10 days after the second inoculation, and stored at  $-20^{\circ}\text{C}$  in aliquots.

#### *Agar gel immunodiffusion test (AGID)*

Soluble CPDV antigen for the AGID test was prepared from scabs obtained from inoculated sheep. Approximately 1 g of tissue was ground with carborundum in 5 ml of PBS. The suspension was centrifuged at 1000 g at  $4^{\circ}\text{C}$  for 30 min. Supernatant was concentrated to 1 ml by counterdialysis with polyethyleneglycol-6000. The dialysate was again centrifuged at 13000 g for 1 h at  $4^{\circ}\text{C}$ . The supernatant was used as an antigen in the AGID test. Soluble antigen from CPV was also prepared as described previously [8] for use in the AGID test.

#### *Western blot analysis*

Proteins of the purified virus preparations were separated on 12.5% SDS-PAGE gel by use of the discontinuous buffer system [15]. After separation, the proteins were transferred to a nitrocellulose membrane (NCM,  $0.45\ \mu\text{m}$  pore size) by the semi-dry method of electro-blotting (Pharmacia Nova-Blot electrophoretic transfer unit). Protein transfer was obtained within 1 h by using  $0.8\ \text{mA}/\text{sq. cm.}$  of gel. The membrane was rinsed in PBS and immersed for 1.5 h in 5% non-fat dry milk powder in PBS to block the unsaturated sites on the NCM. The membrane was incubated with primary antibody (diluted 1/50 in 5% milk powder) at  $37^{\circ}\text{C}$  for 1 h and washed three times for 5 min in 0.05% Tween-20/PBS. The NCM was incubated with rabbit anti-sheep immunoglobulin horse-radish peroxidase enzyme conjugate (1/2000 in 5% milk powder) for 1 h at  $37^{\circ}\text{C}$ , washed as described above and transferred to 50 mM Tris-HCl (pH, 7.5) for 10 min. The membrane was finally incubated in freshly prepared substrate solution (10 mg of diaminobenzidin tetrahydrochloride dissolved in 50 ml of 50 mM Tris-HCl (pH, 7.5) and 20  $\mu\text{l}$  of 30% (v/v) hydrogen peroxide) for approximately 4–5 min. The reaction was stopped by washing with distilled water.

## RESULTS

#### *Agar gel immunodiffusion test*

As described previously cross-analysis of hyperimmune sera from CPV and CPDV with the homologous and heterologous soluble antigens in the AGID test

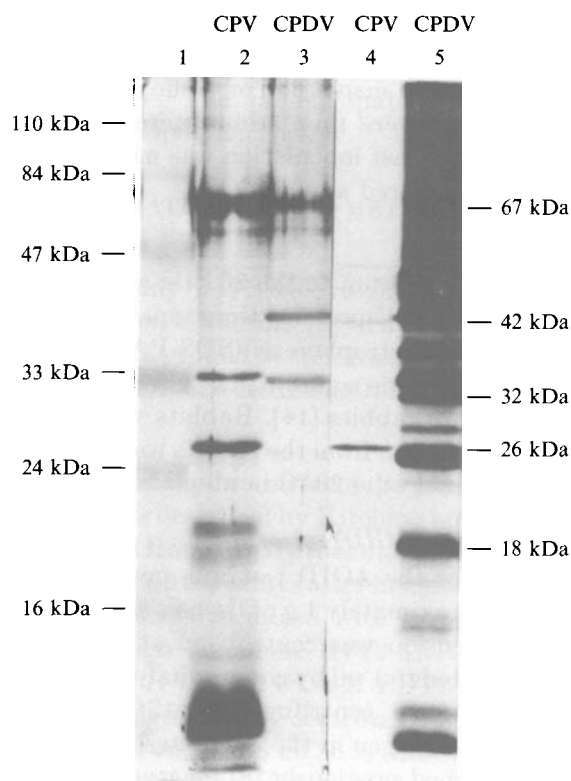


Fig. 1. Results of Western blot analysis of capripoxvirus (CPV) and contagious pustular dermatitis virus (CPDV) proteins, using hyperimmune sera. Lanes 2 and 4 contain CPV proteins and 3 and 5 contain CPDV proteins. Reactions in lanes 2 and 3 with CPV hyperimmune serum, and reactions in lanes 4 and 5 with CPDV hyperimmune serum. Lane 1 contains molecular weight-markers.

revealed serological relationships between the viruses [8]. Sera anti to both CPV and CPDV produced precipitation lines with both antigens. The major precipitation band of CPV homologous antigen-antibody system had a line of identity with a major precipitation band between the CPV soluble antigen and CPDV soluble antigen. Similar results were observed when CPDV soluble antigen was reacted with CPDV hyperimmune serum and CPV hyperimmune serum. Sera obtained from the sheep prior their inoculations showed no reactions with virus soluble antigens.

Serum samples from sheep infected with CPV (Nigeria sheep isolate) also showed positive reactions with CPV and CPDV antigens. Despite the sera having been stored for more than 6 years at  $-20^{\circ}\text{C}$ , the results were similar to those previously reported by Kitching and colleagues [8]. Of the 81 Australian serum samples tested by AGID 41 gave a positive reaction with CPDV antigen, and 31 had a positive reaction with CPV antigen. Many of these samples were haemolysed, but it was possible to detect precipitation lines with both antigens. Visibility of the precipitation lines was improved after 3 days of incubation and three 24 h washes with PBS.

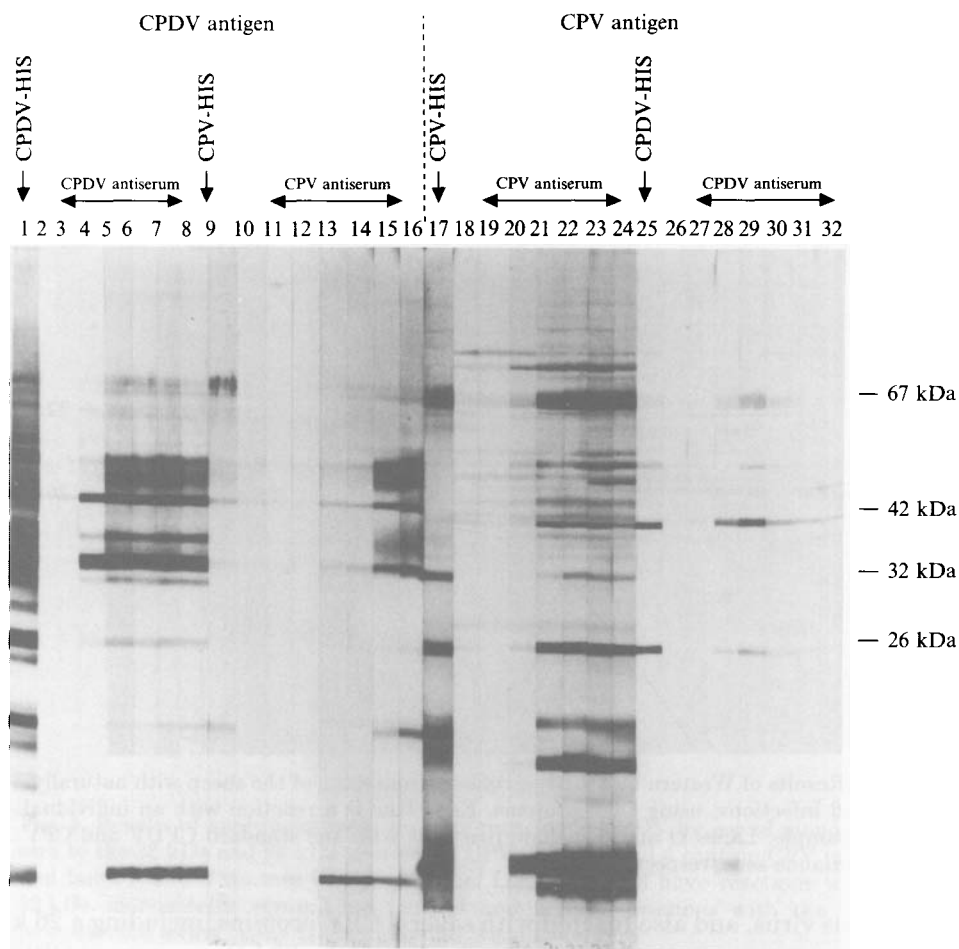


Fig. 2. Results of Western blot analysis of sera obtained weekly from CPDV and CPV-inoculated sheep, using CPDV and CPV proteins. Lanes 1–16 contain CPDV proteins, and 17–32 contain CPV proteins. Lanes 1 and 25 were reacted with CPDV hyperimmune serum and 9 and 17 were reacted with CPV hyperimmune serum. Lanes 3–8 and 27–32 were reacted with sera from CPDV-inoculated sheep, and 11–16 and 19–24 were reacted with sera from CPV-inoculated sheep. Lanes 2, 10, 18 and 26 were reacted with serum obtained from sheep prior to inoculation.

*Western blot analysis*

Western blot analyses of purified preparations of both CPV and CPDV were carried out with the hyperimmune sera (Fig. 1). For comparison, equal amounts of protein of both the viruses (40  $\mu\text{g}/\text{sample}$ ) were separated on SDS-PAGE and transferred to NCM. The analysis revealed reactions of CPV hyperimmune serum with proteins of the homologous virus (67 kDa, 32 kDa, 26 kDa, 19 kDa and 17 kDa) and the heterologous (CPDV) virus (67 kDa, 42 kDa, 32 kDa and 18 kDa). It is important to note that although the CPV hyperimmune serum reacted with CPDV proteins it did not react with any CPDV protein in the 26 kDa range. CPDV hyperimmune serum reacted strongly with proteins of the

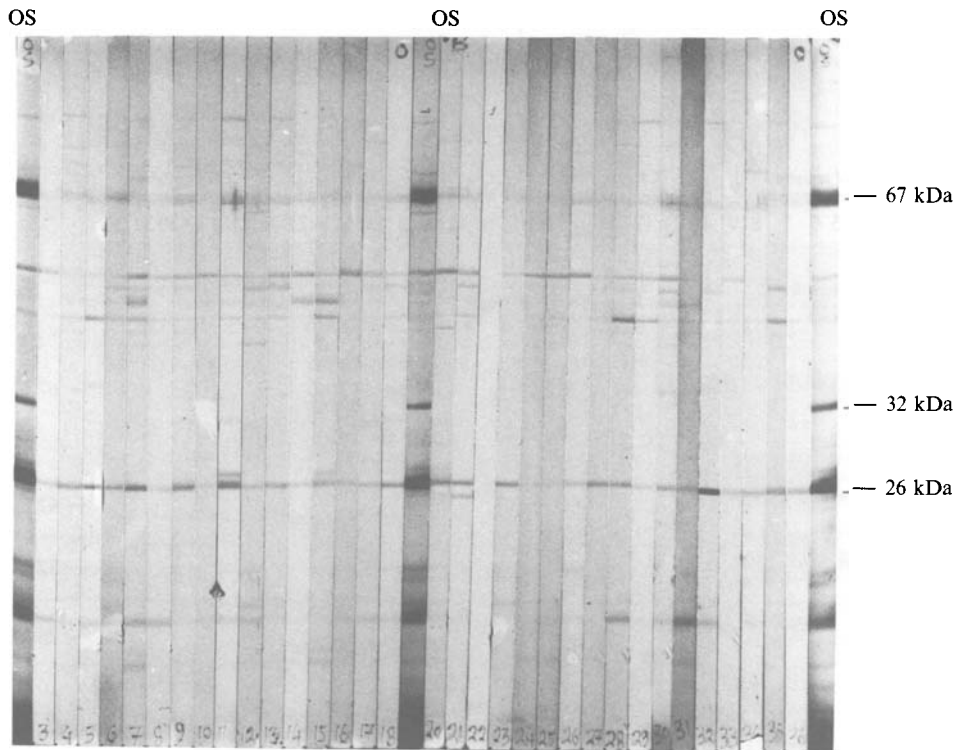


Fig. 3. Results of Western blot analysis of sera from some of the sheep with naturally-acquired infections, using CPV proteins. Each lane is a reaction with an individual serum sample. Lanes O and S include reactions with the standard CPDV and CPV hyperimmune sera, respectively.

homologous virus, and also reacted with several CPV proteins, including a 26 kDa protein. However, no reaction was observed with the 32 kDa protein of CPV.

The results were confirmed by western blot cross analysis of serum samples obtained weekly from sheep inoculated with CPDV or CPV. Possible variation caused by protein migration was eliminated by separating preparations of CPDV and CPV on the same gel, transferring them to NCM, and then cutting the membrane into thin strips so that the proteins could be reacted with individual sera (Fig. 2). Beginning 2 weeks after inoculation CPDV serum samples reacted strongly with CPDV proteins (Fig. 2, lanes 2–8), CPV serum samples (3 weeks after inoculation) reacted weakly with some CPDV proteins (Fig. 2, lanes 10–16). However, virtually no reaction was observed with CPV serum samples with any CPDV protein at the position equivalent to the 26 kDa protein of CPV. CPV serum samples taken 2 weeks after inoculation or later reacted strongly with many of the CPV proteins (Fig. 2, lanes 18–24). None of the CPDV serum samples reacted with the 32 kDa protein of CPV (Fig. 2, lanes 26–32), however, reactions with other proteins (26, 42 and 67 kDa) became apparent 3 weeks after inoculation.

The sera from Australia were tested with CPV antigen. No reaction with the 32 kDa protein of the CPV was observed (Fig. 3). However, most serum samples had reactions with other proteins, including the 26 kDa protein of CPV. The reaction pattern of these serum samples on western blots was similar to that

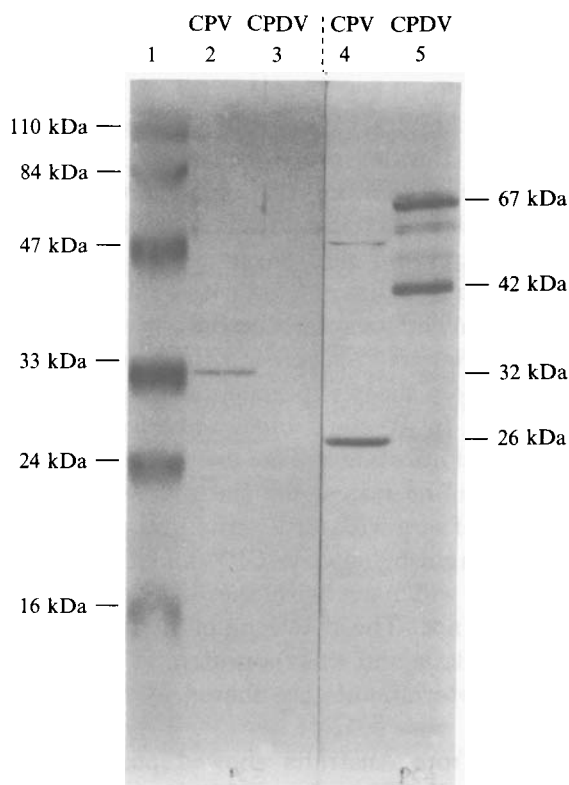


Fig. 4. Results of analysis of cross-reactive proteins of CPDV by use of monospecific sera to the 32 kDa and 26 kDa proteins of CPV. Lanes 2 and 4 contain CPV proteins, and lanes 3 and 5 contain CPDV proteins. Lanes 2 and 3 have reactions with the 32 kDa monospecific serum, and lanes 4 and 5 have reactions with the 26 kDa monospecific serum. Lane 1 has molecular weight-markers.

observed between CPV proteins and serum from CPDV-inoculated sheep. This analysis demonstrated that CPV specific antibodies were not present in these serum samples.

Monospecific polyclonal rabbit sera to the 32 kDa and 26 kDa proteins of CPV were also used to identify putative cross-reactive proteins of CPDV by Western blot analysis. This analysis showed that anti-26 kDa CPV protein antibodies reacted with the 67 kDa and 42 kDa proteins of CPDV, but anti-32 kDa-protein antibodies did not react with any CPDV proteins (Fig. 4). These results clearly suggested that the 26 kDa protein of CPV possessed some antigenic determinants similar to that of 67 kDa and 42 kDa proteins of CPDV, whereas 32 kDa protein is totally CPV specific.

#### DISCUSSION

The results of the AGID tests on sera from sheep with naturally-acquired or experimentally induced capripox and contagious pustular dermatitis showed serological cross-reaction between the two viruses. The soluble antigens used in AGID tests were prepared from tissue cultures and scabs, thus it was difficult to determine whether cross-reactive antibodies were directed against structural or

non-structural proteins of the virus. A major common precipitating antigen (67 kDa) found in all CPV that also reacts with CPDV serum has been reported to be a viral envelope protein [8].

To determine whether the structural proteins were involved in the observed cross serological reaction. Purified preparations of CPV and CPDV were used in the Western-blot analysis. CPV hyperimmune serum did not react with any CPDV protein which corresponds to the 26 kDa protein of CPV, and significantly CPDV hyperimmune serum did not recognize the 32 kDa protein of CPV. However, CPV hyperimmune serum reacted with a 32 kDa protein of CPDV, and CPDV hyperimmune serum had a strong reaction with the 26 kDa protein of CPV. Similar patterns of reaction were observed with Western-blot cross analysis on serum samples obtained from sheep experimentally infected with CPDV or CPV. This pattern of reactions in Western blots provides an easy method for the differentiation of these two infections. The use of both viral preparations for Western-blot analysis is unnecessary for the differentiation of CPV and CPDV infections. The screening of sera with CPV structural proteins, using the Western blot technique, will distinguish between CPV and CPDV infection. Sera from sheep infected with CPV will react with the 32 kDa protein, sera from sheep infected with CPDV will not. The reactions of the monospecific 26 kDa CPV-protein serum with the 67 kDa and 42 kDa protein of CPDV were indicative that cross-reactive antigenic determinants are shared by different proteins of CPDV virus.

Many serum samples from Australia showed positive reactions with both antigens in the AGID test. However, none of these sera reacted with the 32 kDa protein of CPV in western-blot analysis. Most sera did react with the 26 kDa and other proteins of the virus. These results were similar to those obtained by use of sera from CPDV-inoculated sheep or CPDV hyperimmune serum, and clearly indicated that the sheep were infected with CPDV and not CPV. The analysis of antigen-specific antibody responses by western blot analysis represents a reliable method to serologically differentiate CPDV and CPV infections.

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#### REFERENCES

1. Dubey SC, Sawhney AN. Serological relationship between viruses of goat-pox and contagious pustular dermatitis. *Indian J Anim Sci* 1979; **46**: 135-9.
2. Sharma SN, Dhanda MR. Studies on sheep and goat-pox viruses: Relationship with contagious pustular dermatitis and vaccinia viruses. *Indian J Anim Sci* 1971; **41**: 864-7.
3. Subba Rao MW and Malik BS. Cross-neutralization tests on sheep pox, goatpox and contagious pustular dermatitis viruses. *Acta Virol* 1979; **23**: 165-7.
4. Subba Rao MV, Malik BS, Sharma SN. Antigenic relationships among sheep pox, goatpox and contagious pustular dermatitis viruses. *Acta Virol* 1984; **28**: 380-7.
5. Robinson AJ, Balassu T. Contagious pustular dermatitis (orf). *Vet Bull* 1981; **51**: 771-82.
6. Nagington J, Newton AA, Horne RW. The structure of orf virus. *Virology* 1964; **23**: 461-72.



7. Wittek R, Kuenzle CC, Wyler R. High G-C content in parapoxvirus DNA. *J Gen Virol* 1979; **43**: 231–4.
8. Kitching RP, Hammond JM, Black DN. Studies on the major common precipitating antigen of capripox. *J Gen Virol* 1986; **67**: 139–48.
9. Anonymous. Middle East countries refuse “sick” sheep. *New Scientist* 1989; **124** (No: 1686): 24.
10. Esposito JJ, Obijeski JF, Nakano JH. Orthopoxvirus DNA: Strain differentiation by electrophoresis of restriction endonuclease fragmented virion DNA. *Virology* 1978; **89**: 53–66.
11. Robinson AJ, Ellis G, Balassu T. The genome of orf virus: Restriction endonuclease analysis of viral DNA isolated from lesions of Orf in sheep. *Arch Virol* 1982; **71**: 43–55.
12. Joklik WK. The purification of four strains of poxvirus. *Virology* 1962; **18**: 9–18.
13. Lee C, Levin A, Branton D. Copper staining: A five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. *Anal Biochem* 1987; **166**: 308–12.
14. Hallow E, Lane D. *Antibodies: a laboratory manual*. New York: Cold Spring Harbour Laboratory, 1988: 65.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–5.