CHARACTERIZATION OF STRUCTURAL PROTEINS OF CONTAGIOUS PUSTULAR DERMATITIS VIRUS

BY

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HISAR

1997

DEDICATED

TO

MY FATHER

AND

LOVING MEMORIES OF MOTHER

CERTIFICATE-I

This is to certify that this thesis entitled **"Characterization of structural proteins of contagious pustular dermatitis virus"** submitted for the degree of M.V.Sc., in the subject of Veterinary Microbiology, of the CCS Haryana Agricultural University, is a bonafide research work carried out by Mahesh Khatri under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled **"Characterization of structural proteins of contagious pustular dermatitis virus"** submitted by Mahesh Khatri to the CCS Haryana Agricultural University in partial fulfilment of the requirements for the degree of M.V.Sc. in the subject of Veterinary Microbiology, has been approved by the Student's Advisory Committee after an oral examination on the same.

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Hisar
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Mahesh
(Mahesh Khatri)

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CHAPTER-I

INTRODUCTION

Contagious pustular dermatitis (CPD) also called as Orf, Contagious ecthyma or Scabby mouth is highly contagious skin disease of sheep, goats and camel caused by a *Parapoxvirus*. It occasionally infects wild ruminants and also humans (Hessami *et al.*, 1979).

The disease is prevalent in sheep and goats rearing countries including India. It occurs at any time of the year but is more common during spring and summer mainly among lambs and kids (Aynaud, 1923; Glover, 1928; Schmidt and Hardy, 1932).

The disease is characterized by pustular eruptions and crust formation around mouth, on the feet and udder. This disease sometimes also involves the internal organs like gastrointestinal tract, lungs and heart (Darbyshire, 1961). The infection follows a sequence of macule, papule, vesicle, pustule and scab formation. Although the infection normally resolves within 4-6 weeks, persistent infections have been reported (Greig *et al.*, 1984) and latent infections have also been proposed (Robertson, 1976). Morbidity in susceptible population is very high, approaching 100% (Schmidt and Hardy, 1932; Gardiner *et al.*, 1967), but the mortality rate in uncomplicated cases rarely exceeds 1% (Glover, 1928; Schmidt and Hardy, 1932). With secondary complication with either bacteria or parasites, mortality rates may range from 20 to 50% (Aynaud, 1923 and Jacotot, 1924).

CPD virus is the type species of genus parapox in the family *Poxviridae* (Matthews, 1982). Other members of this genus include Bovine papular stomatitis virus, Pseudocowpox virus and Sealpox

virus. These viruses cause skin lesions in their respective hosts. The genome of CPD virus consists of a linear ds DNA about 135 Kbp in size and with relatively high G+C content of 63.5% (Wittek *et al.*, 1979). CPD virus is ovoid in shape with dimensions of 260 nm x 160 nm. A tubular thread like structure 10 to 20 nm wide and about 1000 nm long forms a criss-cross pattern on the surface of CPD virus (Nagington *et al.*, 1967) which gives it a unique appearance and helps in identification of this virus.

Serological cross-reactivity exists between CPD virus and goatpox virus and sheeppox virus and also between vaccinia virus (Webster, 1958; Mercer *et al.*, 1994), but there is no cross protection with either sheepox virus, goatpox virus or vaccinia virus (Sharma and Dhanda, 1971; Renshaw and Dodd, 1978; Mercer *et al.*, 1994).

Contagious pustular dermatitis infection of sheep causes considerable economic losses and was rated as a top health priority problem by US Sheep Industry Development Program in 1976. Production losses from CPD in lambs are due to lesions on the lips, mouth and feet, which interfere with feeding, walking and suckling of the lambs. And ewes with infected teats refuse to nurse their young, subsequently affecting growth rates (Howarth, 1929).

Because of contagious nature and economic importance of the disease, as well as the public health aspect, it is important that effective procedures be developed for its control. Traditional live vaccines prepared from scab material are known to produce a good immunity (Aynaud, 1923; Glover, 1928; Howarth, 1929; Schmidt and

Hardy, 1932; Boughton and Hardy, 1935). But the epizootics of CPD in vaccinated sheep flocks have been reported (Hardy, 1964). Moreover, use of such vaccines pose a potential threat of disease outbreak in sheep. As such there is a need for a new generation of vaccines, preferably a subunit vaccine.

With a view to study CPD virus proteins which are targets of protective immune responses, which in turn may be of help in development of a vaccine, the present study is designed and planned with following objectives :

1. Study of protein profile of CPD virus.
2. Identification of antigenic proteins of CPD virus.

CHAPTER-II

REVIEW OF LITERATURE

Contagious pustular dermatitis is a contagious viral disease of sheep and goats that is transmissible to man. Walley (1890) first described this disease, its contagious nature and referred to it as contagious dermatitis or Orf. The term contagious pustular dermatitis (CPD) was used by Hoare (1913).

Hansen (1879) reported the occurrence of the disease in man where it is commonly referred as Orf. The disease has been reported from many countries including India (Thilakarajan, 1975, Sinha *et al.*, 1986) and considered to be present in sheep raising areas throughout the world (Robinson and Balassu, 1981). CPD virus was first isolated in cell-culture by Greig (1957) using primary embryonic sheep skin cells.

2.1 Classification and nomenclature

On the basis of host range, the International Committee for Taxonomy of Viruses (ICTV) has divided the family *Poxviridae* into two subfamilies i.e. *Chordopoxvirinae* (Poxviruses of vertebrates) and *Entomopoxvirinae* (Poxviruses of insects) (Matthews, 1982). Further, based on cross protection studies in animals, cross-neutralization of infectivity in tissue-culture and cross-hybridisation of genomic DNA from virions, these subfamilies have been classified into several genera. Parapoxvirus is one of the genus which belongs to the subfamily *Chordopoxvirinae* (Francki *et al.*, 1991).

Parapoxviruses were first classified in 1956, when it was stated that the pustular dermatitis virus of sheep and goats and that of milkers' nodules should be included with the poxviruses (Downie and Dumbell,

1956). In 1957, a classification scheme based on a latin binomial system was proposed and under the genus *Poxvirus*, contagious pustular dermatitis was mentioned as being within the group and related immunologically to goatpox virus (Fenner and Burnett, 1957).

In 1976, ICTV named CPD subgroup as parapoxvirus genus within the family *Poxviridae* (Fenner, 1976), the name presumably being derived from 'Paravaccinia'. CPD virus was chosen as the type species, with Bovine papular stomatitis virus, Chamois contagious ecthyma virus, and Pseudocowpox virus being the members. The description of the genus at that time was, "viruses of ungulates that may infect man. Virion is ether sensitive, ovoid of 220 x 300 nm size, external coat and filaments are thicker than in vaccinia virions and are arranged in a regular spiral coil consisting of a single thread. Species show serological cross-reactivity, infected cells do not produce hemagglutinin".

The ICTV reports of 1979 (Matthews, 1979) and 1982 (Matthews, 1982) gave similar descriptions of the parapoxviruses, only cryptogram scheme was omitted. In addition, it was shown that the DNA of parapoxviruses was double stranded with a molecular weight of 85 x 10^6.

The three members of the genus parapoxvirus were separated on the basis of the animal species affected and the pathological character of the disease. The separation of these viruses into three distinct members has been done by animal inoculation tests (Huck, 1966), serology (Rosenbusch, 1983), DNA/DNA hybridization (Gassmann *et al.*, 1985) and restriction endonuclease analysis (Wittek *et al.*, 1980; Gassmann *et al.*, 1985).

Figure 1. Diagrammatic depiction of Parapox virus particle.

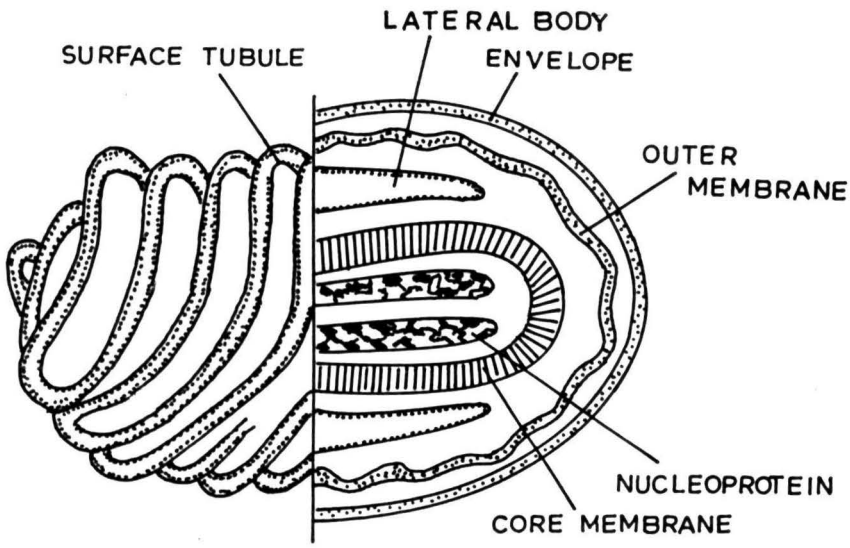

LATERAL BODY
SURFACE TUBULE
ENVELOPE
OUTER
MEMBRANE
NUCLEOPROTEIN
CORE MEMBRANE

Figure 1

2.2 Structure of virus

Poxviruses are the largest amongst the animal viruses and the virions are brick or oval shaped of about 200-400 nm in size. Structurally the virus particles are complex and composed of following components:

a) A biconcave or dumb-bell shaped core

b) two lateral bodies

c) the outer membrane coat, and

d) the outer envelope

A diagrammatic structure of parapox particle is shown in figure 1.

2.2.1 Unique morphology

The viruses of parapox genus have unique morphology when viewed in the electron microscope and this formed the basis for their inclusion in a separate group from other members of poxvirus family. Electron microscopy of CPD virus, reveals an oval shaped virus which is approximately 260 x 160 nm in size (Abdussalam and Cosslett, 1957; Nagington and Horne, 1962; Nagington *et al.*, 1964; Peters *et al.*, 1964). This differs with the other poxviruses which are brick-shaped having an average dimension of 350 x 250 nm.

In negatively stained preparations, like vaccinia virus, parapox viruses also appear in two forms. Where the stain has penetrated the viruses, a finely crenulate membrane appears to surround an amorphous core (C or capsular form) whereas viruses that remain impervious to the stain reveal a regular array of tubule-like structures arranged in a criss-cross manner along the length of the particle (M or mulberry form)

(Nagington and Horne, 1962). In addition, a layer of regular saucer-shaped subunits spanning the interval between the tubules (1.5 to 2 nm thick) has been seen closely associated with the outer surface of intracellular forms of the virus (Rosenbusch and Reed, 1983). Extra-cellular viruses isolated from infected cell-culture media are surrounded by a 9 to 18 nm thick membranous structure (Mitchner, 1969; Rosenbusch and Reed, 1983). By analogy to vaccinia virus, this membrane is likely to be derived from the Golgi apparatus. Shadow casting studies have shown that the criss-cross appearance is due to superimposition of images of the tubule-like structure as it winds in spiral fashion around the particle. Other members of this genus also show similar morphology (Naginton *et al.*, 1962; Friedman-Kein, 1963; Moscovici *et al.*, 1963; Roslyakov, 1972) and electron microscopy still provides a confirmatory diagnosis of parapoxvirus infections.

2.2.2 Proteins of CPD virus

A large number of proteins constitute each component of virus. Buddle *et al.* (1984) analysed structural proteins of 11 CPD virus isolates by SDS-PAGE. They detected 31 proteins with molecular weights ranging from 18Kd to 200Kd. These 11 isolates differed in the 37Kd to 44Kd molecular weight region and on the basis of these differences, the isolates were divided into 4 groups. Treatment of virus particles with Nonidet P-40 and β-mercaptoethanol alongwith sonication and centrifugation resulted into separation of surface components from viral cores. SDS-PAGE analysis of these preparations showed 15 proteins in

surface components. A protein of 37 to 40Kd was thought to be the characteristic virion surface tubule protein. Similar results were also reported by Balassu and Robinson (1987).

Zuo *et al.* (1988) could detect 28 proteins of molecular weights ranging between 14Kd and 190Kd in purified CPD virus preparation by SDS-PAGE. The protein subunits of the viral capsule were composed of 18 proteins. The immunization of lambs with these viral subunits showed that these subunits were immunogenic in nature.

Gonzalez *et al.* (1991) examined several isolates of parapox virus but could detect 4-12 proteins only by SDS-PAGE analysis in their viral preparations.

Chin and Petersen (1995) compared native virus and subunit antigens in ELISA for detection of antibodies against CPD virus. To obtain subunit antigens, SDS-PAGE analysis of CPD virus was carried out and it revealed about 30 proteins of molecular weights ranging between 15Kd and 110Kd. Six proteins of molecular weights 82-84Kd, 43Kd, 38Kd, 36Kd, 25Kd and 23Kd gave strong reaction on Western blot when reacted with CPD hyperimmune serum. They concluded that subunits antigens of CPD virus are better reagents for ELISA than native antigen. In another study, Mercer *et al.* (1994) detected 14 proteins with molecular weights ranging from 14Kd to 73Kd in Western blot analysis of CPD virus. CPD hyperimmune serum also reacted with 3 vaccinia virus antigens in Western blot, but cross-protection between these two viruses was not reported.

2.3 Physico-chemical properties

The virus is relatively thermostable. It is completely inactivated at 60°C for 30 minutes but retains some infectivity when held at 55°C for 30 min. (Sawhney, 1972; Buxton and Fraser, 1977). Infectivity is reduced by 1.5-2 $\log_{10}$ $TCID_{50}$ when virus is kept at 36±0.5°C for one week (Sawhney, 1972). Skin scabs dried over sulphuric acid, powdered and stored in sealed glass tubes in an ice box retained infectivity for 32 months (Boughton and Hardy, 1935). In an experiment infective dried scab material was divided into three portions and stored in sealed glass bottles in the dark at 83°F. One of the three samples lost infectivity within 54 days, another within 64 days, and the third within 120 days. Scab material dried over sulphuric acid and stored in an amber bottle in the refrigerator at 45°F retained infectivity for 22 years, 8 months (Livington and Hardy, 1960). But the virus could not survive for long when exposed to rain, snow and sunshine (McKeever and Reid, 1986).

Freezing-thawing of samples increase the titre of the virus, probably by disruption of viral aggregates and release of virus from cells (Sawhney, 1972). Scabs exposed to direct sunlight remained infective only for a few months, but, when left on ground in shaded areas, retained infectivity for years (Boughton and Hardy, 1935). In laboratory CPD virus remained infective for as long as 15 years at room temperature (Buxton and Fraser, 1977). CPD virus is resistant to glycerol (Aynaud, 1923; Sawhney, 1972) and only slightly sensitive to ether, chloroform, benzene and toluene (Aynaud, 1923; Trueblood and Chow, 1963).

2.4 Epidemiology and clinical signs

CPD virus of sheep has worldwide distribution and it is believed that the spread and maintenance of the disease is related to resistant nature of the virus in the environment and the short-lived immunity to reinfection.

As the virus is very hardy, it persists in the environment and acts as source of infection for other animals (Theiler, 1928; Glover, 1932-33; Boughton and Hardy, 1935). Once an animal in a flock becomes infected, it is likely that infection spreads from the infected animal to other animals. Virus from scabs formed after vaccination is also thought to contribute to the environmental pool.

CPD virus have been found to persist for long time in the lesions occuring on the head of rams thus leading to persistent infection. These persistent infections are believed to be responsible for maintenance and spread of the disease (Greig, 1984; McKeever, 1984). Another important factor in the epidemiology of CPD virus is the short-lived immunity to reinfection, particularly on the udder of ewes. Such sheep which are immune to reinfection on mouth, leg or coronet, remain susceptible to reinfection on udder (Schmidt, 1967).

This view of epidemiology of CPD virus has been questioned and it has been suggested that the virus may be maintained in some animals as a latent infection and these animals act as reservior of infection (Robertson, 1976).

Disease occurs at any time of the year but is more common during spring and summer mainly among kids and lambs (Aynaud, 1923;

Glover, 1928; Schmidt and Hardy, 1932). The incubation period of disease in experimental infection ranges from 24 to 72 hour. The lesions in sheep and goats start as discrete reddened swellings on the lips, followed by papules, vesicles, pustules, and ulcer formation in 3 to 4 days, rarely involving internal organs.Morbidity is very high (approaching 100%) (Schimidt and Hardy 1932; Gardiner *et al.*, 1967), but the mortality in uncomplicated cases rarely exceeds 1% (Glover, 1928). With secondry complications with bacteria and parasites mortality rates range from 20 to 50% (Aynaud, 1923; Jacotot, 1924).

Although clinically contagious purtular dermatitis and capripox virus infections of sheep are indistinguishable but work of Chand *et al.* (1994) showed that these infections could be differentiated serologically on the basis of specific reactions on Western blot observed against 32Kd envelope protein of capripox virus.

2.5 Host-range and pathogenesis of CPD virus

Contagious pustular dermatitis is regarded as natural disease of domestic sheep and goats which can be transmitted to man. It has also been reported to occur naturally in musk ox; reindeer, mountain goats, bighorn sheep, chamois, steenbok and alpas (Connell, 1954; Kummeneje and Krogsrud, 1978-79; Samuel *et al.*, 1975; Robertson, 1976; L'Heureux, 1996). The disease has also been reported from camel and Japnese serrows (Roslyakov, 1972; Dash tseren *et al.*, 1991; Ogino, 1996). Wilkinson *et al.* (1970) observed outbreak of CPD virus in dogs but Buttner (1995) found that dogs are not susceptible to infection with CPD virus.

Several attempts have been made to experimentally infect the common lab animals like rabbits, mice, chicken, kittens, dogs, pigeons, with variable results. Recently, Dobric (1995) showed that mice and rabbits are susceptible to CPD virus infection and lesions could be seen within 6 to 10 days.

Lyell and Miles (1950) and Abdussalam (1957) reported propagation of CPD virus in chick embryos upto certain passages. But other workers (Greig, 1956; Webster, 1958; Darbyshire, 1961) reported that CPD virus can not be propagated in chick embryos. Sawhney (1966) reported growth and multiplication of CPD virus in chorioallantoic membrane of developing chick embyos. Natural occurrence of the disease in man has been reported (Newsom and Cross, 1934; Carne *et al.*, 1946; Blakemore *et al.*, 1948; Hodgson-Jones, 1951; Muir, 1951; Purdy, 1955; Fastier, 1957; Nagington and Whittle, 1961; Hunter, 1964). Experimental infection of volunteers has also been described (Liebermann and Jungi, 1977). Transmission of disease from sheep to man and man to man has been reported (Purdy, 1955 and Lang, 1961).

The pathology of CPD virus is confined to the epithelium and oral mucosa. Skin lesions begin as reddening and swelling around the sites of inoculation and these develop into small vesicles over 24 hours. These vesicles develop and give a pustular appearance over the next few days. Early in infection, eosinophilic, intracytoplasmic inclusion bodies are seen within degenerating epithelial cells. The pustules develop due to a massive infiltration of polymorphonucleocytes. These pustules after few days turn into scabs. The resolution of lesions occurs over a period of 4 to 6 weeks (Aynaud, 1923; Howarth, 1929).

2.6 Relationship with other poxviruses

Serological studies have shown that parapoxviruses share common antigens with other poxviruses. These common antigens can be demonstrated by ager gel diffusion test done in tubes, between CPD virus, vaccinia virus, and ectromelia virus (Webster, 1958). Complement fixation tests have also revealed sharing of antigens between CPD virus and sheep pox and goat pox viruses. (Sharma and Dhanda, 1971). A common antigen shared between bovine papular stomatitis virus, vaccinia virus and myxoma virus was shown to be a nucleoprotein antigen in ring precipitin tests (Woodroofe and Fenner, 1962). On the other hand, fowlpox virus, a member of *Avipoxvirus,* showed no cross-reactivity in tube gel diffusion tests with either CPD virus or orthopox viruses, suggesting that the avipox viruses are distantly related at antigenic level to the other pox virus genera (Webster, 1958).

The relatedness of parapox viruses to orthopox viruses has not always been demonstrated in agar gel diffusion tests. Using CPD virus antiserum, precipitin lines of identity were detected between CPD virus and pseudocowpox virus, but not between CPD virus and cowpox or vaccinia viruses (Huck, 1966; Papadopoulos *et al.*, 1968). In serum neutralization tests, antisera raised in rabbits against vaccinia virus, did not neutralize CPD virus, nor did CPD virus antisera raised in sheep neutralized vaccinia virus (Webster, 1958). Workers have reported neutralization of CPD virus with goatpox antiserum (Sharma and Dhanda, 1971; Dubey and Sawhney, 1979) but cross-neutralization could not be demonstrated (Renshaw and Dodd, 1978).

Figure 2. Diagrammatic representation of multiplication cycle of Poxviruses (Adapted from Fields Virology 3rd edition, 1996)

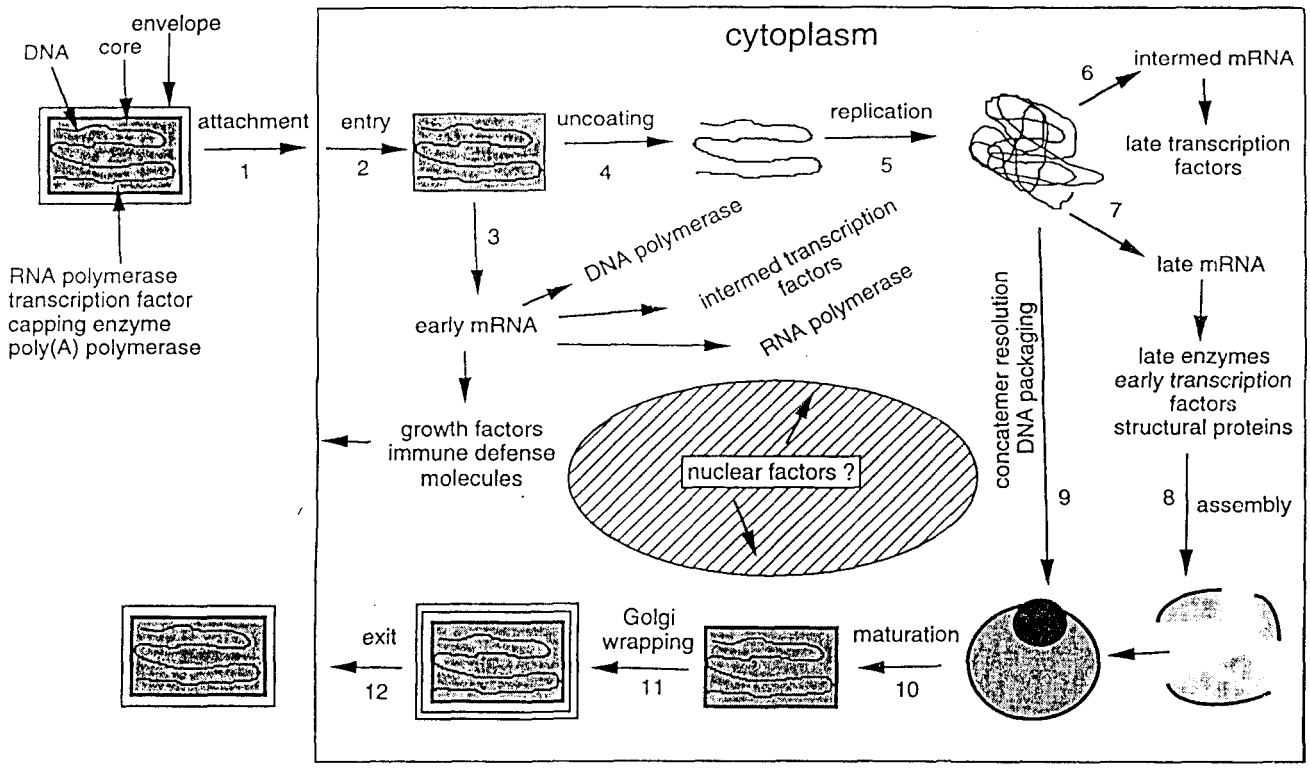

envelope
DNA
core
attachment
1
RNA polymerase
transcription factor
capping enzyme
poly(A) polymerase
cytoplasm
entry
2
uncoating
4
replication
5
6
intermed mRNA
late transcription
factors
7
late mRNA
late enzymes
early transcription
factors
structural proteins
8
assembly
3
DNA polymerase
intermed transcription
factors
RNA polymerase
early mRNA
growth factors
immune defense
molecules
nuclear factors ?
concatemer resolution
DNA packaging
9
exit
12
Golgi
wrapping
11
maturation
10

Figure 2

Gonzalez *et al.* (1991) on the basis of immunodiffusion and counter-immunoelectrophoresis studies, have shown that there is a considerable antigenic variation in CPD viruses from different geographical regions and there are antigenic similarities between CPD virus, bovine papular stomatitis virus and pseudocowpox virus. It is suggested that parapox virus may be a single virus adapted to different species of ruminants.

Despite an apparent serological cross-reactivity and genetic relatedness between CPD virus and vaccinia virus, cross-protection in sheep has not been found (Aynaud, 1923; Robinson and Mercer, 1988).

The CPD hyperimmune serum detected three vaccinia virus antigens on immunoblotting, but there was no cross-protection observed between the two viruses (Mercer *et al.*, 1994). Also cross-protection has not been demonstrated between goatpox virus or sheeppox virus and CPD virus (Sharma and Dhanda, 1971; Renshaw and Dodd, 1978). However, cross-protection has been demonstrated between CPD virus and bovine papular stomatitis virus and between CPD virus and parapox virus of red deer (Mackintosh and Smith, 1987), suggesting that all members of the parapox genus will cross-protect.

2.7 Genome of parapox viruses

Among the parapox viruses, the DNA of bovine papular stomatitis virus was first analysed by restriction endonucleases (Menna *et al.*, 1979). The DNA was shown to be linear, double stranded, with a molecular weight of approximately 85 x 10^6 (135 Kb), and with cross-linked termini. It was reported that the DNAs of CPD virus,

pseudocowpox virus and the virus of bovine papular stomatitis had G + C contents of 63% compared with 36 to 37% for vaccinia virus and rabbitpox virus (Wittek *et al.*, 1979).

CPD virus, bovine papular stomatitis virus, and pseudocowpox virus when compared by restriction endonuclease analysis and DNA/DNA hybridization, the results obtained were in agreement with their classification based on host range and pathology (Wittek *et al.*, 1980; Gassmann *et al.*, 1985). However, analysis with *Eco*RI and *Hin*dIII showed considerabale heterogenicity in the distribution of cleavage sites along the DNA, both between and within isolates of these three viruses (Wittek *et al.*, 1980; Gassmann *et al.*, 1985). Cleavage-site maps confirmed major differences between nine isolates of bovine papular stomatitis virus and two CPD virus and two pseudocowpox virus isolates. DNA/DNA hybridization studies showed that these three viruses shared strong homology in the central regions of their genomes but not at their termini. Hybridization of terminal fragments revealed that the parapox virus genomes were terminally redundant.

The restriction endonuclease analysis of DNA extracted form scab material of different strains of CPD virus isolated in New Zealand showed variation in restriction fragment patterns but contained DNAs of similar size. (Robinson *et al.*, 1982). Similarly Mazur *et al.*, (1991) extracted DNA of CPD virus isolated from scab material. This DNA was used for digestion with several restriction endonucleases which produced parapox-specific patterns. Southern blots with the TK gene of vaccinia virus as probe confirmed the virus as being poxvirus related and showed a preliminary TK gene location for the isolates.

A New Zealand CPD virus isolate, NZ2, was plaque-purified in cell-culture and amplified on sheep skin. It's viral DNA was recovered and maps were produced for the restriction endonucleases *Eco*RI, *Hin*dIII, *Bam*HI, and *Hpa*I. Restriction endonuclease-derived fragments representing complete genome, except for 100 bp at termini, were cloned into various vectors (Mercer *et al.*, 1987). With the help of these clones, a *Kpn*I map of NZ2 isolate and restriction maps for 16 other isolates were deduced. This analysis revealed that restriction endonuclease cleavage sites were conserved between isolates, except for a region spanning 3 to 35 Kbp from the left end. Restriction sites in this region were variably present or absent between isolates and some sites were unique. A section of non-homology in this region, extending for at least 2.75 Kbp was detected in a number of isolates (Robinson *et al.*, 1987).

Restriction endonuclease clevage site maps and gel patterns of New Zealand CPD virus strains were compared with European and North American strains (Wittek *et al.*, 1980; Gassmann *et al.*, 1985; Fraser *et al.*, 1990). These showed differences, but by comparing with *Kpn*I, patterns were same.

DNA from parapox virus of red deer can be distinguished from CPD virus and the cattle parapox viruses by *Kpn*I and *Bam*HI enzymes. DNA/DNA hybridization has shown that the terminal regions of the deer parapox virus do not hybridize to those of CPD virus (Robinson, 1988).

2.7.1 Parapox virus genes and transcription

Portion of the CPD virus genome have been sequenced and a number of open reading frames identified (Fraser *et al.*, 1990;

Naase *et al.*, 1991 and Klemperer *et al.*, 1995). CPD virus genome is the only parapox virus genome for which transcriptional and sequence data are available. Both early and late vaccinia virus-like promoter sequences have been found at 5' to the first ATG in many of these putative genes and the early transcriptional stop motif, T_5NT has been found 3' to those preceeded by early promotor sequences. Transcriptional analysis of the early gene has shown that the 5' end of the message begins 12 to 13 nucleotides down stream from each of two T_5NT motifs (Fleming *et al.*, 1991). The close similarity between CPD virus and vaccinia virus transcriptional control sequences has been analysed further using vaccinia virus recombinants containing this and other early genes (Moss, 1990).

Besides the similarities in transcriptional signals, some of the CPD virus genes have homology to those of vaccinia virus at amino acid level and occupy similar positions on the genome. Homologies have been found between pseudoprotease (dUTPase-like), the 14Kd fusion protein gene, and the vaccinia virus H4, H5, H6 and H7 (topoisomerase) genes (Rosel *et al.*, 1986; Rodriguez and Esteban, 1987; Mercer *et al.*, 1989; Slabaugh *et al.*, 1989; Naase *et al.*, 1991; Klemperer *et al.*, 1995). Mercer *et al.* (1996) found similarity in transcriptional signalling, and homology between the genes of DNA polymerase of CPD virus and vaccinia virus. However, attempts to substitute the activity of the CPD virus DNA polymerase for its vaccinia virus homologue were unsuccesful. Despite difference in G+C content of the two viruses, similarities in transcriptional signals and strong homology between the genes of two viruses, their common evolutionary origin is evident.

The NZ2, CPD virus isolate from New Zealand, revealed a population of variants when serially passaged in primary bovine testis cells. Restriction endonuclease analysis of the DNA extracted showed atleast three distinct mutant forms in which right end of the genome had been duplicated and translocated to the left end accompanied by deletions of sequences at the left end. Sequencing of a single variant isolated from the heterogenous population detected recombination non-homologous sequences; 6.6 Kb of DNA at the left end of the genome had been replaced by 19.3 Kb from the right end. The transposition resulted in the deletion at the left end of 3.3 Kb of DNA encoding 3 genes and the terminal sequence of fourth gene. The three genes completely deleted were a homologue of dUTPase, a gene that encodes a protein containing ankyrin-like repeats and a homologue of the 5Kd protein gene of vaccinia virus WR strain (Fleming *et al.*, 1995). These types of genome rearrangments have also been observed in fowlpox virus and the capripox virus (Drillien *et al.*, 1987; Gershon *et al.*, 1989).

2.8 Immunization

CPD is prevalent in countries that raise sheep and goats. Conventional vaccine which is a live unattenuated vaccine is reported to produce a good immunity (Aynaud, 1923; Glover, 1928; Howarth, 1929; Schmidt and Hardy, 1932). Recurrence of disease in vaccinated animals was reported by Trueblood *et al.* (1963), but it was not known whether this was due to a different strain of CPD virus or improper vaccination technique (Buddle *et al.*,1984). Attempts to produce an effective cell-culture vaccine are on and several workers have advocated the use of

such vaccines both in pregnant ewes and suckling lambs (Pye, 1990; Nettleton *et al.*, 1996 and Marklew, 1997). However, Buddle *et al.* (1984) reported vaccination failure against CPD virus and showed that cell-propagated CPD virus preparation are less effective for vaccination purpose than those propagated in sheep.

A recombinant subunit vaccine against CPD virus has also been developed and shown to be effective in labortory trials (Philbey *et al.*, 1997).

2.9 Immunity to CPD virus infection

Immunity to poxvirus infections is due to humoral and cell-mediated responses of the immune system (Blandeu and Gardner, 1976; Buller and Palumbo, 1991).

Early work showed that serum from immune animals was not protective. The colostrum from immune dams containing neutralizing antibodies when passed onto lambs, it was ineffective in protecting lambs from infection (Aynaud, 1923; Buddle and Pulford, 1984). Workers have also shown that lambs born from immune dams can succumb to natural infection in first few days of life (Boughton and Hardy, 1934; Schmidt, 1967). However, in some experiments, it has been shown that titre of neutralizing antibody in colostrum is directly correlated with the degree of protection transferred to lambs (Poulain *et al.*, 1972; Jan *et al.*, 1978).

Mechanism of protective immune response against CPD virus has been studied by evaluating both humoral and cell-mediated responses

induced after infection. Early work showed that virus neutralizing, agglutinating, precipitating, and complement fixing antibodies can be detected after CPD virus infection (Rottgardt *et al.*, 1949; Abdussalam, 1958; Nagington and Whittle, 1961; Poulain *et al.*, 1972; Jan *et al.*, 1978; Buddle and Pulford, 1984). Cytotoxic antibody measured in complement mediated cytotoxic assay, can be detected in CPD infected sheep, peaking 28 days after infection (DeMartini *et al.*, 1978; Koptopoulos *et al.*, 1982).

Mckeever *et al.* (1987) showed that animals naturally infected with CPD virus responded to 13 virus components on western blot. However, experimentally infected animals responded consistently to only 3 or 4 antigens, one of which appeared to be surface tubule antigen and Ig G class of antibody could be detected at 14 days post-infection by ELISA.

2.9.1 Cell-mediated responses

Antibody responses against CPD virus appear to be of limited significance in overall protection and recovery. It seems likely, therefore that recovery from the disease is the result of cell mediated immune responses.

Cellular responses to CPD infection in sheep have been studied using cells from cannulated efferent ducts of draining lymphnodes. The cells emerging from draining popliteal node increased 10 fold on days 3-4. Large blastic lymphocytes increased 15 to 25 fold over the next 3 days. Antibodies were detected 24 hours after the appearance of blastic cells (Pearson *et al.*, 1979). Similar results were observed by Mckeever and Reid (1987) and Yirrell *et al.* (1991).

Yirrell *et al.* (1989) studied proliferative responses of peripheral blood lymphocytes to CPD virus antigen. Little proliferative response was seen in a primary infection. However, a greater response was seen after a second inoculation. Jenkinson (1992) reported that local scarification coupled with infection with CPD virus induces more number of CD8, CD4 and B-cells within epidermis than the scarification alone. Also, following infection with CPD virus, class II positive dendritic cells develop in dermis and epidermis thus providing local defence (Jenkinson, 1991).

CPD virus infection activates different components of cellular defence mechanism. Cytotoxic T-cells are considered important component in recovery and protection from poxvirus infection. It has been reported that following reinfection with CPD virus, CD8 positive cells get recruited to the site of lesion in skin during the period of viral replication. These cells appear in large number in afferent lymph draining the infection site (Haig *et al.*, 1996). It has also been seen that CPD virus activates both CD45R positive and CD45R negative $\alpha\beta$ receptor T-cell subsets. Among these, $CD45R^-$ subset is predominent in later stage of infection and is principle source of lymphokines in afferent lymph and appear to play important role in recovery from infection (Haig *et al.*, 1996).

CHAPTER-III

MATERIALS AND METHODS

3.1 Animals

Four apparently healthy lambs of either sex between 6-8 months of age were procured from the Department of Animal Breeding of the College of Animal Sciences, CCS HAU, Hisar. The lambs had no previous history of infection with either sheep pox or contagious pustular dermatitis virus. The animals were housed in the experimental animal house of the department and fed *ad libitum.*

The animals were infected in the axilla, groin and flank region by scratching with a 20 gauze needle and then swabbing with the virus infected scabs suspension.

3.2 CPD virus

The contagious pustular dermatitis (CPD) virus originally isolated from an outbreak of CPD in sheep at Central Sheep Breeding Farm, Hisar during 1995 was used in the present study. The virus was maintained in the Pox virus Laboratory of the Department at 4°C in the form of infective skin scabs. A 10% suspension of infected skin scabs in 0.01M phosphate buffer saline, pH 7.2 was prepared by trituration in a pestle and mortar followed by centrifugation at 750 x g at 4°C for 20 min. This suspension was used as virus inoculum for infection of animals.

3.3 Virus stock

For raising the stock virus, the lambs were infected by scarification with CPD virus in the wool free area of groin and axilla.

The rectal temperature of animals was recorded daily. On fourth day post inoculation, small areas of reddening and region of

inflammation were seen along the site of inoculation. The temperature rose to 104°F on 5th day and went upto 105.6°F on 8th day. By this time vesicles had developed at the site of inoculation which turned to scabs during the next 4 to 5 days. By 9th day the temperature started receeding and came to normal by 15th day post inoculation. The skin scabs were harvested on 16th day when they became dry and were about to fall from the skin. These skin scabs were used to obtain purified virus.

3.4 Raising of hyperimmune serum

Hyperimmune serum against CPD virus was raised in lambs. Two of the lambs having no history of CPD virus infection were used for this purpose. An area of about 4 x 4 sq. inches in the flank region was shaved. The skin after thorough cleansing was scratched with a 20 gauze needle. Immediately the scarified area was swabbed with 10% suspension of skin scabs prepared above. The lambs were monitered for the development of clinical signs which started with elevated body (rectal) temperature and reddening and swelling of the skin around the areas of scratching on 4th day post infection. By 7th day distinct small vesicles developed which enlarged to form papules. The development of papules was maximum by 10th day post infection. The papules turned into dried scabs by 15-20 days post-infection. By 22nd day post infection, the scabs formed at the site of infection were sloughed off without leaving any scar. Animals were bled by venipuncture for blood collection and serum was separated for use as convalescent serum. Each animal was further injected i/m with 1 ml of 10% virus scab suspension homogenized with

equal volume of Freund's complete adjuvant. Animals were further boosted twice i/m using 1 ml of 10% scabs suspension mixed with equal quantity of Freund's incomplete adjuvant at 15 days interval. Finally, animals were blood 10 days after the last booster injection, serum was separated, stored at -20°C and used as CPD hyperimmune serum.

3.5 Purification of CPD virus

CPD virus was purified by a modification of the method used by Joklik (1962). Briefly, 2 g of skin scabs infected with CPD virus were triturated in a sterile pestle and mortar. A 10% (w/v) suspension was prepared in TE buffer (0.001M, pH 9). The suspension was sonicated for 30 seconds, followed by centrifugation at 750 x g at 4°C for 20 min. Pellet was discarded and supernatent was layered on to 3 ml of 36% (w/v) sucrose cushion and contrifuged in the swing out rotor (SW-28) of Beckman ultracentrifuge at 65000 x g. at 4°C for 60 min. Supernatant was discarded and the pellet suspended in 30 ml T.E. buffer (0.001 M, pH 9). This suspension was again layered on to 3 ml of 40% (w/v) sucrose cushion and centrifuged using SW-28 rotor of Beckman ultracentrifuge at 65000 x g at 4°C for 60 minutes. The pellet thus obtained was again suspended in 3-6 ml T.E. buffer (0.001 M, pH 9) and layered onto a linear sucrose gradient (40 to 60% w/v). The gradients were centrifuged at 65000 x g at 4°C for 60 min. in of Beckman ultracentrifuge using SW-28 rotor. The diffuse virus band was visible in upper 2/3rd portion of the gradient. This band was collected from the top with the help of a sterile Pasteur pipette and diluted at least five times with T.E. buffer (0.001 M pH 9). The virus was pelleted by

centrifugation at 100,000 x g at 4°C for 60 min. Purified virus pellet was supended in 1 ml of T.E. buffer (0.001M pH 9). Electron microscopy of the viral suspension was done to ascertain the purity of the preparation.

3.6 Electron microscopy

To assess of the purity of the virus preparation obtained after density gradient centrifugation, the virus pellet was suspended in 0.001M T.E. (pH 9) and processed for transmission electron microscopic examination at All India Institute of Medical Sciences, New Delhi. The activated copper grid was placed over a small drop of viral preparation for 20 to 30 seconds. The grid was then transferred over a small drop of staining solution for 15 to 20 seconds and washed twice quickly over drops of phosphate buffer. After air drying, the specimen was examined under the electron microscope (Philips, CM-10).

3.7 Preparation of soluble protein preparation

For obtaining soluble antigen preparation of CPD virus, the infected skin scabs were triturated in a sterile pestle and mortar. A 10% (w/v) suspension was prepared in T.E. buffer (0.001M pH 9). This suspension was freeze thawed twice for mechanical rupture of cells. The suspension was then sonicated for 30 seconds, followed by centrifugation at 750 x g at 4°C for 20 min. Pellet was discarded and supernatant was ultracentrifuged at 100,000 x g at 4°C for 60 min. in SW-28 rotor. The supernatant was collected carefully without disturbing the pellet. This was then concentrated approximately five times by counter dialysis using 20% (w/v) solution of PEG-6000 to get soluble antigen preparation of CPD virus.

3.8 Sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

3.8.1 Sample preparation

The sucrose density gradient purified CPD virus was suspended in T.E.buffer (0.001M pH 9). For carrying out electrophoresis, the samples were mixed with equal volume of 2 x sample buffer, boiled for 3 min. and then loaded onto the SDS-PAGE gel.

3.8.2 SDS-PAGE

Proteins were separated by discontinuous slab gel electrophoresis according to the method of Laemmli (1970). The 12% resolving gel was prepared by cross-linking acrylamide with N-N'- methylene bisacrylamide (29 : 1) in resolving buffer. Polymerization was achieved by the addition of Ammonium persulphate (0.006%, w/v) and TEMED (0.004%, v/v) to the gel solution just before pouring into the gel sandwich, held in a tank containing cold water in order to disperse heat during polymerization. The gel was overlaid carefully with either resolving gel buffer (1/4th strength) or butan-2-ol, to ensure a smooth interface for polymerization. A 5% stacking gel was prepared shortly before sample loading and contained acrylamide cross-linked with N-N'- methylene bisacrylamide (29 : 1) in stacking gel buffer. Wells were made with the help of Teflon combs. About 50 µg sample of each preparations was loaded into wells and electorphoresed in a freshly prepared electrophoresis buffer at a constant voltage of 100V unitll the Bromophenol blue dye front reached upto the bottom of the gel which took approximately 5 to 6 hours.

3.8.3 Coomassie blue staining

After electrophoresis, the gel was transferred to a fixing solution (45% methanol, 9% acetic acid) for 1 hour. Proteins were stained for 2 hours or overnight with 0.025% Coomassie Brilliant Blue-RG 250 prepared in the fixing solution. The destaining of the gel was carried out with a solution containing 30% methanol and 9% acetic acid to remove the background staining of excess stain.

3.9 Western blotting of resolved proteins

Proteins from SDS-PAGE gel were transferred onto nitrocellulose membrane by the semi-dry method of electro-blotting as described by Kyhse-Anderesen (1984) using a Nova Blot Electrophoretic Transfer Unit of Pharmacia. For transferring the proteins following steps were carried out :

The electrode plates of the semi-dry apparatus were rinsed with distilled water. Whatman No 1 absorbant paper sheets (eighteen) and a sheet of nitrocellulose were cut to the size of the gel. The nitrocellulose sheet was soaked in distilled water while whatman filter paper sheets were soaked in transfer buffer. On the bottom plate of the apparatus (anode) nine layers of whatman filter paper soaked in transfer buffer were kept, followed by nitrocellulose sheet, polyacrylamide gel and nine layers of whatman filter paper. Air bubbles trapped if any, were removed by rolling a pipette over the sandwich. Another electrode plate (cathode) was placed on the top of the stack. A current of 0.8 mA/cm^2 of gel was applied for 45 min. to 1 hour. After the run, assembly was carefully disassembled and top of nitrocellulose sheet marked with a pencil for

further processing. The gel was stained with coomassie blue to verify transfer.

After transfer, the nitrocellulose membrane was rinsed in PBS pH 7.2 and immersed in 5% (w/v) non-fat dry milk powder dissolved in PBS pH 7.2 and 0.1% Tween 20 (Blotto) for 1 hour to block unsaturated sites of the nitrocellulose membrane. The nitrocellulose membrane was then incubated with 2% normal rabbit serum in blotto, followed by incubation with diluted CPD hyperimmune serum (1 : 150 in blotto) at 37°C for 1 hour with gentle shaking. After two washing each with PBS-Tween (PBS 0.1% Tween 20), the nitrocellulose membrane was incubated with antisheep immunoglobulin horse radish peroxidase enzyme conjugate (1 : 1500 in Blotto) for 1 hour at 37°C with gentle shaking. The nitrocellulose membrane was washed as described above and placed in 50 mM Tris-HCl (pH 7.6) for 10 minutes. The nitrocellulose membrane was washed and finally incubated in freshly prepared substrate solution (10 mg diaminobenzidine tetra hydrochloride dissolved in 50 ml of 50 mM Tris-HCl buffer pH 7.6 with the addition of 35 µl of 30% v/v H_2O_2) for approximately 4-5 minutes to allow the development of antibody reactive bands. The reaction was stopped by washing the nitrocellulose membrane in distilled water.

3.10 Estimation of molecular weights of proteins

The molecular weights of the proteins separated on SDS-PAGE and Western blot were calculated by using the standard curve (Appendix I).

CHAPTER-IV

RESULTS

4.1 Lesions

All the four sheep which were infected with CPD virus by scarification in wool free areas of axilla and groin and shaved flank area, developed typical lesions of CPD (Fig. 3 and 4). Three days after infection, on 4th day the lesions started developing as reddening and swelling around the sites of inoculation with elevated body temperature. The maximum body temperature was recorded on 8th day post infection. The lesions progressed into small vesicles of approximately 2mm in diameter over next 2 days. The vesicles gradually became pustular and vesiculo-pustular stage of the lesions lasted for about 3 to 4 days. Thereafter, scabs developed by 9 to 10 days post-inoculation, which became thicker with time and started shedding by day 16 to 18. The lesions were resolved, leaving only mild erythema along the lines of scarification between 24 to 28 days. All the sheep infected experimentally, showed almost uniform pattern of development of clinical signs and lesions.

4.2 Electron microscopic examination

CPD virus was purified from skin scabs collected from experimentally infected animals by sucrose density gradient centrifugation. The protein content of purified viral preparation was calculated by measuring the OD of viral suspension at 260 nm. The viral prepartion was found to contain 3.6 mg of protein/ml (10 ODU = 0.65 mg protein, Joklik, 1962). The purity of viral preparation was assessed by electron-microscopy. Negatively stained viral preparation revealed

Figure 3. Photograph shows development of scabs on the wool free skin of axilla region of experimentally infected lambs with contagious pustular dermatitis virus

Figure 4. Photograph shows development of scabs on the wool free shaved flank region of experimentally infected lambs with contagious pustular dermatitis virus

Figure 3

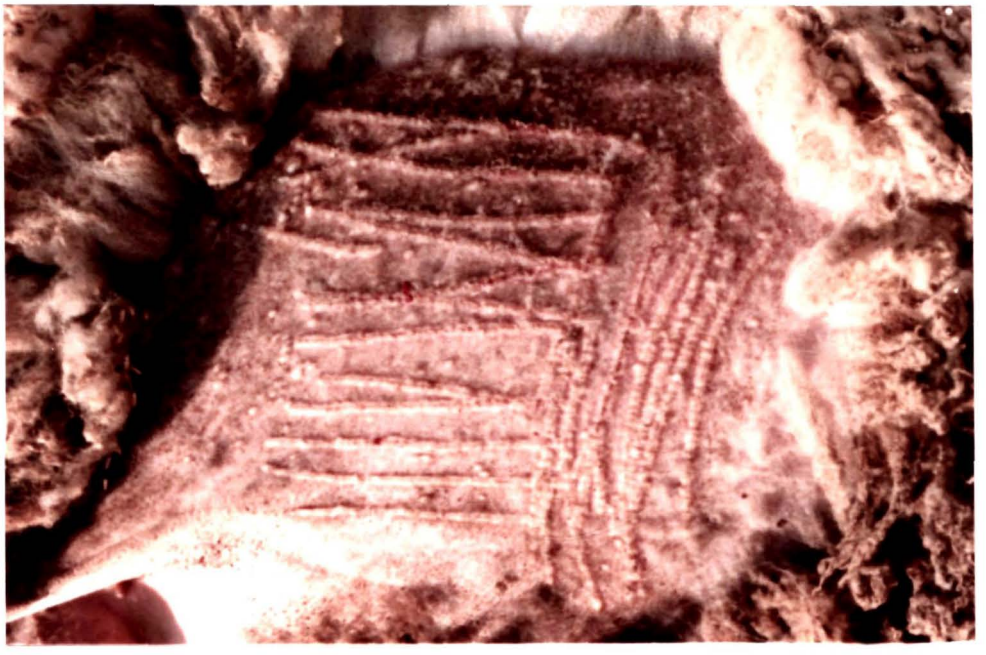


Figure 4

Figure 5. Electron micrograph showing three contagious pustular dermatitis virus particles. The characteristic spiral winding giving unique "ball of yarn" morphology could be seen on the surface of virus particle (X 31000)

Figure 6. Electron micrograph showing several contagious pustular dermatitis virus particles in different planes with a tendency to form aggregates (X 21500)

Figure 5

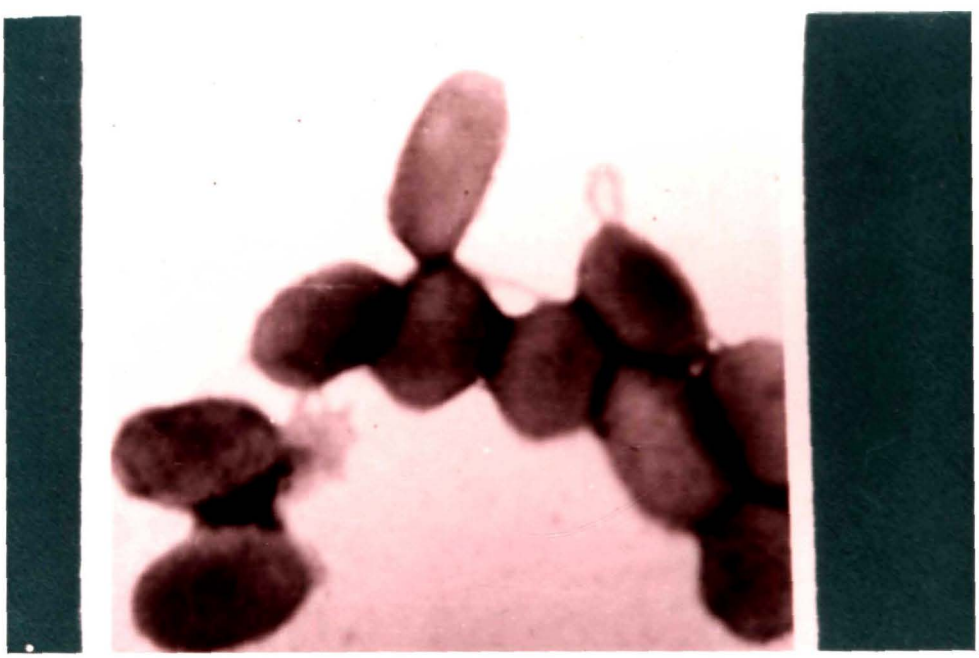


Figure 6

The standard molecular weight markers contained following proteins *viz.* Myosin, β-galactosidase, Phosphorylase b, Fructose-6-phosphate kinase, Bovine serum albumin, Glutamic dehydrogenase, Ovalbumin, Glyceraldehyde-3-Phosphatedehydrogenase, Trypsinogen and Trypsin inhibitor having molecular weights of 205Kd, 116Kd, 97Kd, 84Kd, 66Kd, 55Kd, 45Kd, 36Kd, 24Kd, and 20Kd, respectively. The *regression coefficient* (B) *and correlation coefficient* (γ) *between the* migration pattern of standard molecular weight markers on 8% gel were 48% and 99.8% and on 12% gel were 35% and 99.2%, respectively. Based on these values, the molecular weights of proteins of purified CPD virus and soluble protein preparation resolved on 8% and 12% gels were calculated by applying following equation :

$Y = A + B. \log x$ (Appendix I)

The protein profile of molecular weight marker, mock preparation, purified CPD virus and soluble protein preparation on 8% gel is shown in table 1 and fig. 7. In table 1, the proteins detected in purified CPD virus and soluble protein preparation on 8% gel and in Western blot are shown. The purified CPD virus resolved into 6 proteins of molecular weights ranging from 43Kd to 68Kd and soluble protein preparation separated into 8 proteins of molecular weights ranging from 43Kd to 92Kd. Of 6 proteins detected in purified CPD virus the 53Kd and 43Kd proteins were present in large amounts compared to other proteins and thus appeared to be major structural proteins of purified CPD virus. Similarly out of 8 proteins detected in soluble protein preparation, four proteins (of molecular weights 92Kd, 85Kd, 68Kd and 53Kd) were

Figure 7. SDS - PAGE profile and Western blot analysis of proteins of purified CPD virus and soluble protein preparation seperated on 8% gel. Lanes marked indicate:

Lane -1 Molecular weight marker

I	Myosin	205 Kd
II	β-galactosidase	116 Kd
III	Phsophorylase b	97 Kd
IV	Fructose -6- phosphatekinase	84 Kd
V	Bovine serum albumin	66 Kd
VI	Glutamic dehydrogenase	55 Kd
VII	Ovalbumin	45 Kd
VIII	Glyceraldehyde - 3 - phosphate dehydrogenase	36 Kd
IX	Trypsinogen	24 Kd
X	Trypsin inhibitor	20 Kd

Lane - 2 Soluble protein preparation from uninfected sheep skin (Mock)

Lane - 3 Sucrose gradient purified CPD virus proteins

Lane - 4 Soluble protein preparation obtained from skin scabs collected from infected sheep with CPD virus

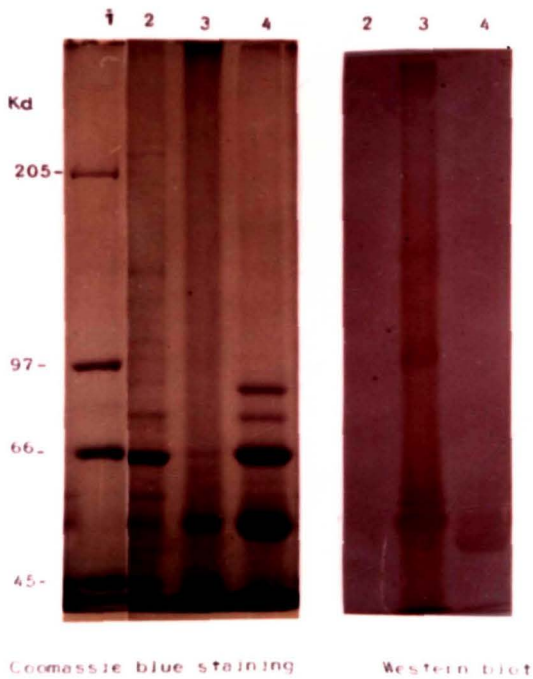

1
2
3
4
2
3
4
Kd
205-
97-
66-
45-
Coomassie blue staining
Western blot

Figure 7

observed to be present in large amounts thus these proteins appeared to be the major components of the soluble protein preparations.

To detect the antigenic nature of individual protein, the proteins separated on SDS-PAGE were transferred onto the nitrocellulose membrane and reacted with CPD hyperimmune serum. The CPD hyperimmune serum reacted with 7 discrete protein bands of purified CPD virus with apparent molecular weights ranging from 43Kd to 116Kd. Similarly four proteins of molecular weights ranging from 53Kd to 92Kd were detected in the soluble protein preparation. The 43Kd protein in purified CPD virus and 53Kd protein in the soluble protein preparation was present in large amounts and also reacted strongly on Western blot.

Proteins resolved on a 12% gel in purified CPD virus preparation and soluble protein preparation and their Western blot are shown in table 2. 18 proteins of molecular weights ranging from 14Kd to 125Kd could be detected in purified CPD virus. The proteins located at 68Kd, 53Kd,43Kd, 21Kd and 14Kd were deeply stained in comparison to the rest of the proteins (fig. 8). These deeply stained proteins appeared to be the major structural proteins of purified CPD virus.

Similarly, soluble protein preparation resolved into 16 proteins of molecular weights ranging from 14Kd to 152Kd on 12% gel. Four proteins of molecular weights 68Kd, 53Kd, 21Kd and 14Kd were present in large amounts, appearing to be the major proteins of soluble protein preparation (table 2).

Figure 8. SDS - PAGE profile and Western blot analysis of proteins of purified CPD virus and soluble protein preparation seperated on 12% gel.Lanes marked indicate:

Lane -1 Molecular weight marker

I	Myosin	205 Kd
II	β-galactosidase	116 Kd
III	Phsophorylase b	97 Kd
IV	Fructose -6- phosphatekinase	84 Kd
V	Bovine serum albumin	66 Kd
VI	Glutamic dehydrogenase	55 Kd
VII	Ovalbumin	45 Kd
VIII	Glyceraldehyde - 3 - phosphate dehydrogenase	36 Kd
IX	Trypsinogen	24 Kd
X	Trypsin inhibitor	20 Kd

Lane - 2 Soluble protein preparation from uninfected sheep skin (Mock)

Lane - 3 Sucrose gradient purified CPD virus proteins

Lane - 4 Soluble protein preparation obtained from skin scabs collected from infected sheep with CPD virus

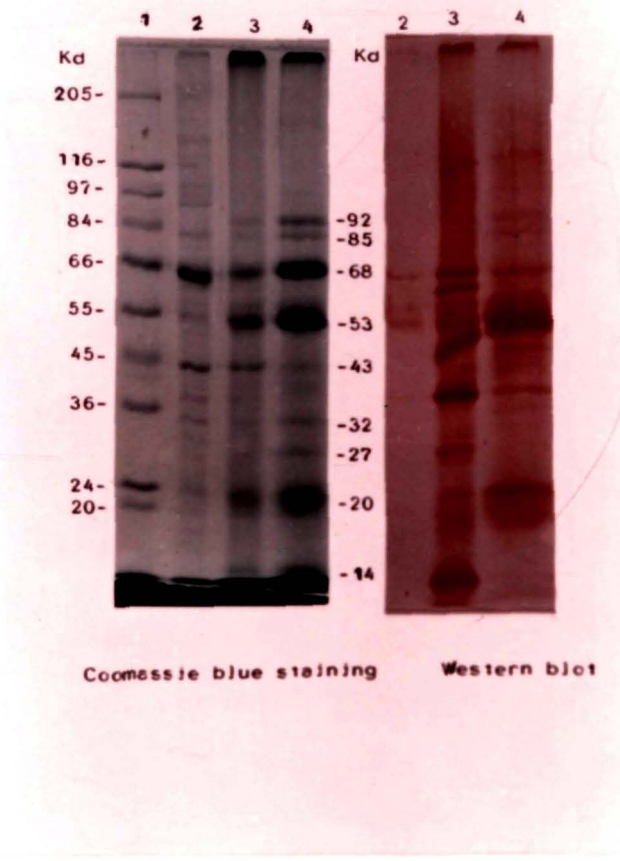
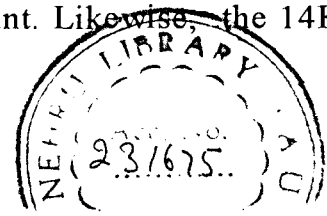

1
2
3
4
Kd
205-
116-
97-
84-
66-
55-
45-
36-
24-
20-
2
3
4
Kd
-92
-85
-68
-53
-43
-32
-27
-20
-14
Coomassie blue staining
Western blot

Figure 8

Western blot of 12% gel showed 12 proteins of CPD virus of apparent molecular weights ranging from 14Kd to 116Kd detectable by reacting with specific antibodies present in CPD hyperimmune serum. Among these proteins, the proteins of molecular weights 68Kd, 67Kd, 53Kd, 38Kd and 14Kd reacted strongly with hyperimmune serum, indicating that these were major antigenic proteins of purified CPD virus. Similarly in soluble protein preparation, 12 proteins reacted with hyperimmune serum (fig. 8). The molecular weights of these proteins were in the range of 21Kd to 123Kd. The proteins of molecular weights 68Kd, 53Kd, 38Kd and 23Kd showed strong reaction compared to other proteins on Western blot, appearing to be the major antigenic proteins of soluble protein preparation. The proteins of molecular weights of 125Kd, 107Kd, 57Kd in purified CPD virus and proteins of molecular weights of 152Kd, 110Kd, 52Kd and 14Kd in soluble protein preparation were detected on SDS-PAGE but did not react with CPD hyperimmune serum on Western blot. The proteins of 116Kd in purified CPD virus and 123Kd, 47Kd and 35Kd in soluble protein preparation were detected on Western blot only. The proteins of 81Kd, 67Kd and 62Kd of purified CPD virus and 85Kd and 59Kd of soluble protein preparation were detected on SDS-PAGE as well as on western blot. The protein of molecular weight 43Kd was present in large amount in purified CPD virus but did not react with CPD hyperimmune serum on Western blot. Whereas the protein of similar molecular weight (43Kd) was detected on both SDS-PAGE and Western blot in soluble protein preparation but it was present in lesser amount. Likewise, the 14Kd protein was present



in large amounts in purified CPD virus and found to produce strong reaction on Western blot. A protein of similar molecular weight (14Kd) was detected in soluble protein preparation on SDS-PAGE which did not react with CPD hyperimmune serum on Western blot.

Besides these, some of the proteins which were detected in both the preparations are shown in table 3.

The proteins of molecular weights of 68Kd and 53Kd were detected in large amounts on SDS-PAGE in both purified CPD virus and soluble protein preparation. In addition these strongly reacted with CPD hyperimmune serum on Western blot. The protein of 38Kd was found to be strongly antigenic despite this protein appeared to be a minor protein on SDS-PAGE in purified CPD virus and soluble protein preparation. The 21Kd protein appeared to be a major protein of both purified CPD virus and soluble protein preparation but reacted weakly with CPD hyperimmune serum on Western blot. The protein of 23Kd was detected on SDS-PAGE and Western blot of both purified CPD virus and soluble protein preparation but was strongly antigenic in soluble protein preparation. The protein of 92Kd was present in both preparations and was found to be antigenic in nature. Three proteins of molecular weights of 100Kd, 32Kd and 27Kd were detected on SDS-PAGE of purified CPD virus and soluble protein preparation but they did not react with CPD hyperimmune serum on Western blot.

Table 1. SDS-PAGE profile and Western blot analysis of purified CPD virus structural proteins and soluble protein preparation on 8% gel

S.No.	Purified CPD Virus		Soluble Protein Preparation	
	SDS-PAGE	Western blot	SDS-PAGE	Western blot
1.		116		
2.			92*	92
3.			85*	
4.	68	68	68*	68
5.	67	67**		
6.			64	
7.	62	62		
8.		60**		
9.			59	59
10.	53*	53	53*	53
11.	45		45	
12.	43*	43	43	
Total	6	7	8	4

* Major Proteins

**Strongly Antigenic Proteins

Table 2. SDS-PAGE profile and Western blot analysis of purified CPD virus structural proteins and soluble protein preparation

S.No.	Purified CPD Virus		Soluble Protein Preparation	
	SDS-PAGE	Western blot	SDS-PAGE	Western blot
1.			152	
2.	125			
3.				123
4.		116		
5.			110	
6.	107			
7.	100		100	
8.	92	92	92	92
9.			85	85
10.	81	81		
11.	68*	68**	68*	68**
12.	67	67**		
13.	62	62		
14.			59	59
15.	57			
16.	53*	53**	53*	53**
17.			52	
18.				47
19.	43*		43	43
20.	38	38**	38	38**
21.				35
22.	32		32	
23.	29	29		
24.	27		27	
25.	23	23	23	23**
26.	21*	21	21*	21
27.	14*	14**	14*	
Total	18	12	16	12

* Major Proteins

**Strongly Antigenic Proteins

Table 3. Antigenic and non-antigenic co-migrating proteins present in purified CPD virus preparation and soluble protein preparation on 12% gel

S.No.	Purified CPD Virus		Soluble Protein Preparation	
	SDS-PAGE	Western blot	SDS-PAGE	Western blot
1.	100		100	
2.	92	92	92	92
3.	68*	68**	68*	68**
4.	53*	53**	53*	53**
5.	38	38**	38	38**
6.	32		32	
7.	27		27	
8.	23	23	23	23**
9.	21*	21	21*	21

* Major Proteins

**Strongly Antigenic Proteins

CHAPTER-V

DISCUSSION

Contagious pustular dermatitis (CPD) is an infectious eruptive skin disease of sheep and goats. The causative agent of CPD disease belongs to the genus *Papapoxvirus* of the family *Poxviridae* (Matthews, 1979). The members of parapoxvirus genus have been reported to be closely related (Gonzalez *et al.*, 1991a). Clinically the disease resembles with that of capripox infection and an antigenic relationship of CPD virus has been reported with that of capripox virus and vaccinia virus on the basis of AGID and complement fixation test (Sharma and Dhanda, 1971; Dubey and Sawhney, 1979; Mercer *et al.*, 1994). The genome of CPD virus extracted from skin scabs collected from clinical cases of the disease has been shown to be a linear, 140 Kb, ds DNA molecule (Robinson *et al.*, 1982). The DNA of CPD virus codes for a large number of structural and non-structural proteins. Analysis of these viral proteins is important for understanding of different biological properties and functions of the virus.

The purified CPD virus, in the present study, was found to resolve into 6 proteins by SDS-PAGE on 8% gel whereas 18 proteins were detected on 12% gel. Hence, 12% gel was used in subsequent studies. The molecular weights of the various viral proteins on resolving by SDS-PAGE when estimated by calculating the Rf values were found to vary between 14Kd and 125Kd. There appears to be no agreement on number of proteins detected in purified CPD virus on SDS-PAGE by different workers. Gonzalez *et al.* (1991b) reported 4 to 12 proteins while comparing the SDS-PAGE profile of different isolates of CPD virus. By metabolic methionine labelling of the virus proteins, Balassu and

Robinson (1987) reported, 25 to 35 proteins in five different isolates of CPD virus. Buddle *et al.* (1984) using the same procedure detected 31 proteins in CPD virus. Zuo *et al.* (1988) reported 28 proteins of molecular weight ranging from 14Kd to 190Kd in purified CPD virus. In the present study, in purified virus 18 viral proteins were detected on SDS-PAGE. The differences in the number of proteins observed by different workers could be attributed to the difference in isolates of CPD virus, methods of detection and source of virus. Nevertheless heterogeneity in genomes of CPD virus isolates is well documented (Buddle *et al.*, 1984). The mobility of different proteins on SDS-PAGE and range of molecular weights of the proteins (14Kd to 125Kd) observed in this study is in agreement with that reported by Balassu and Robinson (1987) who have reported variable number of proteins detected in five different isolates of CPD virus. Majority of the 18 proteins detected in purified CPD virus in the present study had molecular weights similar to that as reported by Balassu and Robinson while studying SDS-PAGE profile of 5 different CPD virus isolates. However, differences in detection of number of proteins between their CPD virus isolates and our CPD virus isolate are evident. This may be due to highly sensitive radio labelling procedure of virus proteins used by the authors.

In the present study, the proteins located at 68Kd, 53Kd, 43Kd, 21Kd and 14Kd in the gel were strongly stained with coomassie blue. These proteins appear to be the major structural proteins of this isolate of CPD virus.

The viral proteins are considered to be good immunogens and are known to elicit antibody responses when inoculated into an animal system. Replication of virus in the host animal induces antibody

production. The specific antibodies produced in response to different viral proteins in the host can be identified using Western blotting technique. The latter allows the transfer of a complex protein mixture resolved on a SDS-PAGE gel to a nitrocellulose membrane, when its components are accessible to immunological detection methods and is a useful tool for the dissection of humoral immune response to a given pathogen (Hering and Sharp, 1984).

The Western blotting of purified CPD virus preparation revealed reaction with 12 proteins of the virus indicating their antigenic nature. The molecular weights of these proteins ranged between 14Kd and 116Kd (table 2). The proteins of molecular weights of 14Kd, 38Kd, 53Kd, 67Kd and 68Kd reacted sharply with the antibodies present in CPD hyperimmune serum indicating their strong antigenic nature. Mercer *et al.* (1994) while studying kinetic of antibody production against CPD virus observed that by using hyperimmune serum 14 discrete proteins with apparent molecular weights ranging from 14Kd to 73Kd were detectable by Western blotting but while using serum from lambs obtained 4 weeks after primary inoculation, reaction with only 5 proteins (36Kd, 36.5Kd, 38Kd, 49Kd and 53Kd) of the 14 bands detectable by hyperimmune serum was observed.

A protein of 116Kd detectable on Western blot (table 2) was not detected on coomassie blue staining of SDS-PAGE gel of CPD virus. This might be due to higher sensitivity of the Western blot as compared to staining with coomassie blue. Of the five major antigenic proteins the 14Kd, 53Kd, 68Kd proteins were expressed in larger amounts as detected on SDS-PAGE (fig. 8).

Chin and Petersen (1995) have reported that out of 30 proteins resolved on SDS-PAGE 5 proteins located at 82-84Kd, 43Kd, 38Kd, 36Kd, 25Kd and 23Kd positions showed strong reactions with sheep hyperimmune serum against CPD virus on Western blotting. During the present study, 12 antigenic proteins were detected and some of them appeared to be similar in electrophoretic mobilities with those reported by Chin and Petersen (1995). Some differences observed in these two studies might be due to differences in hyperimmunization procedure of animals or due to antigenic variation of different CPD virus isolates.

Tiwari and Negi (1994) while working on goatpox virus, observed that virus free soluble protein preparation produced antibodies which had neutralizing effect on virus. Their results indicated that soluble proteins of goatpox virus induce neutralizing antibodies when inoculated in goats. Similar kinds of soluble proteins have been shown in other poxviruses. For example, soluble proteins of sheeppox virus (Sambyal and Singh 1978) and vaccinia (Cohen and Wilcox,1966; Singh, 1972) have been shown to be immunogenic in nature. These soluble proteins could be structural or non-structural components of the virus produced during virus multiplication. The CPD virus soluble protein preparation prepared from skin scabs was anlaysed in the present study to identify the number of proteins and their antigenic nature. The SDS-PAGE profile of soluble protein preparation revealed 16 proteins of molecular weights ranging between 14Kd and 152Kd. The proteins of molecular weights 68Kd, 53Kd, 21Kd and 14Kd were observed in larger amounts indicating that these proteins are synthesized in excess during virus

multiplication. Twelve proteins of different molecular weights between 21Kd and 123Kd showed reaction on Western blot. Four of these proteins of 68Kd, 53Kd, 38Kd and 23Kd molecular weights were found to produce strong immunostaining signal on Western blot showing their strong antigenic nature.

The soluble proteins of 123Kd, 47Kd and 35Kd were seen only on Western blot, indicating that the amount of these proteins present in soluble protein preparation was below the detection limit of SDS-PAGE but Western blotting being a very sensitive technique for detection of antigenic proteins, the amounts in preparation of these proteins was sufficient to produce immunostaining signal on Western blot. The 152Kd, 110Kd and 52Kd proteins were detected on SDS-PAGE only as they did not react with CPD antibodies on Western blot (fig. 8). These proteins could be either host contaminating proteins or if coded by virus, they appear to be non structural proteins which are not antigenic in nature.

The 38Kd and 23Kd proteins appear to be minor proteins of virus present in soluble protein preparation but they show strong reactions on Western blot. Similarly in purified CPD virus, 67Kd and 68Kd proteins are reacting strongly on Western blot but are mildly stained on SDS-PAGE (fig. 8), indicating higher sensitivity of Western blot.

Though the 23Kd protein was detected on SDS-PAGE and Western blot of both purified CPD virus and soluble protein preparation but strong immunostaining signal on Western blot was observed with soluble protein preparation (table 2 and fig. 8). There could be two different

proteins of similar electrophoretic mobility produced by CPD virus. It is likely that both of them are present in soluble protein preparation and one being strongly antigenic and produced strong reactions on Western blot.

The 43Kd protein though appeared to be a major structural protein of CPD virus on SDS-PAGE but it did not show reactions with antibodies on Western blot indicating its non-antigenic nature. However, a protein of similar molecular weight (43Kd) in soluble protein preparation was detected on SDS-PAGE and Western blot. Possibly two different proteins are encoded by CPD virus, one of which appear to be non-antigenic major structural protein of the virus and other non-structural and antigenic in nature. On the other hand the 14Kd protein was found to be a major structural protein of purified CPD virus which also strongly reacted with CPD hyperimmune serum on Western blot but a major protein of similar molecular weight (14Kd) appeared to be present in soluble protein preparation which failed to react with antibodies on Western blot. These results indicate that the protein detected in purified virus was antigenic in nature whereas the protein of similar molecular weight detected in soluble protein preparation was not antigenic in nature. Alternatively the 14Kd protein detected in soluble protein preparation could be host contaminating protein.

The 92Kd, 68Kd, 53Kd, 38Kd, 23Kd and 21Kd proteins were observed on SDS-PAGE and Western blot of CPD virus and soluble protein preparation, showing that all these proteins are produced in excess during virus replication and are capable of inducing antibody response against them.

The proteins of 100Kd, 32Kd and 27Kd could be detected only on SDS-PAGE of both preparations but did not show reactions on Western blot with CPD hyperimmune serum. These proteins appear to be the structural proteins of CPD virus which are non antigenic in nature or alternatively in soluble protein preparation, they could be host contaminating proteins having molecular weights similar to virus induced proteins.

The 85Kd and 59Kd proteins were detected on SDS-PAGE of soluble protein preparation and produced reactions on Western blot but not detected on SDS-PAGE profile of purified virus. Thus, these two proteins appear to be non-structural antigenic proteins of virus.

The proteins of 81Kd, 67Kd, 62Kd and 29Kd were detected only in CPD virus and produced reactions on Western blot but not detected in soluble protein preparation indicating that these proteins are structural antigenic proteins of CPD virus.

Among the surface or envelope proteins of CPD virus a 38Kd protein has been shown to be a structural unit of the surface tubules of the virus. This protein has been shown to be antigenic in nature and slight differences in its molecular weights have been reported (Balassu and Robinson, 1987; McKeever *et al.*, 1987 and Buddle *et al.*, 1984). This protein being present on the surface of virus is considered to be one of the proteins important to induce neutralizing antibodies. In vaccinia virus the 58Kd surface tubule protein elicits neutralizing antibodies which inhibit virus entry into the cell and cell to cell fusion (Stern and Dales, 1976). Likewise, in capripox virus 32Kd protein is the envelope

protein and is responsible for eliciting specific antibody response in capripox virus infection (Chand, 1992). Although surface proteins of CPD virus were not analysed separately in the present study but a 38Kd protein was detected in both CPD virus preparation and soluble protein preparation. This protein produced strong immunostaining signal on Western blot of both the preparations. These results indicate that this antigenic protein is soluble in nature as well. This protein could be a better candidate for developing a suitable immunoassay for the diagnosis of this disease.

Twelve of the structural proteins of CPD virus were found to react with antibodies on Western blot. This indicates that these proteins possess linear antigenic epitopes which are accessible to antibodies. In addition, it is reasonable to infer that these linear epitopes stimulated B-cells in animals to produce antibodies. It will be of interest to study whether these proteins possess T-cell epitopes in addition to B-cell epitopes. The 38Kd protein has been shown to be associated with envelope of the CPD virus and also elicits antibody production under natural infection (Buddle *et al.* 1984; McKeever *et al.*, 1987; Mercer *et al.*, 1994). A protein of similar molecular weight (38Kd) was found to be associated with CPD virus and also detected in soluble protein preparation in the present study. This protein would be a potent candidate for future study of its cell-mediated immune responses.

CHAPTER-VI

SUMMARY

Contagious pustular dermatitis virus a member of genus *Parapox* of the family *Poxviridae* is responsible for causing, a highly contagious eruptive skin disease in sheep and goats Humans and Cattle are incidentally infected with this virus. The virus causes lesions around lips, nostrils, on the feet and udder. The disease is of considerable economic importance to sheep industry. Economic losses occur due to reduced growth rate, trade restrictions, morbidity and mortality. Lesions on mouth and legs interfere with feeding and walking and with suckling of young lambs, which subsequently leads to loss of production. The disease is known to be present in every sheep raising country including India.

In this study, lambs were infected with CPD virus experimentally by scarification and swabbing with infected skin scabs suspension. The lesions produced in experimental lambs were similar to that of natural CPD infection. Scabs were collected from these animals and processed to obtain the purified virus by sucrose density gradient centrifugation. Purified viral suspension was checked for its purity by examining under electron microscope at different magnifications. The virions revealed a characteristic morphology with 'ball of yarn' appearance under the electron microscope. Examination of different fields under electron microscope showed majority of CPD virion particles with very little amount of any other kind of protein indicating that preparation contained only CPD virus particles.

Poxviruses synthesize a large number of proteins needed for their various biological functions. A number of proteins coded by virus,

are perhaps produced in excess and are released from infected cells by mechanical disruption of cells. In the present study purified CPD virus and virus free soluble protein preparation were analysed by SDS-PAGE and Western blotting. Purified CPD virus resolved into 18 proteins of molecular weights ranging from 14Kd to 125Kd on 12% SDS-PAGE gel. The proteins of molecular weights 68Kd, 53Kd, 43Kd, 21Kd and 14Kd were found to be major structural proteins of CPD virus. Soluble protein preparation separated into 16 proteins of molecular weights ranging between 14Kd and 152Kd on 12% gel. Among these 68Kd, 53Kd, 21Kd and 14Kd proteins were detected in larger amounts.

The antigenic nature of proteins of purified CPD virus and soluble protein preparation was assessed by transferring SDS-PAGE resolved proteins onto nitrocellulose membrane and reacting them with CPD hyperimmune serum. Twelve proteins of purified CPD virus reacted with hyperimmune serum on Western blot and among these proteins of molecular weights of 68Kd, 67Kd, 53Kd, 38Kd and 14Kd reacted strongly indicating these are major antigenic proteins of the virus. Similarly, 12 proteins of soluble protein preparation reacted with CPD hyperimmune serum on Western blot. Strong reaction was observed with the proteins of molecular weights of 68Kd, 53Kd, 38Kd and 23Kd, indicating their strong antigenic nature. The 68Kd, 53Kd and 38Kd proteins were major antigenic proteins detected in both purified CPD virus and soluble protein preparation. These results indicate that these antigenic proteins could be better candidates for developing an effective subunit vaccine. In addition these antigenic proteins of CPD virus could be exploited for developing a suitable diagnostic enzyme immuno assay.

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APPENDIX-1

Reagents, buffers and solutions

In all preparations double distilled glass water (DDGW) was used and solutions were sterilized by autoclaving at 15 lb for 15 minutes or by filtration. All chemicals used were purchased from Sigma/SRL/ Centron. The buffers, solutions and reagents used in present study are as follows :

1.1 Phosphate buffer saline (PBS), pH 7.2

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.2 g
DDGW to make	1000 ml
pH 7.2	

1.2 Phosphate buffer, 0.1M (pH 6.0 and pH 7.4)

Stock solution

A : 0.2M solution of monobasic sodium phosphate ($NaHPO_4$. $2H_2O$ 31.2 g in 1000 ml)

B : 0.2 M solution of Na_2HPO_4 (28.39 g in 1000 ml)

Xml of A + Y ml of B, diluted to total of 200 ml

X	Y	pH
87.7	12.3	6.0
19.0	81.0	7.4

pH of these solutions was checked with a pH meter. The solutions were filtered and stored at room temperature.

1.3 36% (w/v) sucrose solution

36 g sucrose was dissolved in 100 ml of DDGW. Solution was kept in waterbath for easy dissolution. Solution was filtered through whatman filterpaper, autoclaved and stored at room temperature.

1.4 40% (w/v) sucrose solution

40% sucrose solution was prepared by dissolving 40 g sucrose in 100 ml of DDGW. Solution was filtered through whatman filter paper, autoclaved and stored at room temperature.

1.5 20% (w/v) Polyethylene Glycol-6000 solution

20% solution of PEG-6000 was prepared by dissolving 20 g of PEG-6000 in 100 ml of DDGW. Solution was filtered through whatman filter paper and stored at room temperature.

1.6 Dialysis tubing

Dialysis tube was boiled in DDGW containing 2mM of EDTA. After that dialysis tube was cut to desired length, filled with sample and tied at both ends before carrying out dialysis.

1.7 Acrylamide-bisacrylamide solution

A 30% stock was prepared by adding

Acrylamide	29.2 g
Bisacrylamide	0.8 g
DDGW to make	100 ml

The contents were mixed and filtered through whatman filter paper No. 3. Solution was stored at 4°C in air tight bottles.

1.8 2 Normal Hydrochloric acid solution (2N HCl)

2N HCl solution was prepared by adding 20 ml of concentrated (11N) HCl in 100 ml of DDGW. Solution was kept at room temperature.

1.9 1.5 M Tris HCl (pH 8.8)

Tris base	18.15 g
DDGW to make	100 ml

Tris base was first dissolved in 60 ml distilled water then the pH was adjusted to 8.8 with 2N HCl. Finally volume was made to 100 ml with DDGW. Solution was filtered through whatman filter paper and sterilized by autoclaving and stored at 4°C in air tight bottles.

1.10 1.0 M Tris HCl (pH 6.8)

First 12.1 g of Tris base was dissolved in 60 ml distilled water and pH was adjusted to 6.8 with 2N HCl. Finally volume was made to 100 ml with DDGW. Solution was filtered and autoclaved, followed by storage at 4°C in air tight bottles.

1.11 IM Tris HCl (pH 7.6)

12.1 g of Tris base was dissolved in 60 ml distilled water, pH was adjusted to 7.6 with 2N HCl and final volume was made to 100 ml with DDGW. Solution was filtered, autoclaved and stored at 4°C in air tight bottles.

1.12 Sodium dodecyl sulphate/sodium lauryl sulphate (SDS)

A 10% stock solution was prepared by adding 10 g of electrophoresis grade SDS in 80 ml of distilled water. Solution was kept in waterbath for easy dissolution. Finally volume was made to 100 ml with DDGW.

1.13 Ammonium per sulphate solution (APS)

APS	100 mg
DDGW to make	1 ml

When ever needed fresh solution of APS was prepared.

1.14 Running buffer

A x 5 stock solution was prepared :

Tris base	15.1 g (25 mM Tris)
Glycine	74 g (250 mM glycine, pH 8.3)
SDS	5 g (0.1% SDS)
DDGW to make	1000 ml

The contents were mixed thoroughly and stored at room temperature. Before use the stock was diluted five times with distilled water.

1.15 Sample buffer/loading buffer/Laemmli lysis buffer

A x 2 sample buffer was prepared by mixing

Tris base	0.15 g
SDS (10%)	4 ml
Glycerol	1 ml
Bromophenol blue	20 mg
β-mercaptoethanol	0.4 ml
DDGW to make	10 ml

Sample buffer was also procured from Sigma.

1.16 Coomassie blue stain (0.25%)

Stain was prepared by adding

Methanol	45 ml
Glacial acetic acid	9 ml
Coomassie Brilliant Blue (R-250)	0.25 g
DDGW to make	100 ml

Solution was kept at room temperature.

1.17 Destain solution

Methanol	45 ml
Glacial acetic acid	9 ml
DDGW to make	100 ml

Solution was kept at room temperature. It was reused after passing through activated charcoal powder.

1.18 Resolving gel/Separating gel

Solution Components	Component volumes (ml) per gel mold volume of 25 ml (8%) ml	(12%) ml
H_2O	11.5	8.2
30% Acrylamide mix	6.7	10.0
1.5M Tris (pH 8.3)	6.3	6.3
10% SDS	0.25	0.25
10% APS	0.25	0.25
TEMED (Sigma)	0.015	0.01

1.19 Stacking gel (5%)

Solution Components	Component volumes (ml) per gel mold volume of 5 ml
H_2O	3.4
30% Acrylamide mix	0.83
1M Tris (pH 6.8)	0.63
10% SDS	0.05
10% APS	0.05
TEMED (Sigma)	0.005

1.20 Blocking buffer (Blotto-T)

Blocking buffer was prepared by adding 5% (w/v) non fat dry milk in PBS pH 7.2 containing 0.1% Tween 20.

1.21 Electroblotting buffer/Transfer buffer

Tris base	5.8 g (48 mM)
Glycine	2.9 g (39 mM)
SDS	0.37 g
Methanol	200 ml
DDGW to make	1000 ml

Solution was kept at room temperature.

1.22 Tris-EDTA (pH 9.0)

EDTA	1 mM
Tris-HCl	10 mM

pH was adjusted to 9.0 with 2N HCl.

1.23 Estimation of molecular weights of proteins

Standard markers (Sigma) of molecular weights ranging from 12Kd to 205Kd were run alongwith samples on SDS-PAGE gel. The Rf values of each marker were calculated by the following relationship :

$$Rf = \frac{\text{Distance travelled by a protein marker}}{\text{Distance travelled by the dye}}$$

Logarithmic regression ($Y = A + B.\log x$) of Rf values was determined by using standard molecular weight markers as reference.

A standard calibration curve was drawn by plotting the relationship of molecular weights and logarithmic regression Rf values. Molecular weights of proteins in the samples were then calculated using standard curve (Weber and Osborn, 1969).

Fig. 9 : Standard curve for molecular weight determination

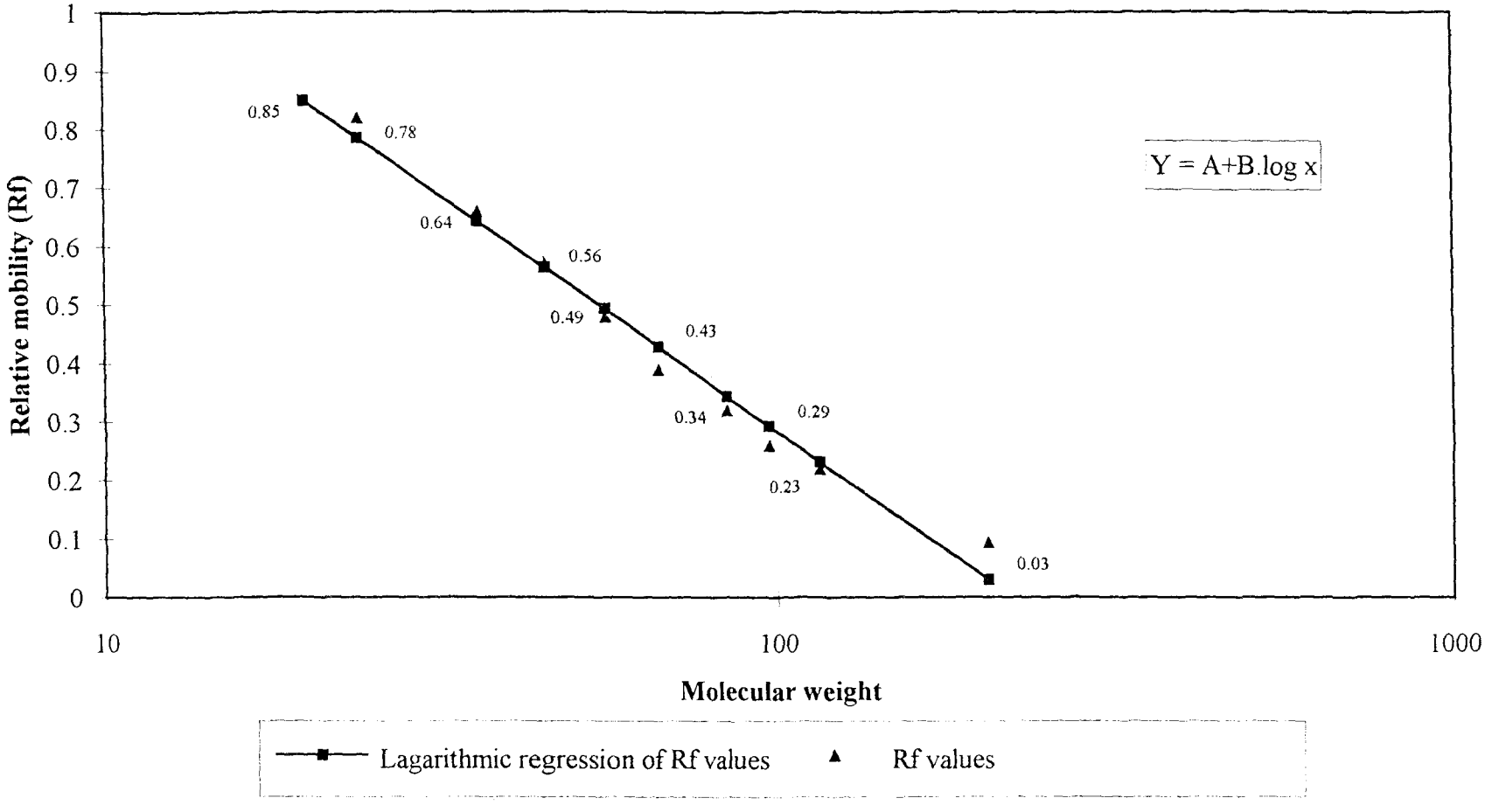

Table 4. Determination of Rf values and logarithmic regression of Rf values of standard molecular weight markers

Standard molecular weight markers	Molecular weight (Kd)	Distance travelled by molecular weight marker	Rf value	Logarithmic regression of Rf values
Myosin	205	1.10	0.094	0.030
β-galactosidase	116	2.60	0.220	0.231
Phosphorylase b	97	3.15	0.260	0.293
Fructose-6-phosphatekinase	84	3.80	0.320	0.344
Bovine serum albumin	66	4.65	0.390	0.429
Glutamic dehydrogenase	55	5.70	0.480	0.493
Ovalbumin	45	6.75	0.570	0.564
Glyceraldehyde-3-phosphate dehydrogenase	36	7.75	0.660	0.642
Trypsinogen	24	9.70	0.820	0.785
Trypsin inhibitor	20	10.05	0.850	0.849
Dye front = 11.7				

Logarithmic regression values
A = 1.902 (constant)
B = -0.8098 (regression coefficient)
γ = -0.992 (correlation coefficient)