IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF IMMUNOGENIC PROTEINS OF CAPRIPOX VIRUS

Thesis

Submitted to the

G. B. Pant University of Agriculture & Technology
PANTNAGAR – 263145, (U.S.NAGAR), UTTARANCHAL, INDIA

By
SANJAY SHAKYA
M.V.Sc.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
(Veterinary Microbiology and Immunology)

SEPTEMBER, 2001

DEDICATED
TO
MY BELOVED PARENTS

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to my advisor Dr. V.D.P.Rao, Professor and Head, Department of Veterinary Microcrobiology for his constant interest, valuable guidance and constructive criticism during the course of this study.

Sincere thanks are due to members of advisory committee; Dr. S.K. Garg, Professor, Department of Veterinary Microbiology; Dr. B.D.Lakhchaura, Professor and Head, Department of Veterinary Biotechnology; Dr. D.C.Thapliyal, Professor and Head, Department of Veterinary Public Health and Dr. Rajesh Chandra, Associate Profesor, Department of Veterinary Microbiology for their constant encouragement and valuable suggestions.

I am thankful to Dr. Amresh Kumar, Dean, College of Veterinary Sciences, Dr. Harpal Singh, Dean, College of Post-graduate studies and Director, Experimentation Station for providing necessary facilities to carry out this work.

I am thankful to Hon'ble Vice-chancellor, Indira Gandhi Krishi Vishwavidhalaya, Raipur for according the study leave for pursuing the present study.

I further extend my regards to Dr. V.D.Sharma and Dr. R.S.Gupta, Professors of Veterinary Microbiology for their time to time help.

I am grateful to Dr. V.V.Suryanarayana, Director, National Biotechnological Centre, Dr. P.K. Gupta and Dr. R.K.Singh, Scientists, IVRI, Izatnagar for providing necessary facilities for purification of virus. I am also

thankful to All India Institute of Medical Sciences, New Delhi for providing necessary facilities for electron microscopic studies.

The facilities and cooperation provided by Dr. G.K.Singh, Professor and Head, Department of Anatomy, Dr. H.C.Sharma, Professor, Irrigation and Soil Water Management and Miss Ragini Saxena, for photography are duly acknowledged.

The willing help and cooperation extended by Dr. Rajesh Kumar and Deepak Rathore, during the tenure of this study is highly appreciable.

I am thankful to Drs. Subodh, Praveen, Sameer, Kapindra, Vikas, Sunil, Shingini, Payal, Pooja, Rajesh and Ashwani for their help during the study.

The author is thankful to Shri Bishan Singh for their assistance and management of laboratory during the course of present study. The help provided by S/Shri J.B. sharma, R.P.Tiwari and S.K.Sharma and other staff is duly acknowledged. I experess my thanks to Shri H.N.Sah for skillful typing of manuscript.

My Special thanks are due to Drs. S.L.Ali, A.K.Gauraha, Jain, Mehta, Wani, Vaid, Guptaji, Sarkar and U.K.Mishra for theirfriendly cooperation.

Words are not enough to express my feelings to my wife Anita for her unceasing moral support and cooperation. I can not forget to mention the sacrifices of my loving daughter Ayushi. I am greatly indebted to my entire family for extending encouragement and help.

(SANJAY SHAKYA)

PANTNAGAR
September,2001

Dr. V.D.P.Rao
Professor and Head

Department of Veterinary Microbiology
College of Veterinary Sciences
G. B. Pant Univ. of Ag. & Tech.
Pantnagar–26 3145, Uttaranchal, India.

CERTIFICATE

This is to certify that the thesis entitled **"IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF IMMUNOGENIC PROTEINS OF CAPRIPOX VIRUS"** submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** with major in **Veterinary Microbiology and Immunology** of the College of Post-Graduate Studies, G. B. Pant University of Agriculture and Technology, Pantnagar, is a record of ***bona fide*** research carried out by **Mr. SANJAY SHAKYA, I.D. No. 25871**, under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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

V.D.P.Rao

(V.D.P.RAO)
Chairman
Advisory Committee

CERTIFICATE

We, the undersigned, members of the Advisory Committee of **Mr. SANJAY SHAKYA, I.D. No. 25871**, a candidate for the degree of **Doctor of Philosophy** with major in **Veterinary Microbiology and Immunology,** and minor in **Veterinary Public Health**, agree that the thesis entitled **"IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF IMMUNOGENIC PROTEINS OF CAPRIPOX VIRUS"** may be submitted in partial fulfillment of the requirements for the degree.

(V. D. P. RAO)
Chairman

(S.K.GARG)
Member

(RAJESH CHANDRA)
Member

(B.D.LAKHCHAURA)
Member

(D.C.THAPLIYAL)
Member

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INTRODUCTION

INTRODUCTION

Capripox, a highly contagious viral disease of sheep and goats, is characterized by fever, ocular and nasal discharge and pox lesions on the skin and respiratory and gastrointestinal mucosa. It is the most severe disease amongst pox virus infections of domestic animals.

Sheep and goats are of great socio-economic importance in agrarian economy. For backward, marginal and landless farmers, these animals are an asset, as their entire livelihood depends upon it. The major source of income from these animals is by sale of kids and lambs and from older culled animals for meat. Other sources of income include skin and hides, wool, milk, manure and "folding" (fee paid to owners by landholders for mannuring of agricultural land by overnight panning of animals). The economics of sheep and goats is adversely affected because of capripox infection. Due to inadequate disease reporting system, it is difficult to estimate the exact economic losses caused by sheep and goat pox (Garner *et al.*, 2000).

Capripox of sheep and goats is enzootic in Africa, Middle East and Turkey, Iran, Afghanistan, Pakistan, India, Nepal, parts of People's Republic of China and Bangladesh. Sheep pox was eradicated from Britain

in 1866 and from France, Spain and Portugal in 1967, 1968 and 1969, respectively. Sporadic outbreaks still occur in Europe for instance in Italy in 1983 and Greece in 1988 and 1989. Morbidity rates vary from 2 to 80% and mortality rates may exceed 90%, particularly if the infection is in association with other disease or bad management (Kitching, 1994).

India has an estimated 56.5 million sheep and 120.6 million goats (FAO, 1998). Sheep pox and goatpox occur in almost all the regions where sheep and goats are reared (ICAR, 1998). The epidemiological pattern is complex with reports of some outbreaks affecting only sheep or only goats, while in other outbreaks both species are affected (Bhowmik *et al.*, 1986; Dubey *et al.*, 1987; Mallick, 1988). Anecdotal evidence suggests that outbreaks occur throughout the year but are more prevalent during rainy season.

The growth of capripox virus strains to high titres in primary culture have been reported by many investigators. However, the information regarding growth of virus to high titre in established cell lines is scanty. Thus, if the virus is adapted to grow with high titre in established cell lines, it will the be most suitable source of viral antigen for vaccine production and other research studies.

The serological differences among capripox virus were not reported, however, possibility of appearance of new isolate can not be ruled out.

Similarly, the variation in immunogenicity within a strain is also possible. Therefore, isolation of capripox virus from field outbreaks and their characterization warrants our attention.

Protection in early life of a newborn animal is afforded by maternally derived antibodies acquired through placental transfer and by intestinal absorption of colostral immunoglulins. On the other hand, higher level of maternal antibodies may interfere with response to vaccination. Therefore, it is essential to determine the duration of protection conferred by maternally acquired antibodies, which will be helpful in planning an effective vaccination regimen.

Indian capripox virus isolates have a greater degree of host preference and cross protection is rarely seen. No systematic work has been carried out for identification and characterization of proteins of goat and sheep pox viruses that are involved in host immune response. In addition, there is not even a single report of lumpy skin disease of cattle in India, inspite of high prevalence of sheep pox and goat pox. This indicates that Indian isolates of capripox virus are different from that of African countries. Therefore, protein analysis of capripox virus, especially Indian isolate, with respect to their molecular, antigenic and immunogenic characterization is necessary to understand the biological behaviour of virus. The characterization of proteins may help to develop a simple

diagnostic test and to identify protein(s) involved in protective immune response.

The diagnosis of capripox virus infection in sheep and goats, on the basis of clinical signs, is confusing and always require laboratory confirmation, which is difficult in rural areas due to unavailability of such facilities. Therefore, there is need for a diagnostic test to confirm capripox virus infection that can be performed under field conditions to allow control measures to be implemented without delay.

Keeping above facts in view, the present work on capripox virus has been undertaken with the following objectives:

1. Isolation and characterization of capripox virus.
2. To study the adaptation and propagation of capripox virus strains in natural host and cell cultures.
3. To characterize proteins of capripox virus.
4. To prepare monospecific antibodies against major proteins and evaluation of their neutralizing ability.
5. To standardize the Western blot technique for detection of capripox virus-specific antigens in infected tissues.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Goatpox and sheep pox are highly contagious viral diseases of goats and sheep characterized by pyrexia, generalised skin and internal pox lesions and lymphadenopathy (Kitching and Taylor, 1985). The morbidity and mortality can be high. These diseases are considered to be the most serious poxvirus diseases of production animals (Carn, 1993). Goatpox and sheep pox are generally host specific for goats and sheep respectively, although in some areas strains of virus have been reported that affect both species to a greater or lesser extent (Carn, 1993; Munz and Dumbell, 1994; Geering *et al.*, 1995).

2.1 Classification

Sheep pox, goat pox and lumpy skin disease viruses are members of the genus Capripoxvirus, sub family Chordopoxvirinae and family Poxviridae. Formerly the members of the genus were classified according to the species from which they were isolated. However, many strains can infect across the species barrier and experimental results have shown that this is true even with strains that do not appear to cross infect naturally. The strains of virus appear to have a differing degree of virulence between species (Kitching and Taylor, 1985). Genome mapping with the use of

restriction enzymes, has provided strong evidence that distinctions between strains are few and that recombination may occur naturally between isolates from different species (Gershon and Black, 1988; Gershon *et al.*,1989).

2.2 Epidemiology

Goatpox and sheeppox have been described in detail in ancient Veterinary medical text, and the diseases have been widespread since early times (Carn, 1993).

Goatpox, reported first time by Henson (1879) from Norway was later recognized in South West Africa (Zeller, 1920), France (Besnoit and Robin, 1923), Turkey (Gerlach, 1933), Cyprus (Levant, 1934), Morocco (Grimpret, 1938), Tajakistan and Sweden (Bakos and Brag, 1957), Iran (Rafyi and Ramyar, 1959), Kenya (Davies, 1976), Western United States (Renshaw and Dodd, 1978), Iraq (Tantawi *et al.*, 1979; Karim, 1983), Sudan (Mohamed *et al.*,1982; Hajer *et al.*, 1988), Oman and Yeman (Kitching *et al.*, 1986a), Bangladesh (Kitching *et al.*, 1987a), Chad (Bidjeh *et al.*, 1990), Jordan (Abo-Shehad, 1990), Netherland (Abo-Shehadas, 1990), Nigeria (Okaiyeto *et al.* , 1995), Egypt (Soad, *et al.*, 1996), Tanzania (Maedamachang and Kaonta, 1996), Algeria, Tunisia, Senegal and Ethopia (OIE, 1998). In India the disease has been reported from Haryana (Lall *et al.* 1947), Uttar Pradesh (Das *et al.*, 1978), West Bengal (Saha *et al.*, 1985; Lodh *et al.*, 1993), Bihar (Somen *et al.*, 1985),

Andhra Pradesh (Rao *et al.*, 1998), TamilNadu (Arya *et al.*, 1998), Madhya Pradesh (Joshi *et al.*, 1999) and Maharastra (Garner, *et al.*,2000).

The earliest record of sheepox dates back to the second century AD (Hutyra *et al.*, 1946). The sheep pox is prevalent in China, Iran, Iraq and Saudi Arabia (Singh *et al.*, 1979), Mali (Maiga and Sarr, 1992), Greece (OIE, 1995), Qatar (OIE, 1996), Jordan (Daoud, 1997), Tunisia, Senegal and Ethiopia (OIE, 1998), Kenya (Peeler and Wanyangu, 1998), Israel (Yeruham *et al.*, 1998), Sudan (Hajer, 1998), Algeria (Achour and Bouguedoar, 1999) and Egypt (Ammar *et al.*, 1999).

In India disease is prevalent in Rajasthan (Dubey *et al.*, 1987; Kataria and Sharma,1992), Haryana (Malik *et al.*, 1998), Tamil Nadu (Arya *et al.*, 1998), Andhra Pradesh (Reddy and Choudhuri, 1999) and Himachal Pradesh (Batta *et al.*, 1999).

In adult goats the disease is mild but in kids it is malignant and showed high mortality rate. Tantawi *et al.* (1974) reported 90% morbidity and 4% mortality rates. The 100% morbidity and 50% mortality rates were recorded by Bandopadhyay *et al.* (1984). Kitching *et al.* (1987a) recorded 50% morbidity and 85% mortality rates in an outbreak in Bangladesh. In kids, morbidity and mortality rates of 81.8% and 100%, respectively were reported by Saha *et al.* (1985). Joshi *et al.* (1999) reported an overall

morbidity of 74.67% and mortality of 48.21% in an outbreak occurred in Madhya Pradesh. In Maharastra the survey conducted on sheep and goats producers showed average morbidity and mortality rates of 63.5% and 49.5%, respectively, due to sheeppox and goatpox (Garner *et al.*, 2000).

The data collected between 1984-85 in Tamilnadu revealed disease prevalence rate of 6% in sheep and 8% in goats with higher rates of 8 and 12% in lambs and kids, respectively. The case fatality rate was over 60% in sheep and around 40% in goats (Arya *et al.* 1998). Kataria and Sharma (1992) reported 38.5% morbidity in an outbreak occurred in sheep flock in Churu (Rajasthan).

2.3 Host range

Goats are natural host for goatpox virus (Rafyi and Ramyar, 1959; Sharma, 1966; Tantawi *et al.*, 1979; Kitching and Taylor, 1985). Bandyopadhya *et al.* (1984) reported that in a mixed farm only goats were affected with goatpox virus (GPV) while other animal species i.e. ovine, bovine and swine, though were in close contact remained unaffected.

Experimentally, sheep infected with GPV failed to develop disease (Besnoit and Robin, 1923; Lall *et al.*, 1947; Sharma, 1966; Adlakha *et al.*, 1971; Sharma and Dhanda, 1971; Das *et al.*, 1978; Tantawi *et al.*, 1980 and Asagba and Obi, 1986). In contrast, some workers reported that GPV can infect sheep as well (Davies, 1976; Kitching and Taylor, 1985)

and successfully transmitted GPV to sheep in experimental infections (Blanc *et al.*, 1929, Slagsvold, 1938; Bakos and Brag, 1957; Mohamed *et al.*, 1982; Kitching and Taylor, 1985 and Kitching *et al.*, 1986a).

Some workers reported that GPV can be transmitted experimentally to horses, dog, poultry (Melanidi and Tzortzaki, 1936), monkey, rabbits and calves (Bennett *et al.*, 1944). However, others failed to transmit disease to calves (Bakos and Brag, 1957), cows and buffaloes (Besnoit and Robin, 1923; Lall *et al.*, 1947); rabbits (Sharma, 1966; Sharma *et al.*, 1966; Adlakha *et al.*, 1971; Sharma and Dhanda, 1971; Das *et al.*, 1978; Tantawi *et al.*, 1980; Bandyopadhyay *et al.*, 1984; Asgaba and Obi, 1986), guinea pigs and hamster (Bakos and Brag, 1957; Tantawi *et al.*, 1980).

2.4 Morphology

Electron microscopic studies revealed that goat pox and sheep pox viruses were morphologically similar to members of orthopox genus. The virus particles measuring, viz., 250-280x212-260 nm (Mohamed *et al.*, 1982), 220 x 230 nm (Tantawi *et al.*, 1979) and 230 x 220 nm (Tantawi and Falluji, 1979) were recorded.

Abdussalam (1957) reported variation in size and shape of GPV strains from that of sheep pox virus. However, Kitching and Smale (1986), could not observe significant differences between external dimensions of M form of sheep pox virus from Nigeria (293 $\pm$ 12 X 252 $\pm$ 14 nm), sheep and

goat pox virus from Kenya (299 ± 22 x 273 ± 21 nm) and lumpy skin disease virus from South Africa (249 ± 20 x 262 ± 22 nm). The morphology and morphogenesis of GPV showed bilayer envelope at an early developmental stage with maximum protein and minimum lipid. The lipid content increased with development of virion (Wang *et al.*, 1987).

2.5 Molecular Biology

Bhatt (1993), characterized the viral DNA of sheep pox viruses; a Kenyan cattle isolate, 3 Kenyan sheep isolates, one Romanian 'Fanar' vaccine isolate, one Russian field isolate, three Indian isolates (Ranipet, Jaipur and Karnal), a goat pox virus field isolate and a buffalopox virus. The estimates of the molecular length of the capripox virus genome based on fragments generated by different restriction enzymes was smaller than buffalopox virus.

Bhatt *et al.* (1993) reported that total length of genome of GPV generated by 5 hexacutter restriction enzymes (Pst I, Bam HI, Bgl II, Ava I and Hind III) varied from 141 kbp to 146 kbp and showed relatedness with sheep pox virus. However, Hind III restriction enzyme profile showed larger differences in typical sheep pox virus isolates from Iraq and India (Bhatt, 1993).

Bhatt (1993) also reported vast dissimilarity between capripox and orthopox genome on the basis of comigratory fragments and demonstrated

a remarkable degree of stability in capripox viruses. A cloned Hind III-S genome fragment with one of the complete ORFs had a gene for thymidine kinase (TK) of Ranipet sheep pox virus DNA indicated positive homology for this region in all sheep pox virus isolates. This region also indicated positive, though weak, hybridization with buffalo pox virus Hind III, Bam HI and Eco RI genomic DNA fragments thereby indicating the homology with orthopox virus.

Cao *et al.* (1995) reported that sequence of the Hind III Q2 fragment near the terminus of KS-1 strain of capripox virus contains 2 complete ORFs and part of a third, encoding a protein related to members of the G protein-coupled chemokine receptor sub family. Copies of this putative G protein-coupled chemokine receptor can be identified in all 3 members of the capripox virus genus (sheep pox, goat pox and lumpy skin disease of cattle) by Southern blotting.

Kitching *et al.* (1986b) reported that major structural polypeptides of sheep pox, goat pox, sheep and goat pox and lumpy skin disease viruses, labelled with (^{35}S) methionine, comigrated on polyacrylamide gel and demonstrated the close biochemical relationship between them. The agar gel immunodiffusion test performed with radio-labelled antigen preparations showed, a major common precipitating antigen, which co-

migrated on polyacrylamide gel with one of the major viral polypeptides of 67000 K molecular weight.

Singh and Rai (1991) reported 18 polypeptides in purified sheep pox virus (Jaipur strain) by polyacrylamide gel electrophoresis. The molecular weights of resolved virion polypeptides were 140k, 120k, 78k, 67k, 64k, 55k, 53k, 47k, 44k, 30k, 27k, 25k, 22k, 21k, 20k, 19k, 18k and 15 k.

The purified vaccinia virus labelled with (^{35}S) methionine showed 56 polypeptides in one-dimensional SDS-PAGE, whereas more than 100 polypeptides were separated by two-dimensional (2-D) gel electrophoresis (Essani and Samual, 1979).

SDS-PAGE analysis of purified fowl pox virus showed 28 viral polypeptides ranging from 10 KD to 132 KD (Obijeski and Palmer, 1973).

Chand *et al.*(1994) identified a 32 Kda protein of capripox virus for differentiation between capripox and contagious pustular dermatitis infections in sheep and goats.

Rao *et al.* (1996) obtained soluble antigen fraction of SPV with 30% ammonium sulphate precipitation and reported that it did not react with antiserum against GPV. This 210 Kda SPV soluble antigen was found to contain 3 polypeptides of 100, 35 and 17 K in SDS-PAGE.

Exclusion chromatographic studies of soluble antigens of purified sheep pox virus revealed 10 proteins of molecular weights 220, 168, 87.3,

71.5, 52.5, 36.7, 31.0, 23.4, 18.3 and 14.2 Kda. Nine of them were found to be precipitinogens and 5 were identified as structural components of virus particle. SDS-PAGE analysis revealed a polypeptide profile of 10 bands with 2 prominent polypeptides of 64 and 42 Kda. However, Western blotting, detected 2 immunogenic polypeptides of 100 and 64 Kda (Rao *et al.*, 1997).

2.6 Purification of capripox virus

Joklik (1962) described the purification of four strains of pox virus (Vaccinia-CL, Rabbit pox - Utrecht, Cowpox - Brighton and Ectromalia - Dohi A), belonging to vaccinia - variola subgroup. The virus source was chorioallantoic membrane of infected developing chicken embryos and the recovery of virus ranged from 33 to 62%. In one particular virus strain over one third of a milligram of virus per infected membrane was isolated.

Kitching *et al.* (1986b) reported the purification of (^{35}S) methionine labelled sheeppox, goatpox, sheep and goat pox and lumpy skin disease viruses by 40-60 % sucrose density gradient. The pelleted viruses were treated with trypsin and DNase for maximum yield.

Chand *et al* (1994) reported the method for purification of contagious pustular dermatitis virus from scab obtained from an experimentally inoculated sheep. The virus pelleted on 36% sucrose cushion was further purified by 25-50% sodium diatrizoate gradient.

Singh and Rai (1991) purified the sheep pox virus Jaipur strain cultivated in vero cells and sheep skin. The clarified virus was further purified by 40% sucrose cushion at 100,000 g.

The soluble antigens of SPV were purified by centrifugation at 25,000 g for 90 min on 30% sucrose bed in TEN buffer and protein content of purified virus was estimated by UV absorption method after taking absorbance at 260 and 280 nm (Rao *et al.*, 1997).

2.7 Physico-chemical properties

2.7.1 Heat

The strains of GPV are generally thermolabile. Residual infectivity of Damoh strain of GPV was recorded after heat treatment at 60^0 C for 1 h (Pandey and Singh, 1970a). Tantawi *et al.*(1980) reported complete inactivation of Sersenk strain of GPV at 70^0 C within 10 min whereas Ranchi strain of GPV was inactivated at 60^0 C within 3 min and a log5 drop in infectivity titre was recorded at 55^0 C (Dutta and Soman, 1991). Tantawi and Falluji (1979) reported that Iranian and Egyptian strains were more heat resistant showing no significant drop in titre after heating at 56^0 C for 1 h, as compared to Iraqi (Sersenk) and Russian (Dushanbe) strains which showed a decline of 4 and 2 log in infectivity titres, respectively.

Sharma *et al.* (1988a) reported complete inactivation at 60^0 C in 30 min, whereas Turkish strain of SPV looses its infectivity at 55^0 C in 1 h

(Koylu and Nitzschke, 1968). The inactivation of the Jaipur strain of SPV at 50^0 C followed a bimodal curve and even after one hour residual infectivity was titreable (Pandey and Singh, 1970a).

2.7.2 pH

Sersenk strain of GPV was found to be sensitive for both acid and alkali. The drop in titre from 2 x 10^7 PFU to 3 x 10^3 PFU/0.2 ml was recorded at pH 5 for 1 h (Tantawi *et al.*, 1980). However, log 5 and log 1 drop in infectivity titres at pH 3 and pH 8, respectively, were recorded with Ranchi strain of GPV. The effect at pH 5 was moderate as log 3 drop in titre was noted (Dutta and Soman, 1991). In SPV the drop of log 4.7 and log 3.4 in infectivity titres were recorded at pH 3 and pH 11, respectively (Ferreira, 1973).

2.7. 3 Lipid solvents

GPV was reported to be sensitive to both ether and chloroform (Pandey and Singh, 1970a; Talhouk and El-Zein, 1986). The infectivity titre 2 x 10^7 PFU/0.2 ml declined to 1.6 x 10^2 PFU/0.2 ml and 2 x 10^3 PFU/0.2 ml after ether and chloroform treatment, respectively (Tantawi *et al.*, 1980), whereas drop of log 6 and log 3 in infectivity titre after ether and chloroform treatment, respectively, were reported with Ranchi strain of GPV (Dutta and Soman, 1991). Sharma *et al.* (1988a) reported complete inactivation of GPV after chloroform treatment. The SPV is also

susceptible to ether (Plowright and Ferris, 1959; Onar *et al.*, 1968; Pandey and Singh, 1970a) and chloroform (Onar *et al.*, 1968; Pandey and Singh, 1970a). However, few strains have been reported to be ether resistant (Bennett *et al.*, 1944).

2.7.3 Other chemicals

GPV is inactivated with 0.03% formalin (Davies,1976) but did not inactivated after treatment with trypsin and EDTA (Talhouk and El-Zein, 1986). The bromodeoxyuridin (BUdR) and Actinomycin D showed inhibitory effect on GPV (Tantawi and Falluji, 1979). The BUdR had greater inhibitory effect on replication of GPV than actinomycin D. The Sersenk strain was found to be more sensitive than Dushanbe, Gorgan and Egyptian strains, to the inhibitory effect of both drugs.

Two percent solutions of hydrochloric acid, sulphuric acid and phenol inactivate the SPV (Perigo strain) in lymph in 15 min.

2.7.4 Antigenic and serological properties

The strains of SPV were indistinguishable by neutralization and cross protection tests (Kitching and Taylor,1985). In cross neutralization test the antiserum of Gorgan strain of GPV neutralised its homologous antigen with a neutralizing index of 10,000 while it neutralized the Sersenk strain with neutralizing index of 100 (Tantawi *et al.*,1980).

The members of capripox genus were indistinguishable by FAT, SNT or AGPT (Kitching, 1986). The complement fixing (CF) antibodies in GPV infected goats were detected by 10^{th} day and persisted for a period of 60 days with maximum titre between 21 to 28 days post infection (Sharma *et al.*, 1966). The CF antibodies were also found in SPV inoculated sheep (Canic, 1935) and maximum titre attained at 14-21 days PI (Uppal,1963).

Bandyopadhyay *et al.* (1984) reported that goats immune to Mukteshwar strain of GPV and those recovered from Sambalpur strain of GPV were protected against challenge with field isolate and reported that both the strains were immunologically similar. The Sersenk, Dushanbe, Gorgan and Egyptian strains of GPV showed at least two common precipitating antigens in AGPT and close antigenic relationship in cross neutralization and pock reduction tests. (Tantawi and Falluji, 1979).

Kitching and Taylor (1985) were unable to distinguish clinical pathology of disease produced by isolates of sheep and goat pox from Yemen, Nigeria, Sudan, Kenya, Arab Republic, Turkey, Pakistan and India. The homologous and heterologous antisera in neutralization test could not distinguish Yemen, Nigerian and Indian isolates. Animals that had recovered from infection with one isolate were resistant to challenge with any of the other isolates.

The Jaipur strain of SPV reacts with homologous rabbit and sheep antiserum producing 4 and 1-2 precipitin bands, respectively (Uppal, 1963; Sharma *et al.*, 1966; Sarkar *et al.*, 1976). It cross-reacts with GPV hyperimmune serum with single identical precipitin band (Bhambani and Krishnamurty, 1963). The double gel diffusion and agar gel immunoelectrophoresis showed five antigenic components of SPV and out of these 2 were shared by GPV (Pandey and Singh, 1972). The SPV convalescent serum could not reduce 100% CPE in lamb testicular cell culture (Plowright and Ferris, 1958)and to over come this problem serum neutralization test (constant serum and variable virus) with unheated normal guinea pig serum was standardized by Martin *et al.* (1973).

2.7.5 Antigenic relationship with other members of Poxviridae

Goatpox and sheep pox viruses were serologically distinguishable with vaccinia, camel pox or horse pox (Davies and Otema, 1981) or with avian pox viruses (Uppal and Nilakantan, 1970) but indistinguishable with parapoxviruses in AGID (Sharma and Dhanda, 1971; Rao *et al.*, 1984) and CFT (Bakos and Brag, 1957).

Bakos and Brag (1957) found no relationship among the viruses of goat pox, vaccinia and CPD by VNT. The CPD antiserum failed to neutralize GPV (Dubey and Swahney, 1979) whereas GPV antiserum neutralize the CPD virus (Sharma and Bhatia, 1959).

2.8 Cultivation

2.8.1 Primary cell cultures

Both GPV and SPV have been successfully cultivated in various primary cell cultures, viz., lamb kidney and testes (Adlakha *et al.*, 1971; Dubey and Sawhney, 1975; Davies, 1976; Tantawi *et al.*, 1979 and 1980; Kitching *et al.*, 1986b), embryonic caprine lung (Dubey and Sawhney, 1975), sheep testes (El-Zein *et al.*, 1983; Talhouk and El-Zein, 1986) sheep thyroid (Nitzschke *et al.*, 1967), sheep kidney (Ramyar, 1966), kid kidney (Joshi, 1988; Katiyar and Soman, 1986), kid kidney and testes (Ramyar, 1966) chicken embryo fibroblasts (Rao and Malik, 1982), sheep embryo dermis (Talhouk and El-Zein, 1986) and calf kidney (Tantawi *et al.*, 1980).

Dubey and Sawhney (1975) found lung cells to be more suitable than kidney cells for propagation of GPV. Goatpox virus could not be propagated in camel kidney (Ahourai, 1971), rabbit testes (El-Zein *et al.*, 1983) and hamster kidney cell culture (Mohamed *et al.*, 1982).

Katiyar and Soman (1987) adapted Mukteshwar and Ranchi strains of GPV in goat kidney. The field isolate of sheep pox virus was successfully adapted in lamb testes cell culture and characteristic CPE were recorded at 3rd passage level (Malik *et al.*, 1998).

2.8.2 Established cell lines

GPV was not adapted to established cell lines, viz., Vero (Mirchasmy and Ahourai (1971), Monkey kidney stable (MS) cell line, HeLa or BHK21 (Ganju *et al.*, 1977).

Davies and Otema (1978) adapted GPV in continuous cell lines prepared from bovine fetal muscle cells and cultivated the virus in the cells upto 40 passages. Davies (1976) reported that GPV, except certain strains, could not produce CPE in BHK cells on primary isolation.

Tantawi *et al.*(1980) reported that Sersenk strain of GPV failed consistently to grow in BHK-21 cells, however, Kitching *et al.* (1987b) observed that Kenyan strain of GPV grew poorly in BHK-21 cells and CPE was only seen after seven days on each passage. The virus titres were reported to be 5.2 $\log_{10}$ $TCID_{50}$/0.2 ml and 4.8 $\log_{10}$ ID_{50}/0.2 ml when titreated in lamb testes cell culture and in goats by intradermal inoculation respectively. The SPV was also adapted to BHK-21 (C13) cell line (Kirubaharan *et al.*, 1993).

Maity *et al.* (1997) adapted Uttarkashi strain of GPV in Vero cells. The CPE appeared on third day between 6^{th} and 7^{th} passage. When inoculated into goats 15^{th} passage virus produced generalized lesions. Sambalpur strain of Goatpox virus was adapted in MDBK (Joshi *et al.*,

1995b) and Vero (Prakash *et al.,* 1994). Singh and Rai (1991) adapted Jaipur strain of sheep pox in vero cells.

2.9 Cytopathic changes in cell culture

GPV produces CPE in goat embryonic lung cells within 24 -48 h PI. The infected culture showed formation of syncytia and intracytoplasmic inclusion bodies of various shape and sizes, mainly eosinophilic but some of them showed basophilic granules (Dubey and Sawhney, 1975). The CPE produced by GPV in lamb kidney, kid kidney and testes cell cultures, included cytoplasmic and nuclear degeneration, intracytoplasmic vacuoles and appearance of intracytoplasmic inclusions (Pandey and Singh, 1970 b)

The Sersenk strain of GPV in primary calf kidney cell culture produced CPE in 30% cells between 7-8 days PI whereas in primary culture of lamb testes, virus induced CPE were manifested by cell rounding and syncytia formation between 3-4 day PI (Tantawi *et al.*, 1980). The virus induced cytoplasmic eosinophilic inclusions and plaques measuring 1-2 mm in diameter having serrated edges and lysed centers were predominant at 48 h PI (Tantawi *et al.*, 1980).

Katiyar and Soman (1986) recorded CPE after 10^{th} passage in goat kidney cell culture and observed cytoplasmic granulation at 48 hr PI. The staining of cells with MGG and H&E recorded increased nuclear size, chromatin fragmentation and intracytoplasmic inclusions with or without

halo between 24 - 48 h PI followed by pyknosis, margination of chromatin and formation of nuclear vacuoles.

Tantawi and Falluji (1979) reported that plaque characteristics were enough to distinguish Dushanbe strain, which induced larger plaques (2-4 mm diameter), from Iraninan and Egyptian strains which induced smaller plaques (0.5 - 0.6 mm diameter) in lamb testes cell culture. The Sersenk strain of GPV produced plaques of intermediate size in lamb testes cell culture (1-3 mm diameter). Chandra *et al.*(1996) reported plaque formation by Karnal strain of sheep pox virus in lamb kidney cell culture.

Malik *et al.*(1998) reported SPV induced cytopathological changes in lamb testes cells. The changes included were rounding, increased refractivity to light, clumping, detachment and formation of intracytoplasmic inclusions.

Joshi *et al.*(1995a) reported that Sambalpur strain of GPV produced CPE in kid kidney cell culture at 2nd passage level. These included the round, spindle shaped cells, clear spaces in the cell monolayer and intracytoplasmic inclusion bodies with or without halo.

Joshi *et al.* (1995b) also recorded the CPE produced by Sambalpur strain of GPV in MDBK cells. The CPE included cytoplasmic vacuolation, syncytia formation and micro or macro plaques. The presence of viral DNA in the cytoplasm of cell was demonstrated by acridine orange staining.

The Sambalpur strain of GPV produced syncytia formation, cytoplasmic vacuolation and intracytoplasmic eosinophilic inclusion bodies in Vero cells (Prakash *et al.*, 1994). The Jaipur strain of SPV also induced CPE in Vero cells which included granularity in cytoplasm, rounding and clustering of cells, microplaques and intracytoplasmic inclusion bodies (Singh and Rai, 1991).

Kirubaharan *et al.* (1993) reported the CPE produced by Ranipet strain of GPV in BHK-21 cell line. At 15th passage rounding was recorded as early as 24 hr PI with spindle shaped cells, syncytia formation and multinucleated giant cells at 72 h PI.

2.9 Pathology

The pathology of Goatpox and sheep pox have been described by several investigators (Plowright *et al.*, 1959; Jubb and Kennedy, 1963; Vegad and Sharma,1970,1971; Murray *et al.*, 1973). Following an incubation period of about one week, hyperaemic macules were observed which lead to the formation of papules in a day or two. Later umblicate vesicles formed which were followed by scab formation. However, Vegad and Sharma (1973) reported the absence of vesicle formation in the development of cutaneous eruptions with Rishikesh strain of SPV, which was capable of provoking a generalized reaction.

The main histopathological changes in skin involved the subepithelial layers, dermis and subcutaneous tissue. Sheep pox cells or cellules claveleuses were characterized by large oval or irregular nuclei with enlarged nucleoli. These cells arise from histocytes and occasionally showed granular cytoplasmic inclusions (Jubb and Kennedy, 1963).

Hutyra *et al.* (1946) reported that SPV produced vesicles and ulcers on mucus membranes of respiratory and alimentary tract and small lymphoma like nodules in kidney and lungs. Regression of lesions observed from 16th day PI (Ramchandran, 1967).

Malik (1999) reported the clinicopathological changes in lamb inoculated with virulent field isolate of sheep pox virus. The clinical signs recorded were rise in rectal temperature, loss of appetite and formation of pox nodules around site of inoculation from day 4 PI. The haematology of infected sheep showed marked leukocytosis, absolute neutrophilia and lymphopenia. The characteristic pathological lesions were seen in skin, lungs, liver and kidney.

Milli *et al.* (1991) described histopathological changes due to sheep pox in lungs. The alveoli were completely lined by proliferating epithelial cells and filled with eosinophilic PAS positive material. The alveolar septa appeared widened by Borrel's sheep pox cells and other mononuclear cells. The nuclei of sheep pox cells were vacuolated and had marginated

chromatin and cytoplasm contained eosinophilic inclusion bodies. Ultrastructural studies revealed that sheep pox cells were predominatly macrophages and rarely monocytes and fibroblast and showed virus replication sites in their cytoplasm. These areas consisted of membranous semi circular and spherical or ovoid immature particles, rectangular or ovoid mature particles and cylindrical structures. Similar type of electron microscopic changes were also reported in sheep pox cells in skin lesions of SPV (Altinsaat and Milli, 1993).

2.10 Immunity

Prasad *et al.* (1986) reported that passive transfer of sensitized lymphocytes and serum collected from sheep that had recovered from infection with virulent Jaipur strain of sheep pox could not transfer immunity and skin lesions appeared in all these sheep within 3-4 days of challenge. However, Kitching (1986) successfully protected the sheep against challenge from virulent strain of CPV after passive transfer of serum from sheep recovered from CPV. The immunity was of short duration. Colostrum also provides passive protection and may interfere with response of vaccination before 6 months of age.

Boulter and Appleyard (1973) reported that in pox viruses most progeny viruses remained inside the infected cells (intracellular naked virus/INV) and that only a small amount released and became extracellular

virus (extracellular enveloped virus, EEV). The local spread of infection was from cell to cell and virus effectively protected from circulating antibody. Dissemination was either through circulation of infected macrophages or extracellular virus in blood stream. The circulating antibody derived through natural infection or vaccination limits spread of virus in the animal but it did not prevent replication at the site of infection.

Boulter *et al.*(1971) reported that high level of antibodies attained through immunization using an inactivated preparation gave only short term protection because immunity to pox viruses was predominantly cell mediated and required a replicating antigen. The immune status of a previously infected or vaccinated animal can not therefore be related to serum level of circulating antibodies (Kitching, 1986).

2.11 Serological tests

2.12.1 Agar gel precipitation test (AGPT)

AGPT was used to demonstrate GPV antigen and antibodies (Bhambani and Krishnamurty, 1963; Sharma, 1966; Sharma and Dhanda, 1971). AGPT has successfully extended to establish the serological relationship among members of the pox group. Bhambani and Krishnamurty (1963) showed six distinct precipitation lines when goatpox cutaneous antigen reacted with hyperimmune serum against goat pox virus produced in rabbits. Sheep pox cutaneous antigen gave three lines against

same sera. The convalescent sera from sheep and goats recovered from sheep pox and goatpox, respectively, when tested against GPV cutaneous antigen in AGPT gave one precipitin line with sheep sera and 2 lines with goat sera.

Pandey and Singh (1972) reported a difference of 3 precipitation lines when antisera were prepared with or without adjuvant. More precipitation lines using antisera prepared with adjuvant was suggestive of enhanced antigenicity of some otherwise weak soluble antigens of sheep and goatpox viruses. The serological relationship between sheep pox and goatpox viruses was established by AGPT (Sharma *et al.*, 1966; Uppal and Nilakantan, 1970; Pandey and Singh, 1972; Tantawi *et al.*, 1980; Rao *et al.*, 1984). Dubey and Sawhney (1979) reported 8 precipitin lines using anti goatpox hyperimmune serum prepared in rabbits; of these six were common for goat pox and contagious ecthyma viruses. Mathew (1975) reported cross reaction of buffalopox virus with fowlpox, sheep pox and goatpox viruses in AGPT test. The sheep pox virus produced three and GPV produced two precipitation lines against antiserum prepared by buffalopox infected skin lesions in rabbits. Kalpana *et al.*(1995) reported that Kenyan sheep and goat pox virus shared 2 soluble antigens with Indian GPV in gel diffusion.

2.12.2 Haemagglutination test (HA)

Goat and sheep pox viruses showed a low titre of haemagglutination activity with fowl and duck erythrocytes and titre did not rise even after treating RBCs with 1:20,000 tannic acid (Sharma and Dhanda, 1971). However, Sersenk strain of GPV did not haemagglutinate the erythrocytes of chicken, duck, sheep, goat, guinea pig, mice or human type 'O' cells (Tantawi *et al.*, 1980). Dutta and Soman (1991) reported that Ranchi strain of GPV did not agglutinate RBCs' of ox, sheep, rabbit, guinea pig or chicken.

Joshi *et al.* (1991) standardized passive haemagglutination test for detection of GPV specific antibodies. The spot agglutination test for detection of GPV antigen was developed using glutaraldehyde treated sheep RBC. The test was equally sensitive as CIE and RPHA (Tiwari *et al.*,1996).

Tiwari *et al.* (1995) developed RPHA test with sheep red blood cells for detection of GPV antigen in skin scab and concluded that RPHA test was more sensitive, more specific, quicker and simpler that AGPT and CIE in field conditions for diagnosis of capripox.

2.12.3 Fluorescent antibody technique (FAT)

Vigario and Ferraz (1967) described the synthesis of sheep pox virus in sheep embryo muscle cells by direct FAT. The viral antigen in the form

of small fluorescent foci was detected in cytoplasm before the appearance of infective virus. The sheep pox virus antigen was demonstrated by FAT in odema fluid (Sarkar *et al.*, 1980), lamb kidney and lamb testes cells (Chand, 1983). Davies and Otema (1981) established the serological relationship between Kenyan strain of GPV, SPV and LSD virus using direct and indirect FAT and found that viruses were identical and showed distant relationship with cow pox virus.

Tantawi and Falluji (1979) used indirect FAT to measure the percentage of GPV infected cells in the monolayer. The serum against Sersenk strain of GPV reacted strongly with homologous antigen and weakly with antigen of reference strain of GPV and SPV in indirect FAT (Tantawi *et al.*, 1980)

Joshi (1988) demonstrated presence of GPV in the cytoplasm of GPV infected kid kidney cells as early as 24 h PI by FAT. The entire cytoplasm showed fluorescence after 96 h PI.

The FAT was successfully used for demonstration of vaccinia virus in cultured human cells (Noyes and Watson, 1955), fowlpox antigen in chicken kidney cell culture (Tripathy *et al.*, 1970) and swine pox virus in PK-15 cells (Garg and Mayor, 1973).

2.12.4 Enzyme linked immunosorbent assay (ELISA)

Sharma *et al.* (1988b) developed ELISA for detection of GPV antigen and antibody using live and chloroform inactivated antigen and hyperimmune convalescent post vaccination and post challenge sera. ELISA gave higher percentage of positive samples as compared to CIE but could not differentiate between goatpox, sheep pox and contagious pustular dermatitis virus. However, Sharma *et al.* (1988a) reported that CIE was slightly better than ELISA for detection of GPV antigen.

Rao *et al.* (1999) evaluated the avidin-biotin ELISA for detection of antibodies against GPV using non infectious soluble antigen as a diagnostic reagent. Out of 90 goat pox suspected sera obtained from field cases only 2 (2.2%) were found positive in CIE while 58 (64.4%) were positive in avidin-biotin ELISA. They concluded that the avidin-biotin ELISA was significantly more efficient than CIE test for detection of GPV antibodies.

Rao and Rao (1999) developed a specific, sensitive and reproducible indirect IgM ELISA for diagnosis of sheep pox virus infection. The evaluation and potential application of immunocapture ELISA in the routine diagnosis of capripox virus disease was reported by Nagichabe *et al.* (1999). The sensitivity and specificity of the test was 92 and 100%, respectively. The positive and negative predictive values of the test were

100 and 64%, respectively. Samples with < 103 $TCID_{50}$ /50 µl of virus could not be detected by the test.

The immuno capture ELISA with 80-100% specificity and 70-86% sensitivity was developed for diagnosis of SPV infection and found to be marginally more sensitive than CIE test (Rao *et al.*, 1997).

Tiwari and Negi (1996) tested 50 samples of scab/skin of goats for GPV antigen by dot ELISA and single radial hemolysis and found that dot ELISA is three times more sensitive and accurate than SRH.

Sudhukhan *et al.* (1998) diagnosed capripox infection in 38 goats by dot ELISA and CIE. The 28 (68.42%) were positive by ELISA and 21 (55.26%) by CIE. With known positive control samples the ELISA showed 100% and CIE 88% correlation. It was concluded that dot-ELISA has high sensitivity and specificity and is suitable for diagnosis of cases in field outbreaks.

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Capripox viruses

3.1.1 Goatpox virus strains

The Sambalpur and Uttarkashi strains of goatpox virus (GPV) were obtained from Divison of Virology, IVRI, Mukteshwar, in the form of skin lesions and maintained by periodical skin to skin transfer in goats.

A local isolate of GPV was initially collected in the form of skin scabs from an outbreak in Rajasthan in 50% buffered glycerol. This isolate has been designated as 'Pantnagar strain'.

3.1.2 Sheep poxvirus

The Jaipur strain of sheep pox virus (SPV) was obtained from Division of Standardization, IVRI, Izatnagar in the form of skin lesions and maintained in the laboratory by periodical skin to skin transfer in lambs as described for GPV.

3.2 Experimental animals

Kids, goats and sheep used in the present investigation were purchased from local supplier. These animals were screened for antibodies against capripox virus and those found negative were used for conducting

the experiment. The testes from kids used for cell culture were removed surgically without sacrifying the animals.

3.3 Cell culture

3.3.1 Secondary kid testes cell culture (KTCC)

Secondary kid testes cell culture, using Glasgow minimum essential medium (GMEM; Sigma) supplemented with 5% newborn calf serum (PAA Laboratories, Austria), was prepared as per the method described by Plowright and Ferris (1958) with minor modifications.

The kid of about 2-3 month of age was surgically operated and testes were aseptically collected in HBSS. After removal of outer layers, the medullary portion was collected and chopped off into small pieces. The minced tissues were washed with HBSS and trypsinized at 37^0C for 15-20 min. The dispersed cells were filtered through muslin cloth and centrifuged at 1000 rpm for 10 min. The packed cell volume was diluted in growth medium to contain 1.5 million cells per ml and seeded in 75 cm^2 tissue culture flask (NUNC) @ 30 ml/flask. After formation of a confluent monolayer, the cells were treated with 3-4 ml of trypsin-versine solution and allowed to act for 2 min. Thereafter, trypsin-versine solution was decanted and bottles were incubated for 2-3 min at 37^0C. The cells were detached by pipetting in 90 ml growth medium and distributed in tissue

culture flasks or Leighton tubes. The secondary monolayer were complete within 48-72 h.

3.3.2 Cell lines

African green monkey kidney (Vero) and Madin Darby bovine kidney (MDBK) cell lines were obtained from CADRAD, IVRI, Izatnagar. The minimum essential medium (MEM; Hyclone, Denmark) supplemented with 0.3% (w/v) tryptose phosphate broth (TPB), 0.03% (w/v) L-glutamine and 5% (v/v) newborn calf serum was used as growth medium. The maintenance medium contained only 2% calf serum.

The tissue culture flask (25 cm^2) containing complete monolayer was decanted and monolayer was treated with 1.0 ml of trypsin-versine solution. The solution was allowed to act for 2 min and then decanted. The bottles were then incubated at 37^0C for few min until the cells started to detach from surface. The detached cells were then dispersed in 30 ml of growth medium by gentle pipetting and dispensed in tissue culture flasks and Leighton tubes @ 10 ml and 2 ml, respectively. The flask and tubes were incubated at 37^0C for formation of monolayers

3.4 Production of hyperimmune serum

The hyperimmune serum was prepared as per the method described by Sharma *et al.* (1988) with minor modifications. The serum was raised in goat by initially injecting 2 ml of viral antigen ($2 \times 10^{4.5}$ GID_{50}/ml) of goat

skin origin by i/d route. On recovery from the disease, the goat was inoculated with 5 ml of above virus suspension mixed with equal volume of Freund's complete adjuvant (Sigma) by s/c route at weekly intervals. The blood was collected 10 days after last inoculation and serum separated from blood clot by centrifugation at 3000 rpm for 20 min at 4^0 C and stored at -20^0 C until used.

The hyperimmune sera against Sambalpur and Uttarkashi strains of GPV and Jaipur strain of SPV, already available in the laboratory, were used.

The serum samples collected during the field outbreaks of goatpox and after experimental infection of goats with Pantnagar strain of GPV were also used during the course of study.

3.5 Purification of globulin from hyperimmune serum

The globulin form hyperimmune serum was purified by ammonium sulphate precipitation as described by Hudson and Hay (1980). The saturated solution of ammonium sulphate (700 g/liter at 0^0 C or 760 g/liter at 25^0 C) was made by dissolving it in hot distilled water. The pH of the clear supernatant was adjusted to 7.0 with ammonia solution and stored in presence of ammonium sulphate crystals.

For precipitation, 10 ml of saturated ammonium sulphate solution was added gradually to 20 ml of serum under constant stirring at room

temperature. The resultant suspension was allowed to stand for 30 min with occasional stirring and precipitate was collected by centrifugation at 3000 rpm for 20 to 30 min. The precipitate was dissolved in 10 ml of distilled water and reprecipitated by gradually adding 5 ml of saturated ammonium sulphate solution. This cycle was repeated twice. The final precipitate was dissolved in 2 ml of 0.15 M sodium chloride solution and ammonium sulphate was removed by dialysis against several changes of 0.15 M sodium chloride solution every 12 h at 4^0C for 3 days. The absence of sulphate ions was checked by 1% solution of barium chloride.

3.6 Propagation/adaptation of capripox viruses

3.6.1 Experimental animals

The Pantnagar, Uttarkashi and Sambalpur strains of GPV and Jaipur strain of SPV were propagated by inoculating the respective viral preparations in experimental goats and sheep. The skin lesions were triturated in pastle and mortar to make a 10% (w/v) suspension in HBSS using sterile sand as an abrasive. After 3 cycles of freezing and thawing, the preparation was centrifuged at 800 g for 20 min. The supernatant was collected and treated with antibiotics (Penicillin 10^3 IU per ml, streptomycin sulphate 1 mg per ml) for 30 min at 37^0C and then used as virus inocula.

The skin area of 100 cm^2 over the last four ribs on each side of sheep and goats was close clipped. The virus suspension was inoculated in 0.2 ml quantity into 5 spots in two vertical rows by i/d route using 26 g needle. The animals were clinically examined daily, their temperatures recorded and diameter of skin lesions measured.

The goat inoculated with Pantnagar strain was humanely destroyed and tissue samples, viz., skin, lung, liver, spleen, kidney and lymph node, were preserved in 10% formal saline for histopathological examination.

3.6.2 Secondary kid testes cell culture

The virus strains, viz., Pantnagar, Sambalpur and Uttarkashi were adapted to kid testes cell culture by adopting the method described by Plowright and Ferris (1958).

After formation of confluent monolayer, the growth medium from each tissue culture flask (25 cm^2) and Leighton tube was decanted. One ml and 0.2 ml of 10% (w/v) virus suspension of infected goat skin origin ($10^{4.5}$ to $10^{5.5}$ GID_{50}/ml) and filtered through 0.45 μm membrane filter were inoculated in tissue culture flasks and Leighton tubes, respectively. After one h of virus adsorption at 37^0C, the excess inoculum was decanted and monolayers washed with 1 ml of HBSS to remove unadsorbed virus. The maintenance medium was then added at the rate of 8 ml and 2 ml in tissue culture flask and tubes, respectively. The uninoculated monolayers were

kept as control. The inoculated tubes and flasks were incubated at 37^{0}C for 7 days. The monolayers were frozen and thawed thrice at different time intervals and centrifuged at 1000 rpm for 10 min. The supernatant thus collected was used for titration of virus and also served as source of virus inocula for subsequent passages.

On day 5 post infection (PI), the maintenance medium was gently decanted from cell culture flask and centrifuged at 1500 rpm for 30 min at 4^{0}C. The supernatant constituting the extracellular virus was titrated and stored at -20^{0}C. Similarly, intracellular virus preparation was also prepared as per the method described by Shrivastava and Singh (1980). After harvesting the extracellular virus, the remaining infected cells still adhering to surface were detached by gentle shaking with PBS and washed 3 times with PBS by repeated centrifugation at 1500 rpm for 30 min at 4^{0}C. The cells from each flask were resuspended to 10 ml of maintenance medium and pooled. The intracellular (cell associated) virus was harvested after 3 cycles of freezing and thawing. The pooled suspension was centrifuged at 1500 rpm for 30 min. The supernatant thus harvested was stored at -20^{0}C until used as source of intracellular virus.

3.6.3 Established cell lines

The 5th passage KTCC adapted Pantnagar strain of GPV with titre of $10^{4.5}$ $TCID_{50}$/ml was used as virus inocula for Vero and MDBK cell lines.

3.7 Study of cytopathic effects

The cytopathic effects (CPE) produced by Pantnagar strain of GPV in KTCC Vero and MDBK cells were studied at different time intervals in unstained, May-Grunwald Giemsa stained and acridine orange stained preparations.

3.7.1 Unstained preparations

The infected monolayers in tissue culture flasks and Leighton tubes were observed daily upto 7 days PI for appearance of CPE. Similarly, coverslip cultures in Leighton tubes were removed at 24 h interval and stained with MGG and acridine orange stains.

3.7.2 May-Grunwald Giemsa staining

The GPV infected and control monolayers were stained with May-Grunwald Giemsa (MGG) stain according to the method described by Merchant *et al.* (1960). The coverslip cultures were washed in prewarmed PBS (pH 7.2) and then fixed in absolute methanol for 20 min at room temperature. The fixed monolayers were stained with May-Grunwald stain for 30 min followed by Giemsa stain for 30 min. The monolayers were then dehydrated using acetone (2 min; 2 changes), acetone and xylene

mixture (1:1) (2 min; 2 changes) and then cleared in xylene (2 min; 2 changes). The coverslips were mounted on glass slides with DPX mount and examined microscopically for CPE.

3.7.3 Acridine Orange staining

The coverslips were also stained with acridine orange as per the method of Mayor and Diwan (1961).

The coverslip cultures were washed thrice with PBS (pH 7.4) and placed for 45 min in Cornoy's fluid for fixation. The cells were then hydrated rapidly in descending series of ethyl alcohol (80, 70, 60 and 50%) and finally dipped in distilled water. The coverslips were transferred to McIlvaine's citric acid buffer (pH 4.9) for 5 min just before staining with 0.01% acridine orange for 5 min. After staining, the coverslips were rinsed in McIlvaine's citric acid buffer and then mounted in the same buffer. The coverslips were stored in moist chamber at 4^0 C until examined under fluorescent microscope.

3.8 Fluorescent Antibody Technique (FAT)

The KTCC, MDBK and Vero cell monolayers infected with GPV (Pantnagar strain) were processed for FAT at 6, 12, 18, 24, 48 72, 96 and 120 h PI. The procedure of Hudson and Hay (1980) was followed with minor modification.

The monolayer on coverslips were washed with prewarmed PBS (pH 7.4) and then fixed in chilled acetone for 20 min at 4^0C. The coverslips were then placed on a glass slide with cell facing upward. Two drops of diluted hyperimmune serum (1:50) was poured on coverslip and incubated in humid chamber for 1 h at 37^0C. After 3 washings with PBS each of 5 min the coverslips were flooded with antigoat FITC conjugate (Sigma) (1:100 diluted with PBS and filtered by 0.22 μm millipore membrane filter) and incubated for 1 h in humid chamber at 37^0C. Thereafter, the coverslips were washed 3 times with PBS and mounted in fluid consisting of 9 parts of glycerol and 1 part of PBS. The control coverslips were processed similarly and examined under fluorescent microscope (Olympus BX 40).

3.9 Electron microscopic studies

The Vero and KT cells infected with GPV were harvested without freezing and thawing. After washing with HBSS, the cells were fixed in 2.5% glutaraldehyde for 48 h. The glutaraldehyde fixed cells were post fixed in 1% OsO_4 for 2 h at 4^0C and then dehydrated in 30, 50, 70, 80, 90 and 95 percent acetone (15 min each) at room temperature. The acetone dehydrated cells were cleared twice by Epoxy propane for 30 min each and then embedded in Epon 812 resin. The thin sections of embedded tissue were cut and stained with uranyl acetate and observed in Philips CM 10 Transmission electron microscope.

3.10 Micro serum neutralization test

The micro serum neutralization test (Micro SNT) was performed in KTCC, Vero and MDBK cells in 96 well microtitre tissue culture plates (NUNC). The constant serum varying virus method, as described by Rai (1985), was followed.

The 10-fold serial dilutions of GPV isolates were prepared and 50 µl of each dilution of virus was dispensed in microtitre plate in triplicate followed by addition of 50 µl of diluted (1/4) serum to each well. This mixture was incubated for 60 min at 37^0C. The 50 µl of cell suspension containing 1.5 x 10^6 cells/ml was then added to each well. The plates were sealed with adhesive tape and incubated at 37^0C. Similarly titration of virus without serum was also carried out. The virus titre in control and virus-serum mixture were calculated as per the method of Reed and Muench (1938). The neutralization indices were expressed as difference between titre of virus-serum mixture and virus alone in control test.

3.11 Assay of viral infectivity

The infectivity titration at different passage levels were carried out in KTCC, Vero and MDBK cell monolayer grown in 96 well microtitre culture plate. The 10-fold serial dilutions of virus isolates were prepared in growth medium and 50 µl of each dilution was then dispensed in each well of microtitre culture plate, in triplicate. The 50 µl of appropriate cell

suspension, containing 1.5 x 10^6 cells/ml was then added to each dilution of virus suspension. These plates were sealed with adhesive tape and incubated at 37^0C for 6-7 days for development of CPE. In control wells 50 µl of cell suspension and equal volume of medium was added.

3.12 Infection of kids with skin adapted virus strains

To confirm the adaptation of Pantnagar strain of GPV in KTCC, MDBK and Vero cells, three healthy kids of about 5-6 month of age were inoculated i/d with 0.2 ml of 15^{th} passage virus separately and observed for development of clinical signs and rise in body temperature.

3.13 Characterization of Pantnagar strain of GPV

3.13.1 Physico-chemical properties

The Pantnagar strain of GPV at 10^{th} passage level with a titre of 5.92 $\log_{10}$ $TCID_{50}$/ml, was subjected to physico-chemical characteri-zation.

3.13.1.1 Sensitivity to heat

The thermal effect on Pantnagar strain was determined according to the method described by Plowright *et al.* (1959) with minor modifications. The 2.0 ml of KTCC adapted virus suspension in screw capped vials were immersed in water bath adjusted to 55^0C, 60^0C and 70^0C separately each for 10 min. The residual infectivity titration was carried out in KT cell monolayers. The $TCID_{50}$ was calculated according to the method of Reed and Muench (1938).

3.13.1.2 Sensitivity to pH

The method described by Dutta and Soman (1991) was followed for evaluation of pH effect on virus. The virus preparations were exposed to pH 3, pH 5 and pH 8 for 1 h at 37^0C. Thereafter, the pH of these samples were adjusted to 7.6 and then titrated in kid testes cell culture for residual infectivity.

3.13.1.3 Sensitivity to diethyl ether

Sensitivity to diethyl ether was determined as per the method described by Andrews and Horstmann (1949). The 1.0 ml of KTCC adapted virus suspension was mixed with 0.2 ml of diethyl ether and kept at 4^0C for 20 h. The mixture was then poured into an uncovered petridish for evaporation of ether. The change in viral infectivity was assayed in KTCC and compared with a suitable control.

3.13.1.4 Sensitivity to chloroform

The method of Feldman and Wang (1961) was employed for determining the effect of chloroform on GPV. The chloroform in 0.1ml quantity was mixed with 1 ml of virus suspension and shaken for 10 min at room temperature and then centrifuged at 1000 rpm for 5 min. The upper layer, which contains the virus was removed and titrated for residual infectivity in KTCC alongwith suitable controls.

3.13.2 Host spectrum

The method of Tantawi *et al.* (1980) was followed for exploring the host range of virus in sheep, chicken, rabbit, mice and buffalo calf. The sheep, buffalo calf and rabbits were exposed through intradermal injections with 1.0 ml of 10% (w/v) suspension of goat skin lesions containing $10^{4.5}GID_{50}$/ml. The mice and chickens were injected by intracerebral and intrafollicular routes, respectively. The inoculated animals were examined daily for 4 weeks.

3.13.3 Haemagglutination

The procedure of Cunnigham (1960) was followed for haemagglutination test. A 10% (w/v) suspension of viral scab was prepared in PBS (pH 7.4) and tested for HA activity using erythrocytes of sheep, goat, pig, rabbit, dog, chicken and human type 'O' cells. The test was performed in 96 well 'U' bottom plate (Tarson). A serial 2-fold dilution using 50 µl of virus suspension was prepared in PBS and 50 µl of 0.8% erythrocyte suspension was added to each well. The control wells received erythrocyte suspension only. The results were recorded after 1 h of incubation at 37^0C.

3.13.4 Growth behaviour

3.13.4.1 On chorio-allantoic membrane of chicken embryos

The 11 to 12 day-old chicken embryos were inoculated with viral suspension containing 10,000 GID_{50}/dose via CAM route. The inoculated eggs were incubated at 37^0C and dead embryos were discarded 24 h post inoculation. The CAM were harvested after 5 days and examined for the presence of lesions.

3.13.4.2 Cell culture

The secondary kid testes cell culture was used for adaptation/propagation of virus. The coverslips containing monolayer were stained with MGG and A.O. for study of cytopathic effects. The infectivity titration was carried out at passage level 5, 10 and 15, as per the method described by Reed and Muench (1938).

3.13.5 Histopathological examination

The kids experimentally infected with Pantnagar strain of GPV was sacrificed and skin, liver, lung, kidney, spleen and lymph node were collected in 10% formal saline for histopathological examination.

3.13.6 Serological characterization

For serological characterization, the AGPT and indirect FAT were performed for determination of antigenic relationship with Sambalpur and Uttarkashi strains of GPV.

3.14 Effect of maternal antibodies in protection of kids against Goatpox

The effect of maternal antibodies in protection of kids against goatpox was assessed. Two healthy goats were immunized against Pantnagar strain of GPV by infecting them initially with 2.0 ml of viral suspension prepared from goat skin (2 x $10^{4.5}$ GID_{50}/ml) by i/d route. After recovery from disease, these goats were inoculated with 5 ml of above virus suspension along with equal volume of Freund's complete adjuvant by s/c route and one week after, 5 ml of PEG concentrated virus suspension was injected along with equal volume of Freund's incomplete adjuvant (FIA).

These immunized goats were allowed to conceive through natural service. The pregnant goats, about 15 days before the kidding, were inoculated with 5 ml of PEG concentrated virus suspension along with 5 ml of FIA by i/m route. The serum samples were collected one week post inoculation and titrated for presence of antibodies against GPV by ELISA and micro-SNT.

The newborn kids were fed colostrum and serum samples collected at intervals of 0, 7, 14, and 21 days. At the age of 21 days, the kids were challenged with 1 ml of virus suspension (2 x $10^{4.5}$ GID_{50}/ml) prepared from infected goat skin. The control kid born from healthy goat, which was free from antibodies against GPV, was also inoculated with same dose of

virus. These animals were closely observed for rise in rectal temperature and development of other clinical signs.

3.15 Enzyme linked immunosorbent assay (ELISA)

ELISA was performed by single dilution method as per the procedure described by Chandrashekhar (1994). The approximate concentration of viral antigen was determined by checkerboard titration. The antigen was diluted 1:1 in carbonate-bicarbonate buffer and 50 µl of this diluted virus was added to each well of 96 well ELISA plate (NUNC). The plate was incubated overnight at 4^0C. Next day, antigen coated plates were washed thrice with PBS-Tween-20 (PBS-T) for 5 min each and tapped thoroughly and then 100 µl of 2% bovine serum albumin was added to each well of plate for blocking. The plates were incubated for 1 h at 37^0C. Again plates were washed as mentioned above.

The single dilution of the test serum was made in an uncoated 96 well microtitre plate by adding 200 µl of dilution buffer and 2 µl of test serum per well resulting in a dilution of 1:100. Positive control serum (against Pantnagar strain of GPV) and negative control serum were also diluted to 1:100 in PBS-T.

The 100 µl of diluted negative control serum was added to the first three wells of the first row and 100 µl of diluted positive serum was added to the last three wells of the last row of ELISA plate and last three wells of

last row were kept as blank. In remaining wells of antigen coated plate, 100 µl of test serum from dilution plate was transferred to each of the three corresponding wells.

The plate was incubated at 37^0C for 30 min and washed thrice with PBS-T. The 100 µl of rabbit antigoat horse radish peroxidase conjugate (Sigma) diluted 1:5000 in PBST was added to each well and plates were incubated at 37^0C for 1 h.

The freshly prepared 100 µl of substrate solution containing orthophenylenediamine dihydrochloride (OPD) in citrate buffer and H_2O_2 was added to each well of plate and then incubated at 37^0C for 15 min in dark. The reaction was stopped by adding 100 µl of stop solution (1N H_2SO_4) in each well of plate. The plate was read at 492 nm in an ELISA reader (ECIL Microscan MS 5605).

3.15.1 Calculation of ELISA titre

The average absorbance of positive and negative control was calculated from the absorbance value of ELISA plate and corrected positive control (CPC) value was determined by subtracting average negative absorbance from average positive absorbance. The specific value (Sp. Value) was calculated by using formula.

$$\text{Sp. value} = \frac{\left[\begin{matrix}\text{Average absorbance}\\ \text{of test sample}\end{matrix}\right] - \left[\begin{matrix}\text{Average absorbance}\\ \text{of negative control}\end{matrix}\right]}{\text{Corrected positive control}}$$

The titre was then calculated by

Log_{10} titre = (1.464 x $\log_{10}$ sp) + 3.197

Titre = Antilog of $\log_{10}$ titre

3.16 Micro serum neutralization test

The serum samples collected during the experiment on role of maternal antibodies in protection of kids against goatpox, were subjected to micro-SNT. The serial 2-fold dilution of inactivated test sera and 100 $TCID_{50}$ of Pantnagar strain of GPV were mixed and incubated for 1 h at 37^0C and then transferred to 96 well tissue culture plate containing monolayer of secondary KTCC. The healthy cell control as well as virus and known sera controls were also kept. After an incubation period of 6-7 days, the results were recorded and neutralizing end point titre was calculated using the method of Reed and Muench (1938).

The serum samples from different goats and kids, collected at particular time interval, were processed separately and mean end point titre was calculated.

3.17 Dot-Enzyme linked immunosorbant assay (Dot-ELISA)

Dot-ELISA was performed according to the method described by Tiwari and Negi (1996) with minor modifications.

The nitrocellulose strips were coated with 2 µl of purified GPV (Pantnagar strain) prepared in carbonate-bicarbonate buffer, pH 9.6 and incubated at 37^0C for 30 min. Thereafter, these strips were placed in 2% bovine serum albumin (SRL) for 30 min for blocking of free sites on membrane. The strips were then washed with PBS-T thrice, each for 2-3 min and incubated in 10-fold diluted antiserum at 37^0C for 30 min. The strips were again washed with PBS-T thrice and incubated in rabbit antigoat horse radish peroxidase conjugate (1:2000 dilution) at 37^0C for 30 min. Thereafter, the strips were washed thrice with PBS-T, dried at room temperature and incubated in freshly prepared substrate solution (0.05% 3 3' diaminobenzidine tetra hydrochloride in 0.1 M citric acid phosphate buffer, pH 5, and 50 µl H_2O_2) for 10-20 min. The appearance of brown spot indicated positive reaction. Known hyperimmune serum and negative serum (as used in plate ELISA) were considered as positive and negative controls, respectively.

3.18 Solid phase aggregation of coated erythrocyte test

The solid phase aggregation of coated erythrocytes (SPACE) test was conducted as per the procedure described by Bansal (1996).

The sheep erythrocytes were collected in Alsever's solution. After 3 washings with PBS, 0.5 ml of packed sheep erythrocytes was mixed with 1.0 ml (5 mg/ml protein concentration) of ammonium sulphate precipitated immunoglobulin (against Pantnagar strain of GPV), 1.0 ml of 0.4% glutaraldehyde and 2.0 ml of PBS. This erythrocyte mixture was kept at room temperature for 2 h and then 10 ml of 2M L-Lysine monohydrochloride, prepared in normal saline, was added to above erythrocyte suspension and kept overnight at 4^0C. Finally, 0.5% suspension of erythrocyte was prepared in PBS and used for performing the test.

For performing the test, hyperimmune serum (HIS) against Pantnagar strain of GPV was diluted 1: 5000 in carbonate- bicarbonate buffer (pH 9.6) and 100 µl of diluted HIS was then dispensed into each well of 96 well microtitre plate (U bottom, Laxbro) and incubated at 4^0C overnight. The plate was washed twice with PBS-T. The serial 2-fold dilution of the test antigen (10% w/v skin scab suspension in PBS) was prepared in PBS-T in microtitre plate and each dilution of antigen was dispensed in HIS coated microtitre plate. Then 0.1 ml of 0.5% suspension of erythrocytes prepared earlier was added to each well.

The plate was incubated at room temperature for 1 to 2 h and results were recorded. The titre of 1:8 and above was considered as positive in the present investigation.

The skin scab collected during experimental infection of goats with known GPV and skin from apparently healthy goats were included as known positive and negative controls, respectively. The unknown test samples of skin were collected during outbreak of disease or from experimentally infected animals.

3.19 Purification of goatpox and sheep pox viruses

3.19.1 Pantnagar strain of GPV grown in KTCC

The Pantnagar strain of GPV, grown in secondary KTCC, was purified by sucrose density gradient as per the method described by Bhatt (1998) and Kitching *et al.* (1986b). The infected KTCC, in 75 cm^2 tissue culture flask were frozen at the stage when approximately 70 -80% cells were showing CPE. After three cycles of freezing and thawing, the suspension was clarified by centrifugation at 600 g for 10 min. The supernatant was then centrifuged at 40,000 g (19,000 rpm, RP -19 rotar, Hitachi centrifuge, SCP 70 H) for 2 h at 4^0C. The resultant pellet was dissolved in 0.5 ml PBS (pH 7.2) by submerging it overnight at 4^0C. The suspension was incubated at 37^0C with DNase (2.5 µg/ml) followed by a further 15 min incubation at 37^0C with trypsin (100 µg/ml). The virus suspension was then cooled to 4^0C and trypsin inhibitor was added to a final concentration of 75 µg/ml. The virus suspension was then layered over a cushion of 2 ml of 36% sucrose (36% sucrose in 50 mM Tris, 1 mM

EDTA, pH 7.5) in a 11 ml polyallomer centrifuge tube (Rotar RPS-40T) and centrifuged at 100,000 g (29,000 rpm) for 75 min at 4^0C. The resultant pellet was dissolved in 0.5 ml Tris EDTA (TE) buffer overnight at 4^0C and then layered onto a 40- 60% sucrose gradient in TE buffer and centrifuged at 85,000 g (28,000 rpm) for 60 min at 4^0C. The virus bands at approximately one third and two third of distance in the tube were removed with Pasteur pipette, mixed and diluted with TE buffer. Finally, the virus was recovered by centrifugation at 85,000 g for 60 min at 4^0C. The pellet of purified virus was suspended in 0.5 ml TE buffer and stored at -20^0C.

The purity of virus was calculated by taking the absorbance at 260 and 280 nm. The presence of virus in bands was confirmed by AGPT.

3.19.2 Sheep pox virus Jaipur strain from skin scab of infected sheep

The sheep pox virus (Jaipur strain) was purified from scab obtained from an experimentally infected sheep. The infected skin scab were collected 10-14 days post infection and processed as described by Kalpana *et al.* (1995). The sample was minced and treated with 3 percent boric acid solution in 1:5 ratio and kept at 4^0C for 48 h. The suspension was frozen and thawed three times and clarified by centrifugation at 500 g for 20 min and purified by the method of Joklik (1962). The resultant supernatant was then pelleted by centrifugation at 15,000 g for 30 min. The pellet was then dissolved in 15 ml Tris buffer (0.001 M, pH 9) and layered onto a cushion

of 3 ml of 36% sucrose and centrifuged at 23000 g for 80 min. The pellet was resuspended in 15 ml Tris buffer and again pelleted on 36% sucrose cushion. The resultant pellet was suspended in 3-6 ml Tris buffer and layered onto sucrose gradient (25-40% sucrose in Tris buffer). The gradient was centrifuged at 23000 g for 45 min. The virus band from 1.5 cm from the bottom of tube was collected and dialysed against two changes of 200 volume each of Tris buffer. The purity of virus was calculated by taking the absorbance at 260 and 280 nm.

3.20 Protein estimation

Protein concentration of purified virus preparations were estimated by spectrophotometeric method as described by Warberg and Christian (1941). The optical density of purified virus suspensions were determined at 260 and 280 nm and protein concentration was calculated by following formula

$$\text{Protein mg/ml} = 1.56 \times OD_{280nm} - 0.76 \times OD_{260\,nm}$$

3.21 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to the method described by Laemmli (1970) with minor modifications.

3.21.1 Preparation of gel

The vertical slab gel electrophoresis apparatus (Atto, Japan) with glass plates of 14 x 14 cm and spacer of 1.5 mm thickness was used for performing SDS-PAGE by discontinuous buffer system. The resolving gel consisted of 10% and 12.5% acrylamide and stacking gel consisted of 3% and 4% acrylamide. The samples were stacked at 60 V till the sample entered the resolving gel and after that it was run at 80 V till the tracking dye reached the bottom of gel.

The molecular weights (mw) of purified virus polypeptides were determined by calculating the RF value and by direct comparison with standard protein MW markers (Sigma) run on the same slab gel according to the procedure described by Weber and Osborn (1969).

3.20.2 Preparation of sample

The samples for SDS-PAGE was prepared by mixing 25 µl of purified virus preparation with the same volume of 2x sample buffer and 2% mercaptoethanol. The suspension was heated at 80^0C for 5 min followed by centrifugation at 8000 rpm for 2 - 3 min. For single well, 50 µl sample (containing 40 µg protein) was used for performing SDS-PAGE.

3.21.3 Staining and Destaining of gel

3.21.3.1 Copper staining

The method of Lee *et al.* (1987) was used for copper staining of SDS-PAGE gels. After electrophoresis, gels were dipped for several seconds in water and then immersed in one smooth motion in 100 ml 0.3 M $CuCl_2$ in a plastic tray. Gels were gently rocked in the copper solution for 5 min at room temperature, washed 2 -3 min in water to remove excess reagent and placed in distilled water for storage.

3.21.3.2 Coomassie Brilliant Blue R staining

The electrophoresed gel were removed carefully and transferred to the 200 ml of CBB staining solution for 12 h at room temperature. The destaining was accomplished by dipping the gel in destaining solution for 2 - 6 h. The gel was than rinsed in distilled water and sealed in plastic bags. After UV treatment for 10 min, on either side of plastic bags containing gels were stored at 4^0C for indefinite time.

3.21.3.3 Silver staining

(a) The electrophoresis gel was first fixed in 400 ml of 40% methanol and 10% acetic acid for 30 min and then fixed twice in 400 ml of 10% ethanol and 5% acetic acid for 15 min.

(b) The Gel was soaked for 3 - 10 min in 200 ml fresh oxidizer (0.0034 M potassium dichromate, 0.0032 N nitric acid) and washed 3 -4 times for 5 -10 min in 400 ml water.

(c) The gel was then soaked in 200 ml fresh silver nitrate solution (0.012 M silver nitrate for 15 - 30 min) and then washed with 400 ml water for 1-2 min.

(d) Finally, the gel was washed for 1 min in developer (0.28 M sodium carbonate, 1.85% paraformaldehyde).

(e) The developer was replaced with fresh solution and incubated for 5 min. The development of colour was stopped with 5% acetic acid.

3.22 Identification of Immunogenic proteins by Western blotting

Polypeptides of purified virus preparation, separated on 12.5% SDS-PAGE using discontinuous buffer system, were transferred onto polyvinylidene fluoride (PVDF) membrane (0.45 μm pore size) by semi-dry method of electroblotting (electrophoretic transfer unit, Atto Corporation, Japan,).

3.22.1 Transfer of proteins with a semi-dry system

The proteins from gel were transferred onto PVDF membrane as per the method described in Millipore literature.

The two sheets of 3 mm thick Whatman filter paper were soaked in anode buffer I and placed onto centre of graphite anode electrode plate. The another sheet, soaked in anode buffer II solution, was placed on top of the first two sheet of filter paper. The anode buffer II soaked PVDF membrane (earlier treated with 100% methanol for 15 seconds followed by distilled water for 2 min) was then placed on the top of filter paper. The gel was then placed on the membrane followed by stacking of three sheets of filter, soaked in cathode buffer. The assembled transfer stack was covered with cathode plate and current of 0.8 mA/cm^2 (184 mA) was applied for 1 h.

To assess the quality of transfer, the membrane were stained with 0.1% Coomassie brilliant blue R for 2 min and destained in destaining solution (in 50% methanol, 7% acetic acid) for 10 min.

3.22.2 Rapid immuno detection of proteins on membrane

The GPV blotted membrane was dried by soaking in 100% methanol for 10 sec and then placed on filter paper for 15 min for evaporation of methanol. The blot was then incubated for 1 h with specific immunoglobulins (ammonium sulphate precipitated hyperimmune serum) diluted 1/50 in blocking buffer. After washing two times with PBS each for 5 min, the blot was again incubated for 30 min with rabbit antigoat HRPO conjugate (diluted 1:2000 in blocking buffer). The blot was washed as described above and transferred to freshly prepared 50 ml of substrate

solution containing 10 mg of diamminobenzidine tetrahydrochloride and 20 µl of 30% (v/v) H_2O_2 for few minutes. The reaction was stopped by washing with distilled water. After drying the membrane was stored in a dark place.

The negative (in AGPT) and unknown serum samples collected from field cases were also used for detection of virus specific antibodies.

The SPV blotted membranes were similarly stained for detection of immunogenic proteins using hyperimmune serum against Jaipur strain of SPV raised in sheep and donkey antisheep HRPO conjugate (Sigma). The procedure described above was followed.

3.23 Affinity purification of monospecific antibodies

The antibodies against immunogenic proteins of virus, blotted on PVDF, were purified from hyperimmune serum against GPV (Pantnagar strain). The methods described by Olmsted (1981) and Maa and Esteban (1987) were followed. The purified virus was transferred to PVDF membrane and location of specific proteins were detected by staining the strip of membrane by CBB stain. The proteins on membrane were separated by cutting the strips horizontally. The strips for specific proteins were then incubated with polyclonal immunoglobulins (ammonium sulphate precipitated) for 1 h at 37^0C. These strips were then washed with PBS followed by 0.5% Triton x -100 in PBS twice each for 10 min. These strips

were then cut into small pieces and transferred to a capped tube containing 3.5 ml of 0.2 M glycine hydrochloride, (pH 2.5) for 2 min with shaking. Thereafter, 1.75 ml of 1 M K_2HPO_4 (pH 9.0) was added to the tube and solution was diluted with 1 volume of 2% bovine serum albumin. The eluted antibodies were tested for specificity on Western blot of purified virus and then concentrated upto 1 ml by lyophilization.

3.24 Micro neutralization with monospecific serum

The monospecific antibodies against different immunogenic proteins were tested for neutralization of viral infectivity in KTCC by micro-SNT. The serial 2-fold dilution of 50 µl of each monospecific sera were prepared in triplicate. The same volume of virus containing 100 $TCID_{50}$ was then added to each well. After 1 h of incubation, these serum virus mixtures were transferred to KTCC monolayer in 96 well tissue culture plate. The appropriate healthy cell control as well as virus and sera controls were also maintained. The plates were incubated at 37^0C for 6-7 days and serum end point titre was calculated following method of Reed and Muench (1938).

3.25 Separation of envelope and core of GPV

The procedure described by Maa and Esteban (1987) was used with slight changes.

Purified Pantnagar strain of GPV was used for separation of viral envelopes and core. The purified virus suspension (5 mg) in 1 ml of 50 mM

Tris hydrochloride (pH 8.5), 10 mM $MgCl_2$, 10 mM dithiothretol, 0.5% Triton x 100 was sonicated at 50 W three times each for 5 second. After incubation at 37^0C for 30 min, the extract was layered on top of a 36% sucrose cushion in the same buffer and centrifuged at 20,000 g for 60 min at 4^0C. The viral envelopes separated in upper phase was collected and dialysed extensively against PBS. The viral cores pelleted in the bottom were suspended in PBS.

The samples of both envelope and cores were prepared as described earlier and immunoblotted using hyperimmune serum against GPV and rabbit antigoat HRPO conjugate.

3.26 Detection of GPV proteins in infected tissues by immuno blotting

The KTCC infected with Pantnagar strain of GPV was used for detection of virus specific proteins by Western blotting as per the method described by Sambrook *et al.* (1989). The KTCC monolayers infected with GPV were washed twice with PBS and lysed by adding 200 µl of hot (85^0C) 2 x gel loading buffer. The cells were scrapped out and boiled for 10 min and centrifuged at 10,000 g for 10 min at room temperature. The supernatant was collected and used as sample for SDS-PAGE and blotting. The uninfected KTCC processed similarly was used as control.

To determine the antigenic relationship between SPV and GPV, the kid testes cells infected with GPV were processed as described above and

proteins were electrophoretically transferred to PVDF membrane. The membrane was then cut into strips. Out of these, one strip was treated with GPV hyperimmune serum raised in goats along with rabbit antigoat HRPO conjugate. The other strip was treated with SPV hyperimmune sera raised in sheep and donkey antisheep HRPO conjugate. Similarly, lamb testes cells infected with SPV were also processed and immunoblotted. The uninfected KTCC and lamb testes cells treated in same manner were included as controls. The virus specific proteins of sheep and goat pox viruses were detected and compared.

RESULTS

RESULTS

4.1 Adaptation/Propagation of capripox virus

4.1.1 Experimental infection of goats

The Pantnagar strain of GPV produced characteristic lesions on intradermal inoculation in goats. The body temperature of infected goats increased gradually from day 4 PI with a peak on day 6 PI and remained almost constant upto day 8 PI. Thereafter, the temperature gradually decreased and became normal by day 16 PI. The skin lesions developed at the site of inoculation within 5 days (Fig 1). The macules were initially seen on hairless areas of body, which later converted into papules of 0.5 - 1.0 cm diameter (Fig 2). These papules changed to scabs 10 days PI and persisted upto 6 weeks in goats. The development of papules was associated with rhinitis, conjunctivitis and enlargement of prescapular lymph node. The secondary lesions developed 10 days after the primary lesions and involved the entire skin of body especially on teats, nose, lips, eyes and ears. The infected goats continued to graze inspite of high temperature and cutaneous lesions.

4.1.2 Secondary kid testes cell culture

The virus strains, viz., Pantnagar, Uttarkashi and Sambalpur, were adapted in secondary KTCC. The multiplication of virus was assessed by appearance of cytopathic effects (CPE). All the strains of GPV were adapted to KTCC at second passage level and showed similar type of CPE. After 7^{th} passage, all strains of virus produced characteristic CPE, which were evident by conglomeration of rounded cells resembling bunches of grapes (Fig 4). All the virus strains were passaged 15 times in KTCC but there were no significant difference in CPE produced by them after 9^{th} passage.

In unstained preparations, the CPE was evident 72 h PI and reached maximum by 120 h PI at 2^{nd} passage level. The virus produced rounding and retraction of cells with increased granularity and distinct cell boundaries at 72 h PI. At 96 h PI, the number of infected cells in monolayer increased and single or clusters of rounded cells were observed just like bunches of grapes, which were often connected by thin cytoplasmic processes (Fig 5). The retraction of the affected cells left long gaps in the monolayer at 120 h PI at 7^{th} passage level (Fig 6). The virus became progressively more cytopathic on serial propagation as evident by early appearance of CPE.

Fig 1. Pox lesions on the head of goat experimentally infected with Pantnagar strain of GPV.

Fig 2. Pox lesions on the abdomen of goat experimentally infected with Pantnagar strain of GPV.

Fig 3. Uninfected kid testes cell culture monolayer at 48 h PI, MGG staining X 100.

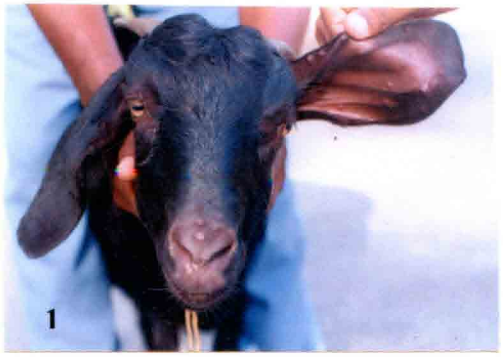
Fig 4. KTCC monolayer infected with Pantnagar strain of GPV showing rounding and clumping of cells at 72 h PI at 7^{th} passage, Unstained, X 50.

Fig 5. KTCC monolayer infected with Pantnagar strain of GPV showing rounding of cells 96 h PI at 2^{nd} passage, unstained, X 50.

Fig 6. KTCC monolayer infected with Pantnagar strain of GPV showing round, spindle shaped cells with marked detachment at 120 h PI at 7^{th} passage Unstained, X 50.

Fig 7. KTCC monolayer infected with Pantnagar strain of GPV showing intracytoplasmic eosinophilic inclusion bodies at 36 h PI at 8^{th} passage level. MGG staining, X 400.

Fig 8. KTCC monolayer infected with Pantnagar strain of GPV showing granular intracytoplasmic inclusions covering entire cytoplasm at 96 h PI at 9^{th} passage level, MGG staining, X 400.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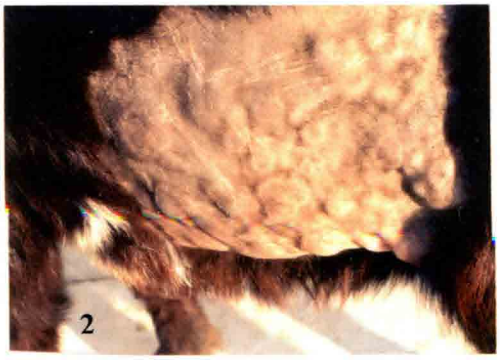
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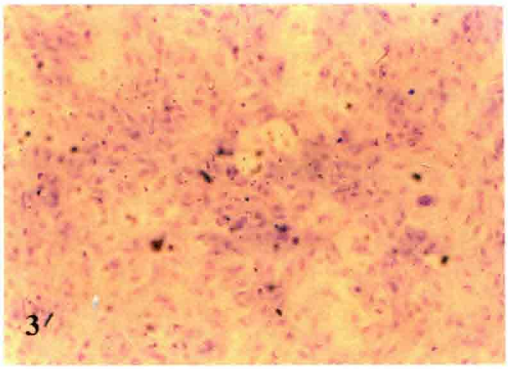
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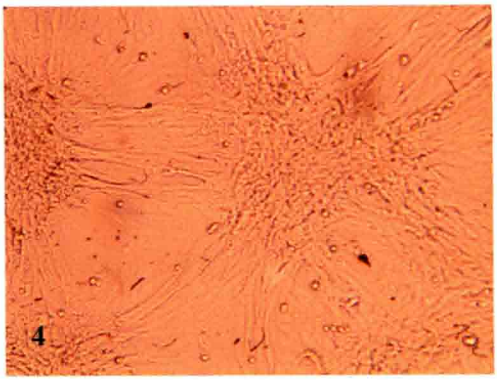
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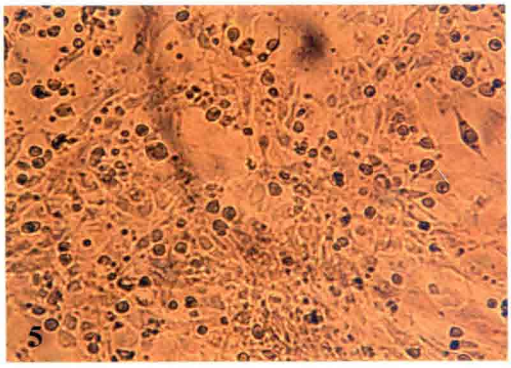
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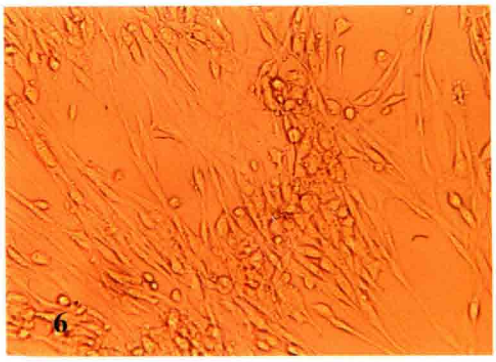
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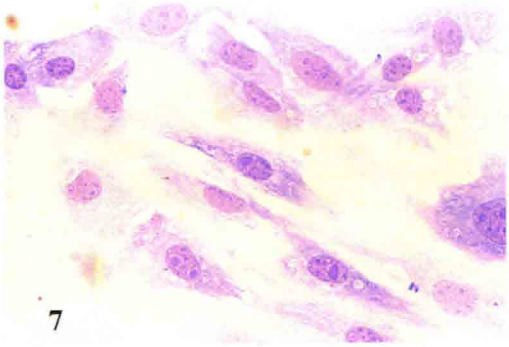
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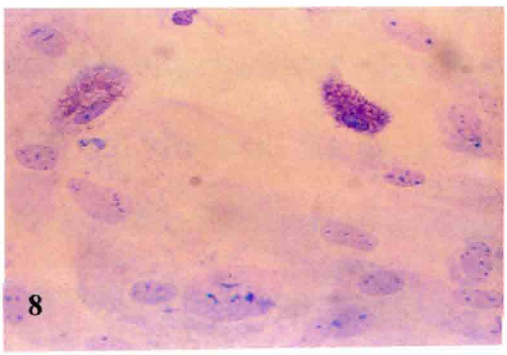
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The MGG staining of infected monolayers revealed intracytoplasmic eosinophilic inclusion bodies of different shape and size at 72 h PI at 3rd passage level. The number of cells showing such inclusion bodies increased significantly at 120 h PI. The inclusions appeared as masses of loose matrix or circumscribed bodies with or without a distinct 'halo' around them (Fig 7). Most of the cells showed more than one inclusion bodies at 120 h PI. The nuclei were pushed to one side and appeared as pyknotic with condensation and margination of chromatin. At 9th passage, some of cells showed intracytoplasmic eosinophilic inclusions, which were in the form of small granular masses covering the entire cytoplasm of the cell (Fig 8). The control cultures did not show any degenerative changes.

On acridine orange staining of control KTCC monolayer, the cells showed brick red cytoplasm with bright greenish yellow nucleus. At third passage level, the monolayers of KTCC infected with strains of GPV showed small greenish bodies in cytoplasm and rounding of cells at 48 h PI. At 72 h PI, cells revealed dense green coloured mass mostly elongated or elliptical in shape, situated at perinuclear area (Fig 14). These masses increased in size at 96 h PI, which is suggestive of presence of viral DNA in the cytoplasm of cells. At later stage of infection, the brick red fluorscence of cytoplasm was replaced by greenish fluorescence of viral DNA. The greenish yellow colour of nucleus did not show any change.

Fig 9. KTCC monolayer infected with Pantnagar strain of GPV showing fluorescence at perinuclear area 12 h PI at 3rd passage, X 100.

Fig 10. KTCC monolayer infected with Pantnagar strain of GPV showing diffuse cytoplasmic fluorescence 48 h PI at 3rd passage, X 400.

Fig 11. KTCC monolayer infected with Pantnagar strain of GPV showing intense cytoplasmic fluorescence 72 h PI at 3rd passage, X 400.

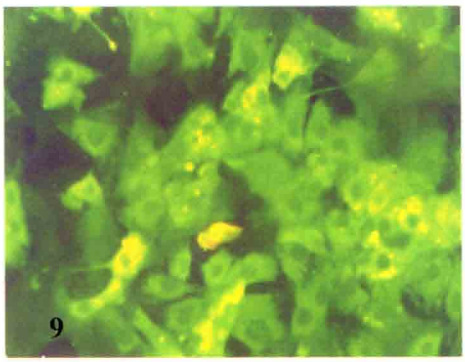
Fig 12. Transmission electron microscopy of kid testes cells infected with Pantnagar strain of GPV showing electron dense virion particles X 4250.

Fig 13. Transmission electron microscopic studies of kid testes cells infected with Pantnagar strain of GPV showing developmental stages of virus particles X 13500.

Fig 14. KTCC infected with Pantnagar strain of GPV showing yellow green cytoplasmic fluorescence and 72 h PI at 3rd passage, AO staining X 400.

Fig 15. Unstained Vero cell monolayer infected with Pantnagar strain of GPV showing rounding of cells at 24 h PI at 5th passage X 50.

Fig 16. Uninfected Vero cell monolayer, Unstained X 50.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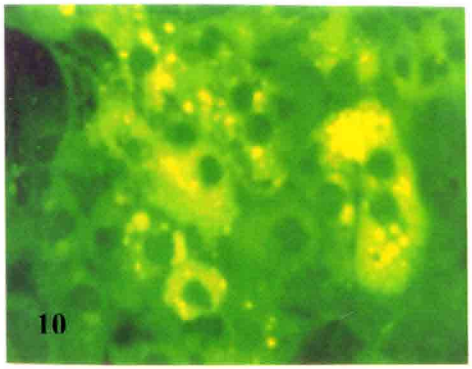
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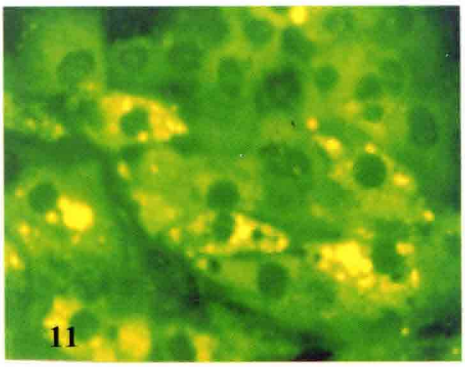
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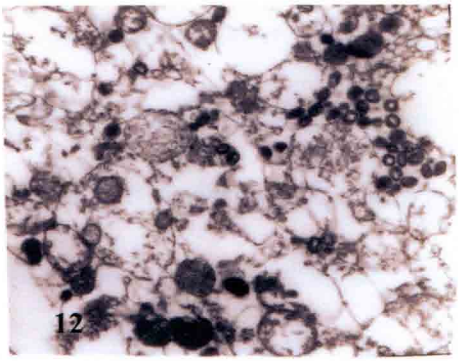
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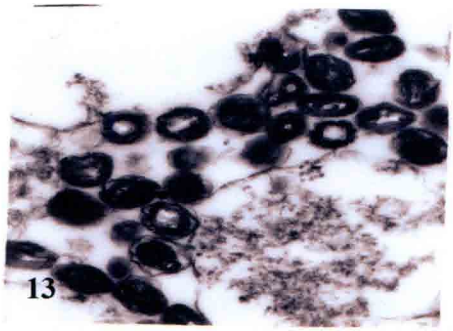
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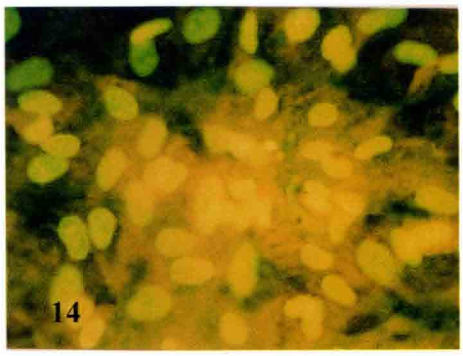
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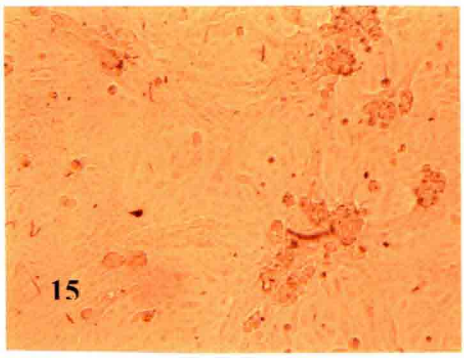
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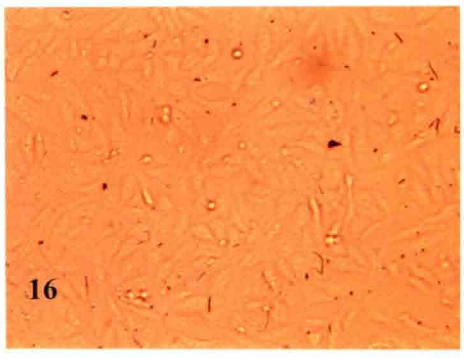
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4.1.3 Vero cell line

The 5th passage KTCC adapted Pantnagar strain of GPV was used as inoculum for infecting Vero cell monolayer. The virus was passaged 15 times in Vero cell culture. The virus produced CPE after 48 h PI at 3rd passage level, characterized by rounding and clumping of cells 72 h PI. At 5th passage, the cell rounding and clumping was more extensive and showed development of microplaques (Fig 15). The virus was fully adapted to Vero cells at 7th passage and induced CPE as early as 24 h PI, which reached to maximum by 96 - 120 h PI. The changes included rounding and clumping of cells with formation of microplaques. The microplaques were about 2 μm in diameter having a sharp boundary of refractile cells. The formation of microplaques reached to maximum after 148 h at 8th passage. After 168 h PI, the cellular degeneration resulted into formation of clear spaces in the cell monolayer.

In MGG staining, the Vero cell monolayers showed CPE at 72 h at 3rd passage level. On serial passages, the virus became progressively more cytopathogenic, as evident by early appearance of CPE and short duration required to produce maximum CPE. At 6th passage level, the infected Vero cells produced syncytia, involving 10-15 cells (Fig 18). At 7th passage level, the virus was fully adapted to Vero cells and produced characteristic CPE, which included early appearance of granularity in the cell cytoplasm,

rounding and clustering of cells and development of microplaques. The clustered cells were connected with cytoplasmic strands. At 96 h PI, the cell cytoplasm showed vacuolation followed by formation of intracytoplasmic inclusion bodies, which were eosinophilic in nature (Fig 17). At 120 h PI, more number of cells showed inclusions, usually at juxta-nuclear position in the cell cytoplasm (Fig 19). These were usually more than one in number in a single cell. The size of these inclusions increased and became circular, elliptical or oval in shape at 144 h PI. The nucleus became swollen with prominent nucleoli in some of the infected cells. During terminal stages, the whole monolayer showed maximum degenerative changes and detachment of degenerated cells resulting into the clear areas in the monolayer.

The acridine orange staining of uninfected Vero cells revealed yellowish green nuclei and reddish yellow nucleoli with brick red cytoplasm (Fig 22). At 24 h PI, some of the infected Vero cells showed cytoplasmic apple green fluorescence, which was further intensified by 48 to 72 h PI at 3rd passage level (Fig 23). The fluorescence further intensified at 48 h PI at 5th passage level involving maximum number of cells. The inclusions in the cell cytoplasm were greenish yellow, irregular and diffused masses of variable size, with or without a halo. The brick red fluorescence of cytoplasm was completely replaced by greenish yellow fluorescence at later stage of infection. Similar changes were recorded in

Fig 17. Vero cell monolayer infected with Pantnagar strain of GPV showing intracytoplasmic inclusion bodies. MGG staining, X 400.

Fig 18. Vero cell monolayer infected with Pantnagar strain of GPV showing syncytia formation at 6^{th} passage, MGG staining X 400.

Fig 19. Vero cell monolayer infected with Pantnagar strain of GPV showing cytoplasmic inclusion bodies 48 h PI at 4^{th} passage. MGG staining X 400.

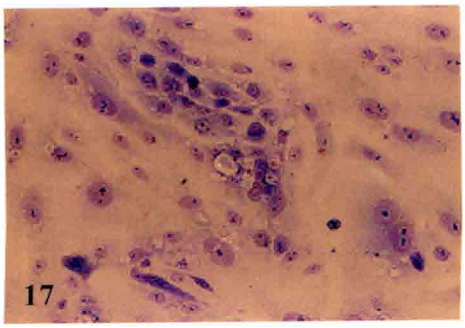
Fig 20. Transmission electron micrograph of Vero cells infected with Pantnagar strain of GPV showing different developmental stages of virus. X 8200.

Fig 21. Electron micrograph of Vero cell infected with Pantnagar strain of GPV showing round or oval virus particles X 21500.

Fig 22. Uninfected Vero cell monolayer showing brick red cytoplasmic fluorescence AO staining. X 400.

Fig 23. Vero cell monolayer infected with Pantnagar strain of GPV showing greenish mass in the cytoplasm at 48 h PI at 3^{rd} passage, AO staining, X 400.

Fig 24. Uninfected MDBK cell monolayer, MGG staining X 100.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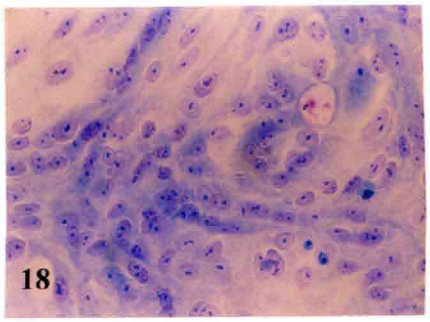
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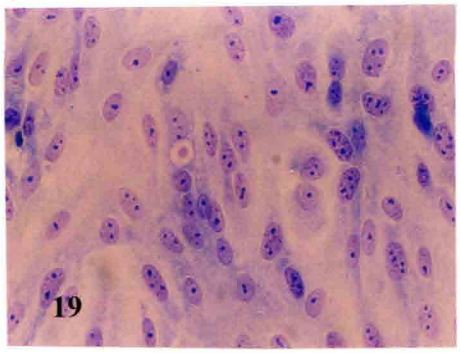
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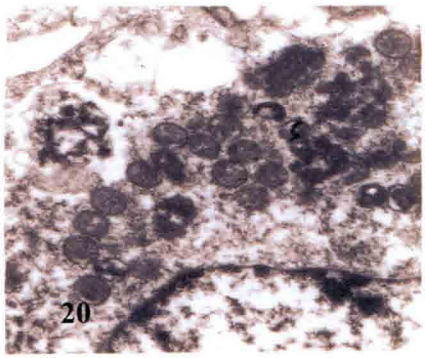
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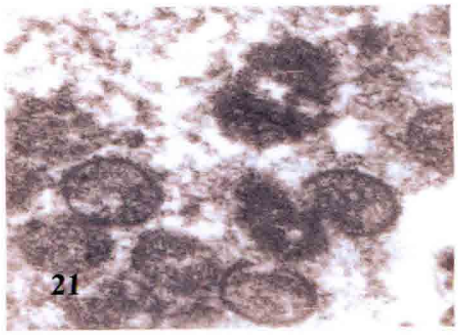
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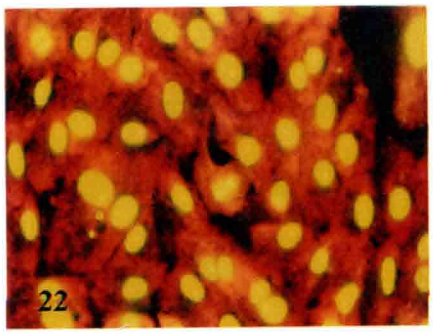
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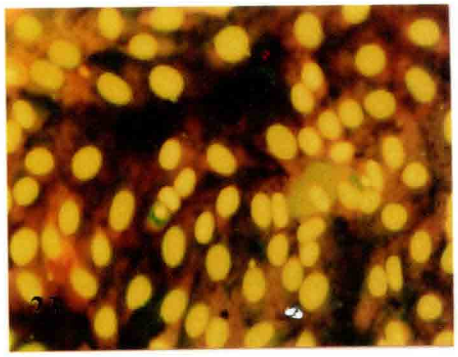

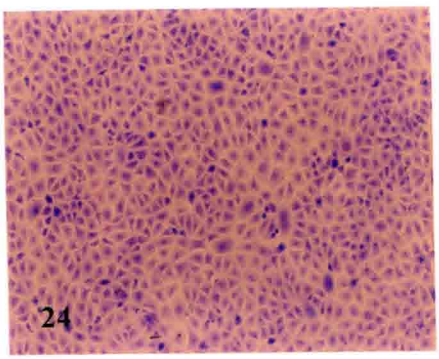
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subsequent passages except that time required for appearance of CPE was reduced.

4.1.4 MDBK cell line

The KTCC adapted Pantnagar strain of GPV was used for adaptation in MDBK cell line. The virus adapted to MDBK cells showed rounding of cells at 72 h PI at 4th passage levels. At 8th passage level, the virus was fully adapted to MDBK cells and produced characteristic cytopathic effects. The virus was passaged 20 times in MDBK cells but there was no significant difference in the CPE after 8th passage except decrease in time for appearance of CPE.

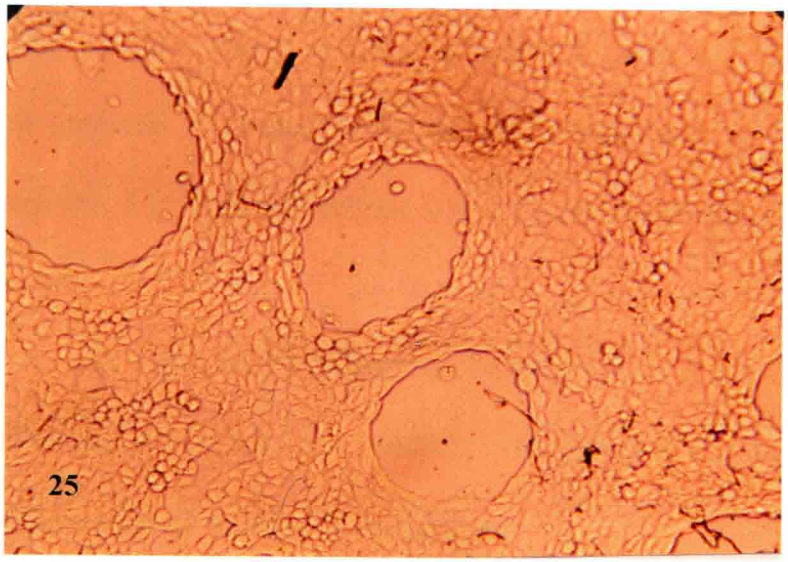
The monolayers stained with MGG showed rounding of cells at 72 h PI at 4th passage, which were clumped together after incubation. At 6th passage level, the rounding and clumping of cells were recorded as early as 24 h PI. At 8th passage level, the CPE was more pronounced, which included granular cell cytoplasm at 24 h PI and vacuolation of entire cytoplasm at 72 h PI. Other changes recorded at this stage were formation of syncytia, microplaques and intracytoplasmic eosinophilic inclusions, which were granular in nature (Fig 25,26). Few infected cells also showed swollen nuclei with margination of chromatin. Further incubation showed degenerative changes and detachment of cells from the surface leaving clear areas of 1-2 mm in diameter.

Fig 25. MDBK cell monolayer infected with Pantnagar strain of GPV showing microplaques. Unstained. X 50.

Fig 26. MDBK cell monolayer infected with Pantnagar strain of GPV showing cytoplasmic inclusion bodies. MGG staining X 400.

Fig 27. Solid phase aggregation of coated erythrocyte test.

A - Positive sample
B - Positive control
C - Negative sample
B - Negative control



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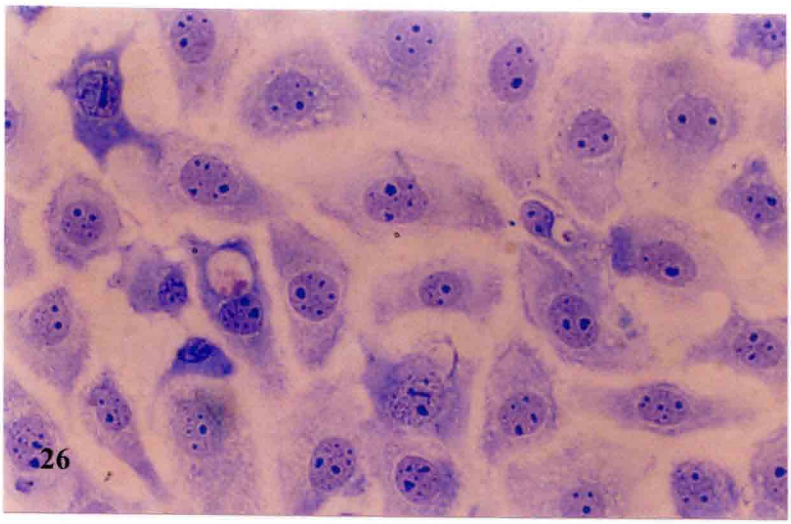

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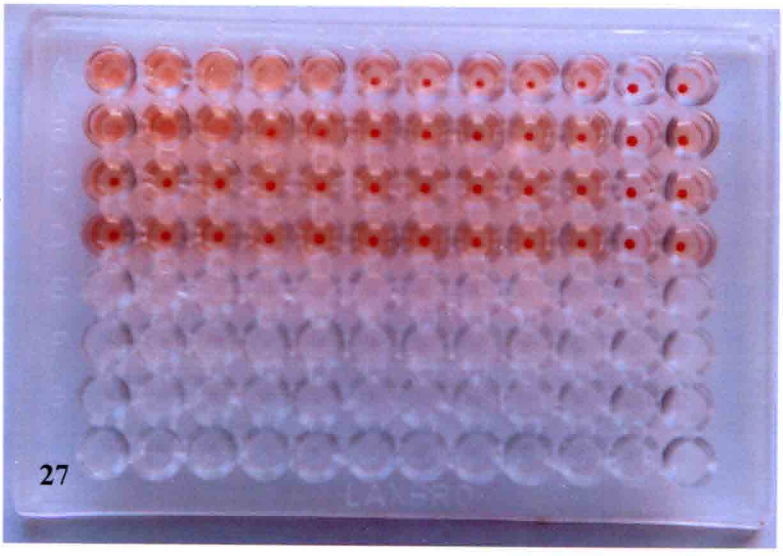

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The acridine orange staining of infected monolayer of MDBK cells revealed greenish fluorescence in the cell cytoplasm at 24 h PI at 4th passage level. The greenish diffuse cytoplasmic inclusions of variable size were recorded at 72 h PI at 6th passage level. In later passages the brick red cytoplasm was replaced by yellowish fluorescence at 96 h PI. The control monolayers showed brick red cytoplasm with greenish nuclei.

4.2 Fluorescent antibody technique

The secondary KTCC infected with Pantnagar, Sambalpur and Uttarkashi strains of GPV showed small areas of fluorescence in the perinuclear areas of cytoplasm at 12 h PI at 3rd passage level (Fig 9). At 48 h PI, the intensity of cytoplasmic fluorescence increased and involved more number of cells (Fig 10). Almost all the cells were found to posses considerable diffused cytoplasmic fluorescence after 72 h PI (Fig 11). The nuclei of infected cells did not exhibit fluorescence at any stage. The uninfected monolayers processed similarly did not show any fluorescence.

The Vero and MDBK cells infected with Pantnagar strain also showed small areas of fluorescence in the cell cytoplasm at 24 h PI at 5th passage level. The intensity of fluorescence was increased at 48 h PI. However, the greenish fluorescence was observed only in the cell cytoplasm.

4.3 Electron microscopic studies

The KTCC and Vero cells infected with Pantnagar strain of GPV when examined electron microscopically showed presence of intracytoplasmic virus particles in the cytoplasmic matrix (Fig 12). The virion particles, which were more electron dense than matrix had a distinct round, oval or elliptical shape (Fig 13). In some cells the cytoplasmic matrix was not seen and virus particles were found distributed throughout the cell cytoplasm (Fig 20, 21).

4.4 Titration of viral infectivity

The titre of GPV (Pantnagar strain) in KTCC was $10^{4.30}$ $TCID_{50}$/ml at 5th passage level, which increased to $10^{6.31}$ $TCID_{50}$/ml at 10th passage level. At 15th passage level, the titre remained almost stablized to $10^{6.57}$ $TCID_{50}$/ml (Table 1).

In Vero cells, the infectivity titre of $10^{2.83}$ $TCID_{50}$/ml was recorded at 5th passage level, which increased to $10^{4.21}$ $TCID_{50}$/ml at 10th passage and remained almost same upto 15th passage. In MDBK cells, the virus titre at different passage levels are presented in Table 1. The virus titre of $10^{2.62}$ $TCID_{50}$/ml was recorded at 5th passage, which increased to its maximum ($10^{4.63}$ $TCID_{50}$/ml) at 15th passage level and thereafter, no significant increase in titre was observed in subsequent passages (Fig 34).

Table 1: Infectivity titres of Pantnagar strain of GPV in cell lines and KTCC

Passage number	Virus titre ($log_{10}TCID_{50}$/ml)		
	KTCC	VERO	MDBK
5	4.30	2.83	2.62
10	6.31	4.21	4.50
15	6.57	4.49	4.63
20	-	-	4.60

Table 2: Infectivity titres of extracellular and intracellular GPV in KTCC

Virus strains	Virus titre (log_{10} $TCID_{50}$/ml)					
	5^{th} passage		10^{th} passage		15^{th} passage	
	EC	IC	EC	IC	EC	IC
Sambalpur	2.67	4.48	4.31	6.31	4.30	6.34
Uttarkashi	2.83	4.30	4.49	6.22	4.21	6.48
Pantnagar	2.62	4.49	4.21	6.59	4.49	6.57

EC = extracellular IC= Intracellular

The infectivity titres of extracellular and intracellular GPV strains, viz., Pantnagar, Sambalpur and Uttarkashi are presented in Table 2. The intracellular virus titre of all the three strains were much higher than the extracellular at different passage levels. The extracellular titre of $10^{6.59}$ $TCID_{50}$/ml was recorded with Pantnagar strain of GPV. The highest intracellular titre of $10^{4.49}$ $TCID_{50}$/ml was also recorded with Pantnagar strain at 15th passage level. The other strains also produced similar titres at different passage levels (Fig 35,36,37).

The adaptation of Pantnagar strain of GPV in KTCC, MDBK and Vero cells was confirmed by inoculation of kids with adapted viruses. The virus inoculated kids showed high rise of temperature (106^0F) 4 days PI, which remained almost constant upto 10 days PI. During this period the kids showed depression, lacrimation and nasal discharge followed by development of papular erythematous, skin lesions at the site of inoculation. The skin lesions reached to its maximum size at 9 days PI and thereafter started declining.

4.5 Micro serum neutralization test

The micro SNT were performed by alpha procedure using constant serum and diluted virus. The hyperimmune serum raised against Pantnagar strain of GPV neutralized the Vero, MDBK and KTCC adopted viruses.

The neutralization indices of KTCC, MDBK and Vero cells adapted Pantnagar strain of GPV were 3.91, 2.98 and 2.66, respectively.

4.6 Characterization of Pantnagar strain of GPV

4.6.1 Physico chemical properties

The 10^{th} passage Pantnagar strain of GPV having titre of 5.92 log_{10} TCID50/ml was used for characterization.

4.6.1.1 Sensitivity to heat

The Pantnagar strain of GPV was completely inactivated at 70^0C in 10 min. The drop of log 2.63 and 3.13 in infectivity titre were recorded at 55^0 and 60^0 C within 10 min, respectively (Table 3).

4.6.1.2 Sensitivity to pH

The Pantnagar strain was found to be sensitive to both alkaline and acidic pH. The drop of log 3.95, 2.79 and 1.25 in infectivity titre were recorded when virus suspensions exposed to pH 3, 5 and 8 for 1 h at 37^0C, respectively (Table 3).

4.6.1.3 Sensitivity to diethyl ether

The Pantnagar strain of GPV was found to be highly susceptible to diethyl ether. The drop of log 4.31 $TCID_{50}$/ml in infectivity titre was recorded after 20 h exposure to ether at 4^0 C.

4.6.1.4 Sensitivity to chloroform

The chloroform treatment of Pantnagar strain resulted into decline in the infectivity titre of virus. The drop of log 3.25 $TCID_{50}$/ml was recorded after treatment with chloroform for 15 min.

4.6.2 Host spectrum

The Pantnagar strain of GPV did not show any sign of infection in sheep, chicken, rabbit, mice and buffalo calf.

4.6.3 Haemagglutination

The Pantnagar strain of GPV did not agglutinate erythrocytes of sheep, goat, pig, rabbit, dog, chicken and human type 'O' cells.

4.6.4 Growth behaviour

The Pantnagar strain of GPV produced pock lesions on CAM of developing chicken embryos. The lesions were seen after 3rd passage, which were nonhaemorrhagic and approximately 2 mm in diameter.

The secondary KTCC infected with Pantnagar strain of GPV produced rounding and clumping of cells, vacuolation of cell cytoplasm, syncytia formation, intracytoplasmic eosinophilic inclusions after 3rd passage. The infectivity titres of $10^{4.30}$, $10^{6.31}$ and $10^{6.37}$ $TCID_{50}$/ml were recorded at 5, 10 and 15 passage levels, respectively.

Table 3: Effect of Physico-chemical agents on infectivity titre of GPV (Pantnagar strain)

Treatment	Duration	*Virus titre after treatment (log_{10} $TCID_{50}$/ml)	Drop in infectivity titre (log_{10} $TCID_{50}$/ml)
Heat			
55^0C	10 min	3.29	2.63
60^0C	10 min	2.79	3.13
70^0C	10 min	Inactivated	
pH			
3	60 min	1.97	3.95
5	60 min	3.13	2.79
8	60 min	4.67	1.25
Diethyl ether	20 hours at 4^0C	1.61	4.31
Chloroform	10 min	2.67	3.25

*Original infectivity titre was 5.92 log_{10} $TCID_{50}$/ml

4.6.5 Histopathological examination

The histopathological examination of kid skin infected with GPV (Pantnagar strain) showed characteristic changes. The epithelial hyperplasia, coagulation necrosis and accumulation of histocytes around papules were the predominant lesions. The nuclei of infected cells showed margination of chromatin. Infected epidermal cells showed hydropic degeneration and presence of eosinophilic intracytoplasmic inclusion bodies.

4.6.6 Serological characterization

The Pantnagar strain produced two precipitation bands against homologous serum in AGPT, however, only one precipitation band was recorded with antiserum against Sambalpur and Uttarkashi strains of GPV.

The indirect fluorescent antibody technique applied for detection of antigen (Pantnagar strain of GPV) revealed intense cytoplasmic fluorescence with homologous sera. However, weak fluorescence was seen with antiserum against Sambalpur and Uttarkashi strains of GPV.

4.7 Role of maternal antibody in protection of kids

Maternal antibodies protected the kids against GPV infection. The serum samples collected from dam prior to kidding showed mean ELISA antibody titre of 3.29 log10 and serum end point titre of 150. The mean

Table 4: Mean serum antibody titre of kids born to GPV immunized goats

Days after birth	ELISA titre (log_{10})	Serum end point titre (micro SNT)
0	3.26	212
7	3.43	355
14	3.40	316
21	3.39	299

ELISA and serum neutrilizing antibody titres of kids at different time intervals are given in Table 4.

The kids born to immunized goats, when challenged with GPV at the age of 21 days, showed slight rise in body temperature upto 104^0F on day 3 PI but returned to normal on 4 day PI. On 5^{th} day PI, nodules of about 4-5 mm diameter appeared at the site of inoculation. These lesions subsided within 6-7 days PI. Besides this, kids remained healthy without showing any clinical sign of disease upto 60 days PI.

The control kid from unimmunized goat, free from antibodies against GPV, showed typical signs of disease after infection with GPV. The body temperature reached to 106^0 F on day 4 PI with symptoms of depression, lacrimation and nasal discharge followed by papular erythematous skin lesions at the site of inoculation. These lesions reached to its maximum severity on day 14 PI and then started regressing.

4.8 Solid phase aggregation of coated erythrocytes test

The results of SPACE test, performed for detection of GPV antigen in skin scabs are given in Table 5 and Fig 27.

The wells showing aggregation of erythrocytes in the bottom were considered as negative, whereas positive reaction showed uniform distribution of erythrocytes over the bottom of the well. The titre was calculated as reciprocal of highest dilution showing positive reaction.

Table 5: Comparative efficacy of SPACE and AGPT for detection of GPV antigen

Sample	Total No. of sample tested	Samples positive in AGPT	Number of positive samples with SPACE test (titre in log_2)								
			3	4	5	6	7	8	9	10	Total
Known positive skin scab	10	10	-	1	4	3	2	-	-	-	10
Known positive cell culture fluid	20	16	2	7	6	2	3	-	-	-	20
Known negative skin samples	10	-	-	-	-	-	-	-	-	-	0
Skin scab samples	20	14	1	4	5	2	3	2	-	-	17

Out of 20 known samples (positive cell culture fluid), only 16 were found positive in AGPT, whereas SPACE test detected anitgen in all the 20 positive samples. Similarly, out of 20 unknown skin scab samples, AGPT detected only 14 samples as positive, whereas SPACE test detected GPV antigen in 17 samples. The test did not show positive reaction with known negative sample.

4.9 Purification of viruses and analysis of viral proteins by SDS-PAGE

4.9.1 Pantnagar strain of GPV

The Pantnagar strain of GPV, grown in secondary KTCC, was purified by sucrose density gradient. The absorbance ratio for purified virus band at 260 and 280 nm was found to be 0.59. On testing the purified virus with known antiserum in AGPT, it showed only one sharp precipitation band. The protein content of purified virus was estimated and diluted to 40 μg/50 μl for SDS-PAGE analysis.

The polypeptides of purified virus were resolved by SDS-PAGE using discontinuous buffer system. In 10% and 12.5% acrylamide gels, the purified virus yielded 53 and 43 polypeptides, respectively, which were visualized on Coomassie brilliant blue staining (Fig 28). A total of 55 polypeptides, ranging from 209 KD to 15 KD, were resolved (Table 6). The silver staining of gels also revealed same number of polypeptides but

Fig 28. SDS-PAGE of purified Pantnagar strain of GPV in 10% resolving gel, CBB staining.

Lane A - Purified GPV

Lane B - MW markers from top to bottom - 205 KD, 116 KD, 97 KD, 84 KD, 66 KD, 55KD, 45 KD and 36 KD.

Lane C - MW markers from top to bottom - 66 KD, 45 KD, 36 KD, 29 KD, 24 KD and 20 KD.

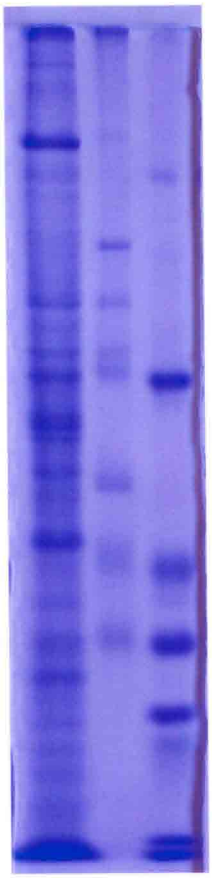

A B C

Fig 29. SDS-PAGE of purified GPV in 10% resolving gel. Silver staining.

Lane A - MW markers from top to bottom- 205 KD, 116 KD, 97 KD, 84 KD, 66 KD, 55 KD, 45 KD, 36 KD, 29 KD, 24 KD and 20 KD

Lane B - Purified GPV

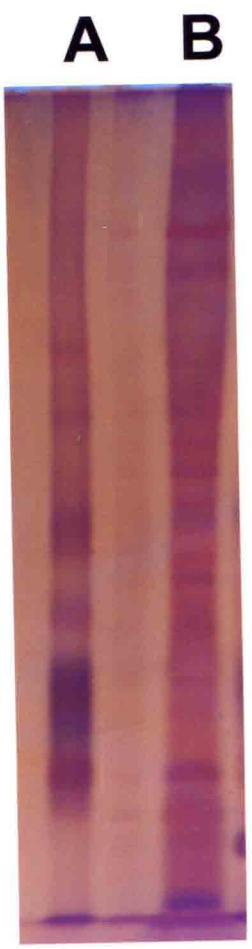

A
B

Table 6: Polypeptides of goatpox virus (Pantnagar strain) resolved by SDS-PAGE

S. No.	Approximate molecular weight in kilodaltons		
	10% resolving gel	12.5% resolving gel	Total number of polypeptides resolved
1	209	-	209
2	207	-	207
3	195	-	195
4	191	191	191
5	-	186	186
6	180	180	180
7	178	-	178
8	174	174	174
9	168	-	168
10	166	166	166
11	158	-	158
12	-	155	155
13	151	151	151
14	-	147	147
15	143	143	143
16	141	141	141
17	-	138	138
18	-	135	135
19	-	129	129
20	127	127	127
21	124	-	124
22	-	122	122
23	118	-	118
24	115	-	115
25	-	112	112

.....contd.

S. No.	Approximate molecular weight in kilodaltons		
	10% resolving gel	12.5% resolving gel	Total number of polypeptides resolved
26	-	108	108
27	-	105	105
28	103	-	103
29	100	100	100
30	97	97	97
31	93	93	93
32	91	-	91
33	87	87	87
34	85	-	85
35	-	81	81
36	78	78	78
37	76	76	76
38	72	72	72
39	68	68	68
40	64	-	64
41	58	58	58
42	55	55	55
43	52	52	52
44	48	48	48
45	45	45	45
46	43	43	43
47	40	40	40
48	38	38	38
49	-	35	35
50	32	-	32
51	28	-	28
52	26	-	26
53	22	-	22
54	-	20	20
55	-	15	15

with better visibility (Fig 29). Copper staining performed for rapid analysis of polypeptides in SDS-PAGE was as sensitive as CBB staining.

4.9.2 Jaipur strain of SPV

The Jaipur strain of SPV was purified from skin scabs collected from infected sheep. The purified virus preparation also showed positive reaction in AGPT. The absorbance ratio for purified virus band at 260 and 280 nm was found to be 0.93. The protein content was estimated by spectrophotometeric method and diluted to yield 40 µg/50 µl.

The polypeptides of purified virus, resolved by discontinuous buffer system, showed 23 polypeptide bands in 10% acrylamide gel after staining with CBB (Fig 30). The molecular weights of polypeptides were calculated by determining RF value and comparing it with standard protein mw markers run on the same gel. The mw thus calculated ranged between 209 KD to 13 KD (Table 7).

4.10 Immunoblot analysis of capripox viruses

The purified GPV, separated on 12.5% gel and transferred onto PVDF membrane (0.45 µm) by semidry system, revealed 14 immunogenic proteins in western blotting (Table 8). Out of these 8 intensely stained proteins were considered as major immunogenic proteins (Table 8). The molecular weights of these polypeptides were 97 KD, 85 KD, 72 KD, 58 KD, 43 KD, 35 KD 32 KD and 26 KD (Fig 31). The convalescent sera

Fig 30. SDS-PAGE of purified SPV in 10% resolving gel, CBB staining

Lane A - MW markers from top to bottom- 66 KD, 55KD, 36 KD, 29 KD, 24 KD and 20 KD.

Lane B - Purified SPV

Lane C - MW marker from top to bottom 205 KD, 116 KD, [illegible] 97 KD, 84 KD, 66KD, 55 KD,45 KD, 36KD, 29KD and 24 KD

A B C

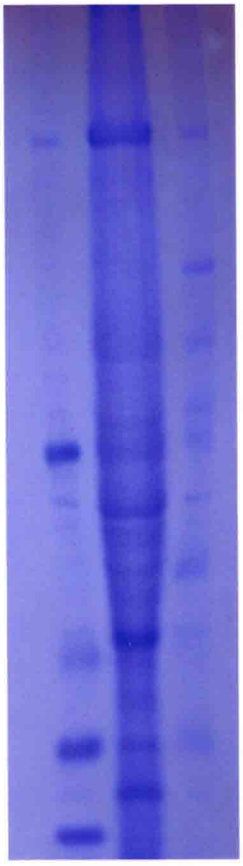


Table 7: Polypeptides of SPV (Jaipur strain) resolved by 10% SDS-PAGE

Virion polypeptide	Approximate molecular weight in K.D.
1	209
2	182
3	158
4	91
5	83
6	71*
7	59*
8	55
9	49
10	47
11	43*
12	39
13	35*
14	30*
15	28
16	24
17	22
18	20
19	19
20	17
21	15
22	14
23	13

* Major immunogenic polypeptides in Western blotting

Fig 31. Western blotting of purified Pantnagar strain of GPV

Lane A - MW markers stained with CBB from top to bottom- 205 KD, 116 KD, 97 KD, 84 KD, 66 KD, 55 KD, 45 KD, 36 KD, 29 KD and 24 KD.

Lane B - Purified GPV polypeptides stained with CBB

Lane C - Immunostained purifed GPV showing 8 major immunogenic polypeptides

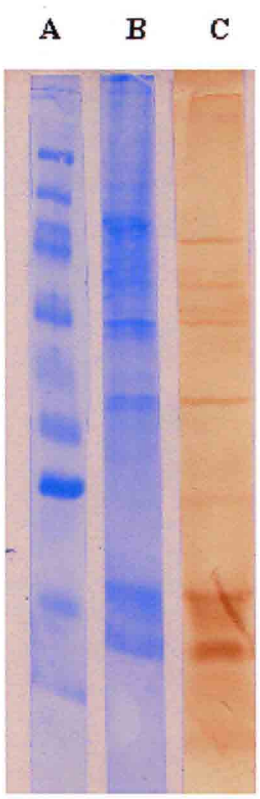

A
B
C

Table 8: Immunogenic polypeptides of GPV (Pantnagar strain) detected by Western blotting

Polypeptide	Molecular weight in KD
1	112
2	97*
3	87
4	85*
5	76
6	72*
7	58*
8	52
9	49
10	43*
11	40
12	35*
13	32*
14	26*

* Major immunogenic polypeptides

collected during field outbreaks of goat pox and after experimental infection of goats with GPV (Pantnagar strain) also revealed similar pattern of immunogenic polypeptides in western blotting (Table 9).

The polypeptides of purified SPV resolved on 12.5% gel was transferred onto PVDF membrane by semidry method. Western blotting using hyperimmune serum against SPV (Jaipur strain) and donkey antisheep HRPO conjugate showed 5 major immunogenic proteins with mw of 71 KD, 59 KD, 43 KD, 35 KD and 30 KD (Table 7).

4.11 Affinity purification of monospecific antibodies and their neutralizing end point titre

Monospecific serum against major immunogenic polypeptides of GPV were purified and tested for their specificity by Western blotting. The neutralizing end point titre of monospecific sera, assayed in secondary KTCC, are presented in Table 10. The polypeptides of 58 KD and 35 KD showed highest neutralizing end point titre of 47 and 45, respectively. The polypeptides of 97 KD, 85 KD, 72 KD and 26 KD showed lower end point titres.

4.12 Immunoblotting of purified core and envelope polypeptides

The purified virus suspension of Pantnagar strain of GPV was separated into core and envelope. Each of the two were then resolved in 12.5% gel and immunoblotted. The immunoblot of these samples revealed that envelope contains major 35 KD protein in association with lower

Fig 32. Western blotting of purified core and envelope of Pantnagar strain of GPV

Lane A - CBB stained MW markers

Lane B - Immunostained purified GPV polypeptides

Lane C - Immunostained purified core polypeptides

Lane D - Immunostained purified envelope Polypeptides

Lane E - CBB stained MW markers

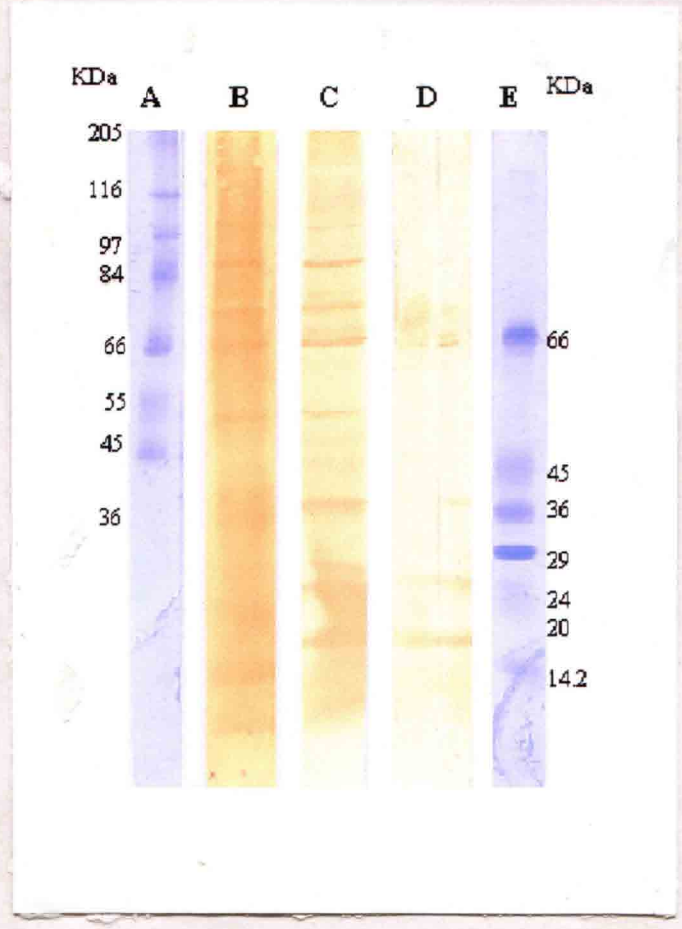

KDa
A
B
C
D
E
KDa
205
116
97
84
66
55
45
36
66
45
36
29
24
20
14.2

Table 9: Comparative evaluation of Western blot and AGPT for detection of GPV specific antibodies

S.No.	Serum samples	Number of samples	Samples positive in	
			AGPT	W.B.
1	Known GPV positive	15	15 (100)	15 (100)
2	Known GPV negative in AGPT	10	-	4 (40)
3	Unknown	25	18 (72)	21 (85)
	Total	**50**	**33 (66)**	**40 (80)**

Table 10: Neutralizing end point titre of major immunogenic proteins of GPV

S. No.	Molecular weight of polypeptides (KD)	eutralizing index	Neutralizing end point titre
1	97	0.67	5
2	85	0.35	2
3	72	0.65	4
4	58	1.67	47
5	43	1.12	13
6	35	1.65	45
7	32	1.57	37
8	26	0.65	4

concentration of 85 KD, 76 KD and 26 KD proteins (Fig 32). The immunoblot of core revealed polypeptide profile similar to those of purified GPV except with very faint band of 35 KD protein.

4.13 Detection of GPV specific proteins in infected KTCC by immunoblotting

The KTCC infected with GPV (Pantnagar strain) were used for detection of virus specific proteins by immunoblotting. The infected KTCC showed virus specific polypeptide after 48 h PI when compared with control. The intensity of virus specific proteins was increased when immunoblots were performed with GPV infected KTCC after 72 h PI. The polypeptides identified were comparable to purified virus of GPV.

The results of immunoblot cross analysis between GPV and SPV are shown in Fig 33. The GPV in infected KTCC was detected by both hyperimmune serum against Pantnagar strain of GPV and Jaipur strain of SPV. Likewise, SPV in lamb testes cell culture was detected by using hyperimmune serum against Jaipur strain of SPV and Pantnagar strain of GPV. The uninfected KTCC and lamb testes cells used as controls, did not show any protein band.

Fig 33. Western blotting of Pantnagar strain of GPV infected cell culture lysates.

Lane A - CBB stained MW markers
Lane B - KTCC lysate infected with GPV and immunostained using hyperimmune serum against SPV.
Lane C - KTCC lysate infected with GPV and immunostained using hyperimmune serum against GPV.
Lane D - LT cell lysate infected with SPV and immunostained using hyperimmune serum against SPV
Lane E - LT cell lysate infected with SPV and immunostained using hyerimmune serum against SPV.
Lane F - Uninfected KTCC cell lysate
Lane G - Uninfected LT cell lysate
Lane H - CBB stained MW markers

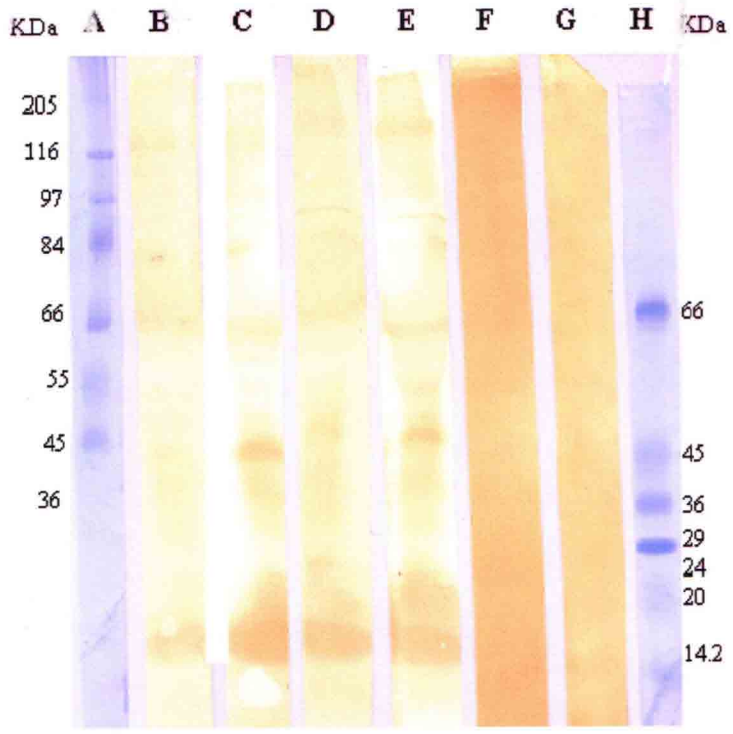

KDa
A
B
C
D
E
F
G
H
KDa
205
116
97
84
66
55
45
36
66
45
36
29
24
20
14.2

Fig 34. Infectivity titres of Pantnagar strain of GPV in cell lines and KTCC

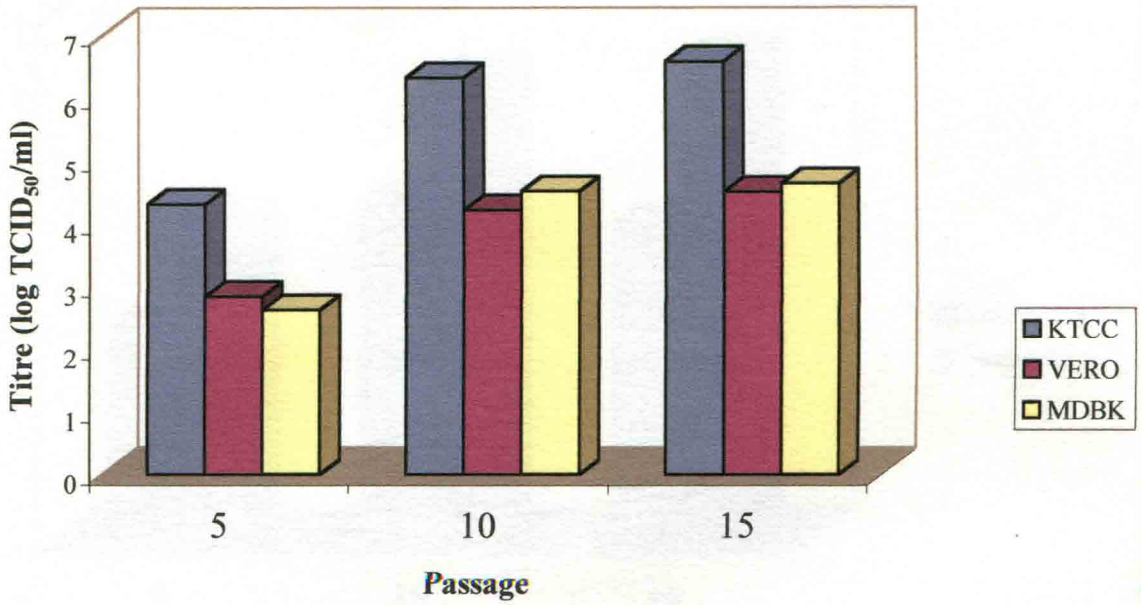


Fig 35. Infectivity titres of Sambalpur strain of GPV in KTCC

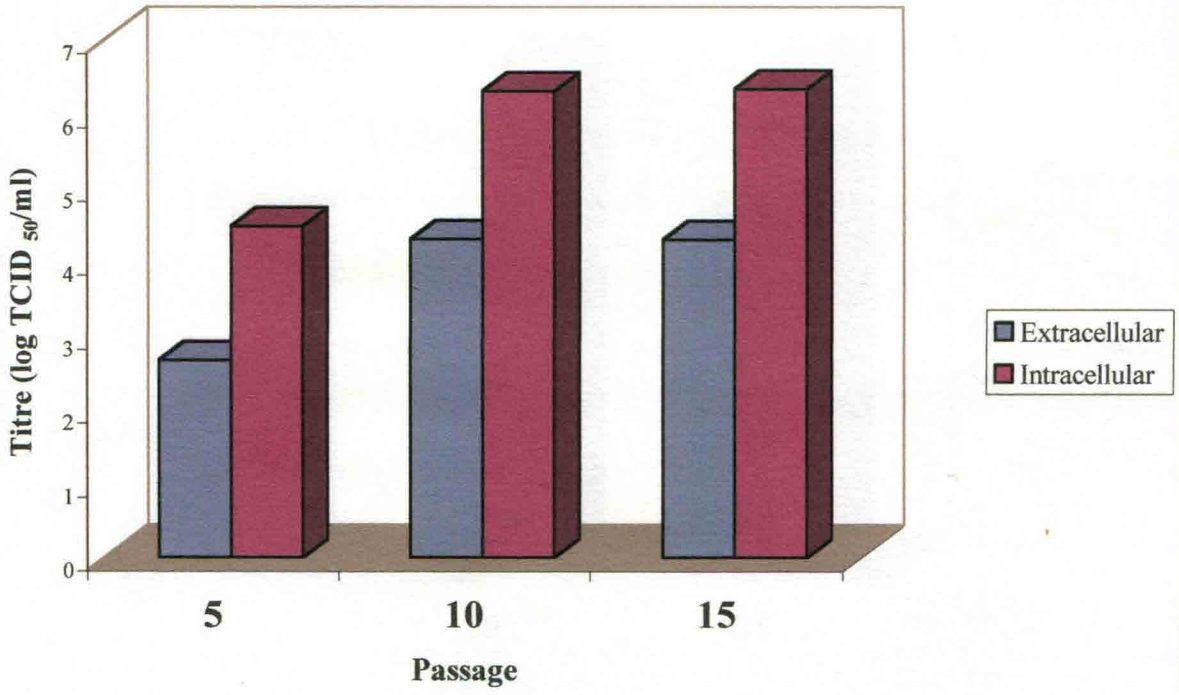


Fig 36. Infectivity titres of Uttarkashi strain of GPV in KTCC

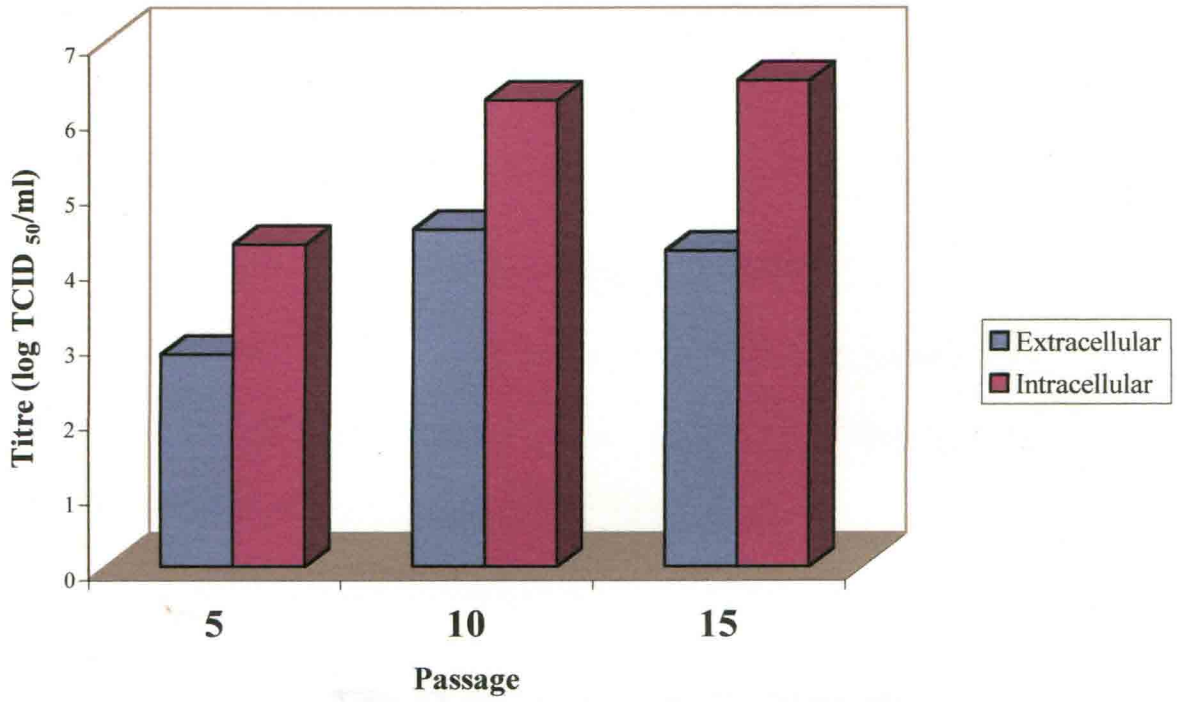
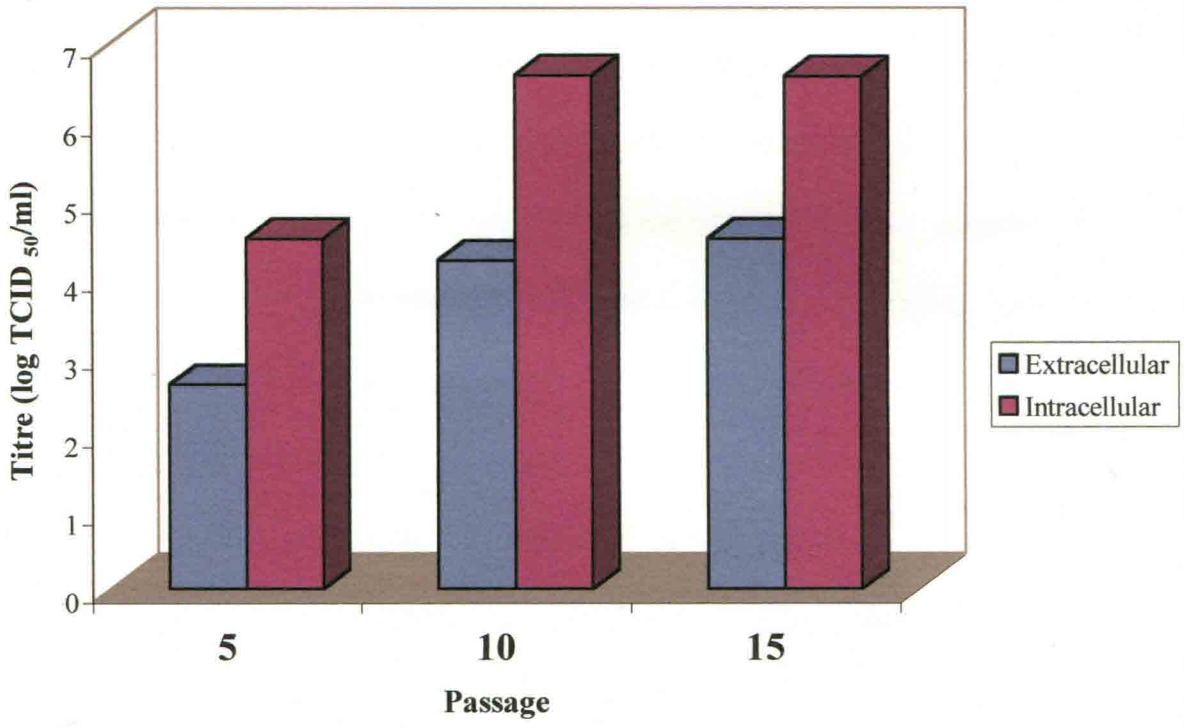


Fig 37. Infectivity titres of Pantnagar strain of GPV in KTCC



DISCUSSION

DISCUSSION

Inspite of competitive limitations, which have arisen from the advantages and wide use of tissue culture and embryonated eggs, natural host is still considered a useful method for studying the clinical manifestations and pathogenesis of animal viruses.

In the present investigation, goats inoculated with GPV (Pantnagar strain) showed development of skin lesions at the site of inoculation that included developmental stages, viz., macules, papules followed by scab formation. However, vesicular stage has not been recorded. The absence of vesicular stage has also been reported in experimental infection of goats with GPV isolated from Orissa (Bandyopadhyay *et al.*, 1984). Kitching (1994) also reported that papules on the skin of animals directly changed into scab with no vesicular or pustular stage. The large vesicles are generally produced by cleavage of necrotic epidermis from underlying inflamed, but viable, dermis (Jubb and Kennedy, 1963). In present study, the absence of vesicles may be due to the low doses of virus used for infection, which results in mild local lesions with only epidermal proliferation. The absence of pustular stage in experimentally inoculated

animals may be due to the use of antibiotics in inoculum and hygienic conditions maintained during the course of infection.

The modern diagnostic laboratories widely use cell culture system for isolating, identifying and propagating viruses and for assaying neutralizing antibodies. It is a relatively homogenous population of cells, free of immunological and humoral influences that affect replication of virus. It can also be used as a model for the comparative cytopathogenicity of various virus strains.

Due to these advantages of cell culture, secondary KTCC, Vero and MDBK cell cultures were used in the present investigation for adaptation and propagation of capripox viruses.

Pantnagar, Uttarkashi and Sambalpur strains of GPV were adapted to KTCC at 7th passage level. The changes reported at this passage level were rounding and aggregation of cells which appeared as bunches of grapes and generally connected by thin cytoplasmic processes. These findings are in agreement with those of Rao and Malik (1982) who reported rounding, retraction and granularity of cells with distinct cell boundaries in KTCC infected with GPV. Shrivastava and Singh (1980) also reported granulation of cytoplasm, rounding and clumping of cells which were connected with cytoplasmic bridges in SPV infected lamb testicular cell culture after 7th passage. The MGG staining of infected KTCC showed

intracytoplasmic inclusion bodies of different shape and size with or without halo at 72 h PI at 3rd passage. Similar types of intracytoplasmic inclusions with distinct halo were also reported in SPV infected kid testes cells (Dubey and Sawhney, 1975; Jassim and Keshavmurthy, 1982). Pandey *et al.* (1969) opined that the halo, seen around inclusions in MGG stained preparation was absent in haematoxylin and eosin stained preparations. It seems that presence of halo is not an essential feature of inclusion bodies and may be an artifact of fixation.

The cytoplasm of infected cells showed vacuolation along with pyknosis, condensation and margination of chromatin in nuclei. Similar type of cytoplasmic and nuclear alterations were earlier reported by Rao and Malik (1982). In the present study, the small granular intracytoplasmic inclusions were also recorded after 9th passage level, which is in agreement with findings of Shrivastava and Singh (1980).

Acridine orange staining of GPV infected KTCC showed viral DNA as a small greenish bodies in the cytoplasm which are mostly elongated or elliptical in shape and situated at perinuclear areas. Similar changes in acridine orange staining of SPV infected lamb testes cells were also recorded by Soman and Singh (1980). The infectivity titre of Pantnagar strain of GPV in KTCC was recorded as $10^{6.57}TCID_{50}$/ml, which is comparable with infectivity titre recorded in GPV infected KTCC (Rao and

Malik, 1982) and SPV infected lamb testes cells (Das and Malik, 1984). However, lower infectivity titre of $10^{4.87}$ $TCID_{50}$/ml was reported by Rao and Malik (1982). This variation in infectivity titre may be due to the use of secondary KTCC for present investigation, which are considered better for propagation of CPV, possibly due to more susceptibility of CPV to fibroblastic than epithelial cells as suggested by Jassim and Keshavmurthy (1982). The extracellular and intracellular titres of GPV (Pantnagar strain) at 15th passage were recorded as $10^{4.49}$ and $10^{6.57}$ $TCID_{50}$/ml, respectively. These results corroborate the findings of Rao and Malik (1982). Shrivastava and Singh (1980) reported that only 3% of virus was released upto day 5 PI and 97% remained cell associated in SPV infected lamb testes cells. Similarly, Carn (1993) reported that vaccines prepared after freezing and thawing of the infected culture that contain majority of intracellular, unenveloped viruses, confer partial immunity against natural infection of CPV because intracellular viruses are immunologically distinct from extracellular enveloped viruses.

The Pantnagar strain of GPV was adapted to Vero cells at 7th passage. The CPE recorded as early as 24 h PI, attained maximum severity by 96-120 h PI. The changes included rounding and clumping of cells with formation of microplaques. Prakash *et al.* (1994) also reported adaptation of Sambalpur strain of GPV in Vero cells at 4th passage with characteristic

changes like clumping of cells alongwith formation of microplaques. Adaptation of SPV in Vero cells at 5th passage level was described by Singh and Rai (1991). The characteristic changes recorded were early appearance of the granularity in cell cytoplasm, rounding of infected cells and development of microplaques. The MGG staining of Vero cells revealed syncytia formation and intracytoplasmic eosinophilic inclusion bodies at juxta nuclear position. These inclusions were multiple and usually circular, elliptical or oval in shape. Similar findings have also been reported by other investigators (Singh and Rai, 1991; Parkash *et al.*, 1994). Acridine orange staining of infected Vero cells showed apple green fluorescence in cell cytoplasm with greenish yellow irregular diffuse masses of variable size with or without halo. Similar type of changes were also recorded in GPV infected Vero cells and kid kidney cells (Joshi, 1993), SPV infected lamb kidney cells (Soman and Singh, 1980) and swine pox infected PK-15 cells (Garg and Mayor, 1973). The infectivity titre of GPV in Vero cells was $10^{4.49}$ $TCID_{50}$/ml at 15th passage level, which is comparable to the infectivity titre of 5 x $10^{5.17}$ $TCID_{50}$/ml as recorded by Joshi (1993). However, higher infectivity titre of $10^{6.3}$ $TCID_{50}$/ml was recorded in SPV infected Vero cells (Singh and Rai 1991). The variation in titres may be due to strain differences and number of passages in Vero cells. The findings of present investigation suggested that Pantnagar strain of GPV is well

adapted to Vero cells which is contrary to the findings of Mirchasmy and Ahourai (1971). This may be due to strain variation.

The Pantnagar strain of GPV adapted to MDBK cells at 4th passage level and produced characteristic CPE at 8th passage level that included granulation of cytoplasm, cytoplasmic vacuolation, formation of syncytia, microplaques and intracytoplasmic eosinophilic inclusions. Few infected cells showed swollen nuclei with margination of chromatin. The acridine orange staining showed greenish fluorescence in cell cytoplasm along with intracytoplasmic inclusions. Joshi *et al.*(1995b) also reported similar observations with Sambalpur strain of GPV in MDBK cells. The infectivity titre $10^{4.60}$ $TCID_{50}$/ml was recorded in GPV infected MDBK cells at 20th passage level, which is comparable to the titre recorded by Joshi *et al.* (1995b).

The KTCC, Vero and MDBK cells infected with Pantnagar strain of GPV showed small areas of cytoplasmic fluorescence in the perinuclear areas of cytoplasm within 24 h PI. The intensity of fluorescence increased with involvement of greater number of cells at 48 h PI. At later stages, diffuse cytoplasmic fluorescence without involvement of nuclei were seen in infected cells. Tantawi and Falluji (1979) also demonstrated GPV specific antigen in lamb testes cell culture at 12 h PI by indirect immunofluorescence. Earlier, FAT was used for localization of intracellular

antigen in GPV infected lamb testes cells (Tantawi *et al.*, 1980), demonstration of swine pox virus (Garg and Mayor, 1973) and fowl pox virus (Tripathy *et al.*, 1970).

Transmission electron microscopy of infected KTCC and Vero cells showed presence of GPV in cytoplasmic matrix. The virion particles were round, oval or elliptical in shape and were more electron dense as compared to cytoplasmic matrix, which is in accordance with observations of Joshi and Garg (1997). Moss (1978) demonstrated two types of pox particles. The electron transparent particles, termed M for mulberry, with a ridged or beaded surface and more electron dense particles, termed C for capsule, exhibiting some internal structure. It appears that in the C particles the integrity of a lipoprotein layer at the surface particle has been destroyed, leading to penetration of the stain. Kitching and Smale (1986) also reported that isolates of sheep pox virus, sheep and goatpox virus and lumpy skin disease virus could not be differentiated on the basis of electron microscopy.

The Pantnagar strain of GPV adapted to KTCC, MDBK and Vero cells showed neutralization indices of 3.91, 2.98 and 2.66, respectively. Rao and Malik (1982) also obtained neutralizing antibody titre of 200 with Poona strain of GPV, using homologous hyperimmune serum assayed in KTCC. Similar findings were also recorded in serum neutralization test

performed for SPV in lamb testicular cells. Sera from animals hyperimmunized with seed virus, extracellular live virus, extracellular inactivated virus and intracellular inactivated virus revealed antibody titre of 80, 80, 80 and 20, respectively, when titrated with extracellular live virus (Shrivastava and Singh, 1980). Singh and Rai (1991) also reported SNI of 4.0 for SPV in Vero cells and confirmed the adaptation of SPV in this cell line.

The Pantnagar strain of GPV was completely inactivated at 70^0C in 10 min. The drop of log 3.13 and 2.63 $TCID_{50}$/ml in infectivity titre were recroded after treatment at 60^0C and 55^0C for 10 min, respectively. These results are in agreement to the earlier findings of Dutta and Soman (1991) who reported that GPV is completely inactivated at 60^0C within 3 min and infectivity titre drops to log 5 after treatment at 55^0C. Similar findings were also recorded by Joshi (2001).

In the present investigation Pantnagar strain of GPV was found to be sensitive to both alkaline and acidic pH. The drop of log 3.95, 2.79 and 1.25 $TCID_{50}$/ml in infectivity titre were recorded when virus suspensions exposed to pH 3, 5 and 8 for 1 h at 37^0C, respectively. These results confirm the earlier report of Joshi (2001), whereas Dutta and Soman (1991) recorded log 5 drop in infectivity titre at pH 3 and log 1 drop at pH 8. The Pantnagar strain of GPV is found to be sensitive to lipid solvent. The drop

of log 4.31 and 3.25 $TCID_{50}$/ml in infectivity titre were recorded after treatment with ether and chloroform, respectively. These corroborate the findings recorded by Tantawi *et al.* (1980) and Joshi (2001).

In the present investigation sheep, chicken, rabbit, mice and buffalo calf were found refractory for infection to GPV infection. Several investigators also failed to transmit the disease to calves (Bakos and Brag, 1957), cows, buffaloes (Besnoit and Robin, 1923; Lall *et al.*, 1947), rabbits (Sharma, 1966; Bandyopadhyay *et al.*, 1984) and fowl (Bakos and Brag, 1957). In contrast there are reports that GPV can be transmitted to sheep (Mohamed *et al.*, 1982; Kitching and Taylor, 1985) monkey, rabbit and calves (Bennett *et al.*, 1944).

The Pantnagar strain of GPV, did not agglutinate the erythrocytes of sheep, goat, pig, rabbit, dog, chicken and human type 'O' cells. These findings are in agreement with the reports that GPV did not haemagglutinate erythrocytes, of chicken, duck, sheep, goat, guinea pig, mice or human type 'O' cells (Davies, 1976; Tantawi *et al.*, 1980; Dutta and Soman, 1991). The absence of haemagglutination property in GPV may be due to nonavailability of specific receptors over the surface of erythrocytes of these species.

Pock lesions produced by Pantnagar strain of GPV on CAM of developing chicken embryos were non-haemorrhagic and about 2 mm in

diameter. Similar findings were also reported by Tantawi *et al.* (1980) who grew Sersenk strain of GPV on CAM with large pocks of 2 to 3 mm in diameter after 3 to 4 days of incubation. However, Ranchi and Mukteshwar strains of GPV could not be adapted to CAM (Sharma *et al.*, 1966; Adlakha *et al.*, 1971; Sharma and Dhanda, 1971 and Dutta and Soman, 1991).Tantawi and Falluji (1979) also reported that Sersenk strain grew easily on CAM, while Iranian and Egyptian strains grew weakly and Dushanbe strain failed to grow on CAM. In the present study, Pantnagar strain produced 2 precipitating lines against homologous serum in AGPT. However, only one precipitating line was recorded when tested with antiserum against Sambalpur and Uttarkashi strain of GPV. Similarly Sersenk strain of GPV also produced two precipitating bands with homologous serum and shared common precipitating lines with reference strain of GPV and SPV (Tantawi *et al.*, 1980). Bhambani and Krishnamurthy (1963) and Sen (1968) reported that convalescent sera from goats recovered from GPV gave two precipitating lines in AGPT. The IFAT performed with Pantnagar strain of GPV produced intense fluorescence with homologous sera, whereas weak fluorescence was recorded with antiserum against Sambalpur and Uttarkashi strains. Similar findings were also recorded with Sersenk strain of GPV, which reacted strongly with homologous antigen and weakly with antigens of Dushanbe

and Romanian strains of GPV (Tantawi *et al.*, 1980). Davies and Otema (1981) established the serological relationship between Kenyan strains of GPV, SPV and LSD using direct and indirect FAT and found that these viruses were identical.

Goats inoculated with Pantnagar strain of GPV showed mean ELISA antibody titre of 3.29 $\log_{10}$ and serum end point titre of 150, which are lower than the ELISA antibody titre of 3.36 $\log_{10}$ and serum end point titre of 212, recorded day 0 of birth of new born kids. This may be due to draining of antibodies from goat to fully developed kids in uterus. Kids born to such immunized goats acquired maternal antibodies and were protected against challenge with Pantnagar strain of GPV. Kitching (1986) also reported that lambs born to sheep previously infected with isolates of capripox from Sudan, India and Nigeria were also protected against challenge with Yeman goat pox. However, passive transfer of sensitized lymphocytes and serum collected from sheep that had recovered from infection with sheep pox could not transfer immunity and skin lesions developed in sheep within 3-4 days post challenge (Prasad *et al.*, 1986).

In the present study, the SPACE test was found to be more sensitive than conventional AGPT for detection of GPV-specific antigen in skin scabs and infected cell culture fluid. The test did not show positive reaction with known negative samples. As this test is based on the principles of

ELISA and haemagglutination test, it has advantage of reading the results without the aid of a microplate ELISA reader and does not require costly reagents.

The KTCC grown Pantnagar strain of GPV was purified by sucrose density gradient. Presence of virus in sharp bands, obtained about 2/3 and 1/3 of the way down, gave one precipitation line in AGPT. The sharpness of band in sucrose gradient is suggestive of purity of virus as described by Joklik (1962). Purity of virus suspension was also checked by absorbance ratio at 260 nm and 280 nm. In the present investigation, this ratio is 0.59, which indicates that virus is purified to good extent by 40-60% sucrose gradient. Hawk *et al.* (1947) also demonstrated purity of virus suspension on the basis of absorbance ratio and speculated that decreased ratio of purified suspension might be due to exclusion of cellular proteins from the virus suspension. Similarly, Kitching *et al.* (1986) also used 40-60% sucrose gradient for purification of virus. The SPV was purified from skin scab after pelleting on 36% sucrose twice and finally, the virus band was recovered by 25-40% sucrose gradient. The purity of virus was evidenced by sharp precipitation line in AGPT and low (0.93) ratio of OD at 260 and 280 nm. Similar concentration of sucrose gradient (25-40%) was also used by Joklik (1962) for purification of four strains of pox virus from CAM of

developing chicken embryos. Chand *et al.* (1994) also purified CPD virus from skin scab in 25-50% sodium diatrizoate composite gradient.

By SDS-PAGE, the MW of polypeptides may be determined with an accuracy of atleast ± 10% (Weber and Osborn,1969). In the present investigation purified GPV revealed 53 polypeptides in 10% resolving gel and 43 in 12.5% gel after CBB staining. In total, 55 polypeptides ranging from 209 KD to 15 KD were recorded. These observations are supported by the demonstration of 56 polypeptides in purified vaccinia virus in 11% acrylamide gel. The number of polypeptides were further increased to 111 by two dimensional (2D) gel electrophoresis (Essani and Dales, 1979). The 28 and 31 polypeptides of Fowl pox and vaccinia virus, respectively, were resolved in 10% separating gel by discontinuous buffer system (Obijeski and Palmer, 1973). However, only 18 polypeptides ranging from 15 KD to 140 KD in 7.5 and 10% separating gel were recorded by Singh and Rai (1991). The variation in number of polypeptides in SDS-PAGE were dependent upon various physical conditions.

The Western blot analysis of proteins of purified GPV separated on 12.5% gel showed 14 immunogenic polypeptides. Out of these, 8 polypeptides, which stained more intensely, were considered as major immunogenic polypeptides. Bhatt (1993) also reported 14 immunogenic polypeptides ranging from 18 KD to 83 KD in capripox virus and out of

these, 5-6 were found major immunogen. In the present study, SPV also revealed 5 major immunogenic proteins ranging from 30 KD to 71 KD. Also there was no significant difference among immunogenic polypeptides of GPV and SPV. Therefore, both viruses can not be differentiated on the basis of protein profile.

Sambrook *et al.* (1989) reported that the Western blotting is extremely useful for identification and quantitation of specific proteins in a complex mixture of proteins that are not radiolabeled. The technique is almost as sensitive as standard solid phase radioimmunoassay. Further more, because electrophoretic separation of proteins is always carried out under denaturing conditions, the problems of solublization, aggregation and coprecipitation of target proteins with adventitious proteins are eliminated.

The monospecific sera against 8 major immunogenic proteins were separated and tested by Western blot for its specificity. Maa and Esteban (1987) also selected monospecific antibodies from Lambda gt 11 recombinant phage using NC membranes. Olmsted (1981) described in detail about affinity purification of antibodies using protein samples that have been electrophoretically transferred to diazotized paper and concluded that elution depends on affinity of antibody for antigen and altered time of incubation with glycine HCl. The technique is based on principle that antibody retain their binding properties after removal from conventional

affinity column by pH shock. Similarly, proteins of blots were shaken with glycine HCl buffer for few minutes and then reequiliberated to maintain the pH for purification of antibodies.

The monospecific sera against polypeptides of MW 32 KD, 35 KD and 58 KD were found to neutralize the corresponding antigen however, monospecific sera against 97 KD, 85 KD, 72 KD and 26 KD polypeptides failed to produce sufficient neutralizing end point titres. Similar findings were also recorded with SPV polypeptides. The protein of mw 64-67 KD induced serum neutralizing antibody in rabbits with an end point titre of 128 and 47 KD polypeptide induced SN antibody with an end point titre of 16, while 44 KD, 30 KD and 22 KD polypeptides failed to induce SN antibody (Singh and Rai, 1991). However, the criteria for selection of these polypeptides for study of their neutralizing activity was not clearly mentioned. Chand (1992) reported that 32 KD envelope protein of capripox also induces neutralizing antibodies in rabbit. The 14 KD envelope protein of vaccinia virus also produced a high level of virus neutralizing antibodies in mice and showed protection when challenged with lethal doses of wild type vaccinia virus (Lai *et al.*, 1991).

In order to identify the protein of GPV envelope, the purified virions were treated with detergent and 2 mercaptoethanol and by centrifugation, core and envelope fractions were separated and examined by Western

blotting. This experiment was undertaken with the aim that proteins on envelope play major role in host immune response. Therefore, their identification and characterization is of immense value for development of vaccine and diagnostics. Atleast 4 major immunogenic proteins were found to be associated with virus envelope in the present study, which corroborate the earlier findings that 6 polypeptides were released when vaccinia virions were treated with anionic detergent and reducing agents (Moss, 1978).

Western blot technique was standardized for detection of small amount of CPV proteins in infected KTCC by probing with specific antibodies and other reagents. The technique identified the GPV polypeptides in cell lysate of infected KTCC as early as 48 h PI. This method can be exploited for demonstration of GPV in field specimen. Similarly, adenovirus infected KB cells, electrophoretically transferred on diazo paper followed by immunoradiography demonstrated the presence of virus specific antigen (Symington *et al.*, 1981). The murine leukemia virus antigen was also demonstrated in complex cellular lysate by electrophorectically transferring them on nitrocellulose membrane followed by detection with antibody and radioiodinated protein A (Burnette, 1981).

The findings of present investigation concludes that Pantnagar strain of GPV, isolated from field outbreak, is adapted to secondary KTCC as

well as cell lines. The virus strains conferred protection in new born kids upto 21 days. The immunogenic protein profile of Pantnagar strain of GPV is indistinguishable from Jaipur strain of SPV. The monospecific sera against 58 KD and 35 KD polypeptides of purified GPV showed highest neutralizing end point titres. The Western blot can be used for the detection of capripox specific antigen in testes cell lysates.

SUMMARY

SUMMARY

The capripox, a severe pox disease of sheep, goats and cattle, is associated with serious economic consequences in terms of mortality, loss of productivity, hide damage and loss of revenue associated with trade restrictions to a disease-free country.

The experimental infection of goats with Pantnagar strain of GPV showed development of skin lesions at the site of inoculation that included developmental stages, viz., macules and papules followed by scab formation. The development of papules was associated with rhinitis, conjunctivitis and enlargement of prescapular lymph node. The secondary lesions were observed 10 days after the primary lesions and involved the entire skin of body especially on teats, nose, lips, eyes and ears.

The GPV strains, viz., Pantnagar, Uttarkashi and Sambalpur, were adapted to KTCC at 2nd passage level. The changes observed were rounding and aggregation of cells. These aggregates of cells appeared as bunches of grapes generally connected by thin cytoplasmic processes. The MGG staining of infected KTCC showed intracytoplasmic inclusion bodies of different shape and size with or without a halo 72 h PI at 3rd passage. The

cytoplasm of infected cells showed vacuolation along with pyknosis, condensation and margination of chromatin in the nuclei. The AO staining of GPV infected cells showed viral DNA as small greenish bodies in the cytoplasm, mostly elongated or elliptical in shape and situated at perinuclear areas. The infectivity titre of $10^{6.57}$ $TCID_{50}$/ml was recorded at 15^{th} passage level. The extracellular and intracellular titres of Pantnagar strain of GPV were $10^{4.49}$ and $10^{6.57}$ $TCID_{50}$/ml, respectively, at 15^{th} passage level.

The Pantnagar strain of GPV was adapted to Vero cells after 7^{th} passage and produced CPE characterized by rounding and clumping of cells and formation of microplaques. The MGG staining of Vero cells revealed syncytia formation and intracytoplasmic eosnophilic inclusion bodies at juxta nuclear position. A.O. staining of infected Vero cells showed greenish yellow and irregular diffuse masses of variable size with or without halo. The GPV (Pantnagar strain) infected Vero cells showed infectivity titre of $10^{4.49}$ $TCID_{50}$/ml at 15^{th} passage.

The Pantnagar strain of GPV adapted to MDBK cells produced granulation and vacuolation of cytoplasm, formation of syncytia, microplaques and intracytoplasmic inclusions. A.O. staining showed greenish fluorescence in the cell cytoplasm along with intracytoplasmic

inclusions. The infectivity titre of $10^{4.63}$ $TCID_{50}$/ml was recorded at 15th passage.

The KTCC, Vero and MDBK cells, infected with Pantnagar strain of GPV, showed small areas of fluorescence in the cell cytoplasm. The intensity of cytoplasmic fluorescence increased and involved more number of cells 48 h PI at 3rd passage level.

The electron microscopic studies of KTCC and Vero cells infected with Pantnagar strain of GPV showed presence of intracytoplasmic virus particles in the cytoplasmic matrix. The virion particles, which were more electron dense than matrix had a distinct round, oval or elliptical shape.

The serum neutralization of Pantnagar strain of GPV adapted to KTCC, MDBK and Vero cells showed indices of 3.91, 2.98 and 2.66, respectively.

The Pantnagar strain of GPV was inactivated completely within 10 min at 70^0 C. A drop of log 3.13 and 2.63 were recorded at 60^0 and 55^0 C for 10 min, respectively. The drop of log 3.95, 2.79 and 1.25 in infectivity titre after exposure to pH 3, 5 and 8 for 1 h at 37^0 C, respectively, were recorded. The Pantnagar strain of GPV was found sensitive to lipid solvents with a drop of log 4.31 and 3.25 in infectivity titres after exposure to ether and chloroform, respectively. The sheep, chicken, rabbit, mice and buffalo calf were found to be refractory to infection. The Pantnagar strain did not

agglutinate the erythrocytes of sheep, goat, pig, rabbit, dog, chicken and human type 'O' cells.

The pock lesions produced on CAM of developing chicken embryos were non-haemorrhagic and about 2 mm in diameter. The strain produced 2 precipitation bands in AGPT with homologous sera and 1 precipitation band with sera against Sambalpur and Uttarkashi strains of GPV.

The maternal antibodies protected the kids born to immunized goats when challenged with GPV at the age of 21 days and did not show clinical signs of disease upto 60 days PI.

The SPACE test detected GPV specific antigen in skin scab and tissue culture fluids. The test was found to be more sensitive than AGPT. The Pantnagar strain of GPV, grown in secondary KTCC, was purified by sucrose density gradient. The polypeptides of purified virus were resolved by SDS-PAGE using discontinuous buffer system. In 10% and 12.5% acrylamide gels, the purified virus yielded 53 and 43 polypeptides, respectively. A total of 55 polypeptides, ranging from 209 KD to 15 KD, were resolved.

The Jaipur strain of SPV was purified from skin scabs collected from infected sheep. The polypeptides of purified virus, resolved by discontinuous buffer system, showed 23 polypeptide bands in 10%

acrylamide gel after staining with CBB. The molecular weights of polypeptides ranged between 209 to 13 KD.

Immunoblot analysis of purified GPV revealed 14 immunogenic proteins. Out of these, 8 intensely stained proteins were considered as major immunogenic protein. The MW of these polypeptides were 97 KD, 85 KD, 72 KD, 58 KD, 43 KD, 35 KD, 32 KD and 26 KD. The Western blotting of purified SPV showed 5 major immunogenic proteins with MW of 71 KD, 59 KD, 43 KD, 35 KD and 30 KD.

The monospecific sera against major immunogenic polypeptides of GPV showed neutralizing end point titres of 47 and 45 with 58 KD and 35 KD polypeptides, respectively. The polypeptides of 97 KD, 85 KD, 72 KD and 26 KD showed lower end point titres. The purified virus suspension of Pantnagar strain of GPV was separated into core and envelope. The immunoblot revealed that envelope contains major 35 KD protein in association with lower concentration of 85 KD, 76 KD and 26 KD proteins. KTCC infected with Pantnagar strain of GPV showed virus specific polypeptides 48 h PI by immunoblotting. The intensity of virus specific proteins was increased when immunoblots were performed with GPV infected KTCC after 72 h PI.

LITERATURE CITED

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Abdussalam, M. 1957. Contagious pustular dermatitis III. Experimental infection. *Journal of Comperative Pathology.* **67**: 305-319.

Abo-Shehada, M.M. 1990. Vaccine-induced goat pox in an imported Sanen goat flock in Jordan. *Preventive Veterinary Medicine.* **9**: 159-161.

Achour, H.A. and Bongnedour, R. 1999, Epidemiology of sheep pox in Algeria. *Revue-Scientifique-et-Technique-Office-Inter-national-des-Epizootics.* **18**(3): 606-17.

Adlakha, S.C.; Bansal, M.P. and Malik, B.S. 1971. Studies on sheep and goat pox viruses. Adaptation of the viruses in laboratory animals and tissue culture. *Indian Journal of Animal Sciences.* **41**: 171-175.

Altinsaat, M.S. and Milli, , U.H. 1993. Light and electron microscopic studies on strain lesions in naturally occurring sheep pox. *Doga, -Turk-Veterinerlik-ve-Hayvancilik-Dergisi.* **17** (2): 97-103.

Ammar, K.M.; Al-Gabbary, M.H.; Abou-Rawash, A.A.; Foad, F.M.; El-Amrousi, S.A. and Youssef, M.S. 1999. Clinical, Epidemiological and histopathological studies on sheep pox in some in Egypt. *In*: Proceedings of 5th Scientific Congress Egyptian Society for Cattle disease, Egypt. 60-72.

Andrews, C.H. and Horstmann, D.M. 1949. The susceptibility of viruses to diethyl ether. *Journal of General Microbiology.* **3**: 290.

Arya, S.N.; Mathur, D.C. and Bhatia, D.K. 1998. Disease profile of sheep and goats in Tiruchirapalli district of Tamilnadu. *Indian Journal of Animal Sciences.* **68**(II): 1103-1105.

Asagba, M.O. and Obi, T.U. 1986. Isolation and identification of goat pox virus in Nigeria. *In*: Bulletin of Animal Health and Production in Africa. **34**: 35-37.

Bakos, K. and Brag, S. 1957. Studies of goat pox in Sweden. *Nordisk Veterinar Medicin* **9:** 431-449.

Bandyopadhyay, S.K.; Gajendragad, M.R.; Dhal, N.K.; Gupta, A.R. and Yadav, M.P. 1984. Some observations on a goat-pox virus isolated from an outbreak in Orissa. *Indian Journal of Animal Science*. **54**: 961-964.

Bansal, R.P. 1996. Techniques in diagnostic virology. Indian Council of Agricultural Research, New Delhi.

Batta, M.M.; Katoch, R.C.; Sharma, M.; Joshi, V.B. and Nagal, K.B. 1999. Epidemiological observation on goat pox in Himachal Pradesh. *Indian Veterinary Journal.* **76**(8): 683-684.

Bennett, S.C.J.; Horgan, E.S. and Haseeb, M.A. 1944. The pox diseases of sheep and goats. *Journal of Comparative Pathology*. **54**: 131-159.

Besnoit, C. and Robin, V. 1923. Variola in the goat. *Revue Veterinaire*. **75**: 685.

Bhambani, B.D. and Krishnamurty, D. 1963. An immuno-diffusion test for laboratory diagnosis of sheep pox and goat pox. *Journal of Comparative Pathology*. **73**: 349-357.

Bhatt, P.P. 1993. Molecular characterization of pox (viral) DNA. *Indian Journal of Animal Science*. **63**(8): 857-862.

Bhatt, P.P.; Bhat, P.N.; Mishra, B.P.; Kumar, S. and Singh, R.K. 1993. A comparison of the genomes of goat, sheep and buffalo pox virus isolates. *Indian Journal of Animal Sciences*. **63**(10): 1057-1078.

Bhatt, P.P. 1998. Genome mapping of viruses-A practical manual. Indian Council of Agricultural Research, New Delhi.

Bhowmik, M.K.; Ghosh, R.K.; Bhattacharya, B.; Chakraborty, A.K. 1986. Goat pox in West Bengal. *Indian Journal of Animal Health*. **25**: 15-18.

Bidjeh, K; Ganda, K.; Diguimbaye, C. and Idriss, A. 1990. Note on goat pox in chad: study of outbreaks. *Revue-d'Elevage-et-Medicine-Veterinaire-des-Pays-Tropicaux.* **43**(1):31-33.

Bidjeh, K.; Gand, K. and Diguimbaye,C. 1991. Vaxiole caprine an Tchad: elude du pouvoir pathogene du virus chez les ovins el les caprins. *Rev Elev Med Vet Pays Trop.* **44** (1): 33-36.

Blanc, G.; Melanidi, C. and Stylianopoulo, M. 1929. Variola in goat in Greece. *Archives de l'Institut Pasteur Hellenique.* **2**: 129.

Boulter, E.A. ; Zwartouw, H.T.; Titmuss, D.H.J. and Maber, H.B. 1971. The nature of immune state produced by inactivated vaccinia virus in rabbits. *American Journal of Epidemiology* **94**: 612-620.

Boulter, E.A. and Appleyard, G. 1973. Differences between extracellular and intracellular forms of poxvirus and their implications. *Prog. Med. Virol.* **16**: 86-108.

Burnette, W. Neal 1981. "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry.* **112**: 195-203.

Canic, R. 1935. Vezivanje komplementa ovojih boginja. *Vet. Glasn.* **15**: 419-23.

Cao, J.X.; Greshon, P.D. and Black, D.N. 1995. Sequence analysis of Hind III Q2 fragment of capripox virus reveals a putative gene encoding a G-protein-coupled chemokine receptor homologue. *Virology.* **209**(1): 207-212.

Carn, V.M. 1993. Control of capripox virus infections. *Vaccine.* **11**(13): 1275-1279.

Chand, P. 1983. Serological studies on sheep pox virus M.V.Sc. thesis submitted to G.B.P.U. A & T., Pantnagar, U.P. India.

Chand, P. 1992. Molecular and Immunological characterization of a major envelope protein of capripox virus. Ph.D. thesis, Univeristy of Surrey.

Chand, P.; Kitching, R.P. and Black, D.N. 1994. Western blot analysis of virus specific antibody responses for capripox and contagious pustular dermatitis viral infections in sheep. *Epidemiology and Infection.* **113**: 377-385.

Chandra, R.; Rao, V.D.P. and Garg, S.K. 1996. Plaque formation by Karnal strain of sheep pox virus adapted to lamb kidney cell culture. *Indian Veterinary Journal* **73**: 607-10.

Chandrashekhar, P. 1994. Studies on Immuno modulatory effect of MDP; Poly I: C and Interferon gamma in chicks immunized with IBD vaccine. M.V.Sc. thesis, IVRI, Izatnagar.

Cunningham, C.H. 1960. A Laboratory Manual. Burgess Publishing Co. Minneapolis, USA.

Daoud, J.A.H. 1997. Sheep pox among Australian sheep in Jordan. *Tropical Animal Health and Production.* **29**(4): 281-282.

Das, S.K.; Pandey, A.K.; Mallick, B.B. 1978. A note on the natural goat pox outbreak in Garhwal hills of Uttar Pradesh. *Indian Veterinary Journal.* **55**: 671-673.

Das, S.K. and Mallick, B.B. 1984. Susceptibility of various cell culture system to sheep pox virus strain. *Indian Journal of Experimental Biology.* **22** : 205-208.

Davies, F.G. 1976. Characteristics of a virus causing a pox disease in sheep and goats in Kenya, with observations on the epidemiology and control. *Journal of Hygiene*. **76**: 163-170.

Davies, F.G. and Otema, C. 1978. The antibody response in sheep infected with a Kenya sheep and goat pox virus. *Journal of Comparative Pathology*. **88**: 205-210.

Davies, F.G. and Otema, C. 1981. Relationship of capripox viruses found in Kenya with two Middle Eastern strains and some orthopox viruses. *Research in Veterinary Sciences*. **31**: 253-255.

Dubey, S.C. and Sawhney, A.N. 1975. Propagation of goat-pox virus in monolayer cultures of embryonic caprine lung. *Indian Journal of Experimental Biology*. **13**: 304-305.

Dubey, S.C. and Sawhney, A.N. 1979. Serological relationship between viruses of goat-pox and contagious pustular dermatitis. *Indian Journal of Animal Science*. **49**: 135-139.

Dubey, S.C.; Srivastava, C.P.; Maru, A. and Lonkar, P.S. 1987. Some observations on sheep pox outbreaks in vaccinated flocks at an

organised farm. *Indian Journal of Comperative Microbiology, Immunology and Infectious Diseases.* **15**: 836-840.

Dutta, S. and Soman, J.P. 1991. Host range and physico-chemical characterization of "Ranchi" strain of goat pox virus. *Indian Journal of Animal Sciences.* **61**: 955-957.

El-Zein, A.; Mehme, S. and Singh, K.V. 1983. Preparation and testing of a goat pox vaccine from a pathogenic field isolate attenuated in cell culture. *Zentalblatt fur Veterinarmedizin.* **30B**: 341-348.

Essani, K. and Dales, S. 1979. Biogenesis of vaccinia: Evidence of more than 100 polypeptides in the virion. *Virology.* **95**: 385-394.

FAO.1998. FAO STAT Agriculture Data.

Feldman, H.A. and Wang, S.S. 1961. Sensitivity to various viruses to chloroform. *Proc. Sco. Exp. Biol. Med.* **106**: 736.

Ferreira, C. 1973. Compartmento do virus da variola ovina sob paccao de alguns agentes fisicos e quinicos. *Anais Esc. Med. Vet. Lisboa.* **15**; 7-40.

Ganju, A.K.; Shukla, D.C. and Chawla, S.K. 1977. Replication and cellular reaction of certain DNA and RNA viruses in established cell lines. *Indian Journal of Animal Science.* **47**: 582-586.

Garg, S.K. and Mayor, R.C. 1973. Studies on swine pox virus: fluorescent and light microscopy of infected cell culture. *Research in Veterinary Sciences.* **14**: 218-219.

Garner, M.G.; Sawarker, S.D.; Brett, E.K.; Edwards, J.R.; Kulkarni, V.B.; Boyle, D.B. and Singh, S.N. 2000. The extent and impact of sheep pox and goat pox in the state of Maharastra, India. *Tropical Animal Health and Production.* **32**: 205-223.

Geering, W.A.; Forman, A.J. and Nunn, M.J. 1995. Exotic diseases of animals. A field guide for Australian Veterinarians, Australian Government Publishing Service, Canberra.

Gerlach, P. 1933. Contagious diseases of animals in Turkey and their control. *Wiener Lierarztliche Monatsschrift.* **20**: 193 and 241.

Greshon, D.D. and Black, D.N. 1988. A comparison of the genome of capripox virus isolates of sheep, goats and cattle. *Virology*. **164**: 341-349.

Greshon, P.D.; Kitching, R.P.; Hammond, J.M. and Black, D.N. 1989. Pox virus genetic recombination during natural virus transmission. *Journal of General Virology*. **70**: 485-489.

Grimpret, J. 1938. An outbreak of goat pox in Moracco. *Recueil de medecine veterinaire exotique*. **11**: 105-112.

Hajer, I.; Abbas, B. and Samra, M.T.A. 1988. Capripox virus in sheep and goats in Sudan. *Revu d' Elevage et de Medicine Veterinaire des Pays Tropicaux*. **41**: 125-128.

Hansen, 1879. Cited by Rafyi and Ramyar, 1959.

Hawk, P.B.; Oser, B.L. and Summerson, W.H. 1947. Blood analysis colorimetery and photometry. *In*: Practical Physiological Chemistry. pp. 450-608.

Hudson, L. and Hay, F.C. 1980. Practical Immunology. Black well scientific publication. Oxford. London.

Hutyra, F.; Marek, J. and Manninger, R. 1946. Special pathology and therapeutics of the disease of domestic animals. 5th Ed. Bailliere, Tindall and Cox London. pp 353-366.

Indian Council of Agricultural Research 1998. All India Coordinated Research Project on Development of a System of Monitoring Surveillance and Forecasting of some Important Animal Diseases. Annual Report. pp. 97.

Jassim, F. A.; Keshvamurthy, B.S. 1982. Propagation of sheep pox virus in cell culture. *Acta Virologica*. **26**: 190-192.

Joklik, W.K. 1962. The purification of four strains of pox virus. *Virology*.**18**: 9-18.

Joshi, R.K. 1988. Immunological characterization of goat pox virus. M.V.Sc. Thesis submitted to G.B.Pant University of Agriculture and Technology, Pantnagar, Nainital, U.P., India.

Joshi, R.K.; Chandra, R.; Rao, V.D.P. and Garg, S.K. 1991. Passive haemagglutination test with goat pox virus. *Indian Journal of Veterinary Medicine*. **11**: 56-57.

Joshi, R.K. 1993. Immunological characterization of goatpox virus. Ph.D. thesis, G.B.Pant University of Agri. Tech. , Pantnagar.

Joshi, R.K.; Chandra, R. ; Rao, V.D.P. and Garg, S.K. 1995a. Cytopathogenicity of goat pox virus. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases.* **15**: 35-36.

Joshi, R.K.; Garg, S.K.; Chandra, R. and Sharma, V.D. 1995b. Growth and cytopathogenecity of goatpox virus in MDBK cell line. *Indian Journal of Virology*. **11**(: 31-33.

Joshi, R.K. and Garg, S.K. 1997. The morphology and morphogenesis of goat pox virus isolate. *Indian Journal of Virology.* **13**: 121-123.

Joshi, R.K.; Ali, S.L.; Shakya, S. and Rao, V.N. 1999. Clinico epidemiological studies on a natural outbreak of goatpox in Madhya Pradesh. *Indian Veterinary Journal.* **76** (. . 279-281.

Joshi, R.K. 2001. Physico-chemical characterization of 'Durg' isolate of goatpox virus. *Indian Veterinary Journal.* **78**: 369-370.

Jubb, K.V.F. and Kennedy, D.C. 1963. Pathology of domestic animals Vol II. Academic press, New York. pp 525-526.

Kalpana, G.; Krishnamurthy, G.V. and Raghvan, R. 1995. Antigenic relationship between the goatpox virus and Kenyan sheep and goat pox virus. *Indian Veterinary Journal.* **72**: 675-679.

Karim, M.A. 1983. Pox among Sannen goats in Iraq. *Tropical Animal health and production.* **15**: 62.

Kataria, A.K. and Sharma, K.N. 1992. A note on natural outbreak of sheep pox in Churu (Rajasthan). *Indian Journal of Animal Health.* **31**(2):165.

Katiyar, M.C. and Soman, J.P. 1986. Cytopathogenic studies of goat pox virus propagated in cell culture. *Indian Journal of Virology.* **2**: 33-40.

Katiyar, M.C. and Soman, J.P. 1987. Studies on adaptation of goat pox virus in goat kidney cell cultures. *Indian Veterinary Journal.* **64**: 261-265.

Kirubaharan, J.J.; Sugirtha, A.P.G. and Padmanaban, V.D. 1993. Titeration of sheep pox virus in BHK-21 cell line. *Indian Veterinary Journal.* **70** (11): 1073-1074.

Kitching, R.P. and Taylor, W.P. 1985. Clinical and antigenic relationship between isolates of sheep and goat pox viruses. *Tropical Animal Health and Production.* **17**: 64-74.

Kitching, R.P. 1986. Passive protection of sheep against capripox virus. *Research in Veterinary Science.* **41**: 247-250.

Kitching, R.P. and Smale, C. 1986. Comparison of the external dimensions of capripox isolates. *Research in Veterinary Science.* **41**: 425-427.

Kitching, R.P.; McGrane, J.J. and Taylor, W.P. 1986a. Capripox in the Yemen Arab Republic and the sultanate of Oman. *Tropical Animal Health and Production.* **18**: 115-122.

Kitching, R.P.; Hammond, J.M. and Black, D.N. 1986b. Studies on the major common precipitating antigen of capripox virus. *Journal of General Virology.* **67**: 139-148.

Kitching, R.P.; McGrane, J.J.; Hammond, J.M.; Miah, A.H.; Mustafa, A.H.M. and Majumder, J.R. 1987a. Capripox in Bangladesh. *Tropical Animal Health and Production.* **19**: 203-208.

Kitching, R.P.; Hammond, J.M. and Taylor, W.P. 1987b. A single vaccine for the control of capripox infection in sheep and goats. *Research in Veterinary Science.* **42**: 53-60.

Kitching, R.P. 1994. Sheep and goat pox viruses. In: Encyclopedia of Virology. Vol. 3. Academic Press, New Delhi. pp. 1160-65.

Koylu, A. and Nitzschke, E. 1968. Characteristics of sheep and goat pox viruses from Turkey. I. Studies on thermostability. *Pendik Veteriner Kontrol VE Arasitma Enstitusu Dergi Istanbul.* **1**: 57-66.

Laemmli, U.K. 1970. Cleavage of structural proteins dueing the assembly of the head of bacteriophage T4. *Nature*. **227**: 680.

Lai, C.; Gong, S. and Esteban, M. 1991. *Journal of Virology*. **48**: 135-47.Cited by Chand, P.).

Lall, H.K.; Singh, G. and Singh, J. 1947. An outbreak of goat pox in Hissar district (Punjab). *Indian Journal of Veterinary Science*. **17**: 243-246.

Lee, Chris; Andrew Levin and Branton, Daniel 1987. Copper staining: A five minute protein stain for sodium dodecyl sulphate-polyacrylamide gels. *Analytical Biochemistry*. **166**: 308-312.

Levant, 1934. Report of the Army Veterinary Research Laboratory, Levent for 1933. *Revue veterinarire militaire*. **18**: 127.

Lodh, C.; Chakrabarti, A. and Mukhopadhyay, S. 1993. Factors affecting kid mortality in West Bengal. *Indian Veterinary Journal*. **70** (1): 48-50.

Maa, J.S. and Esteban, Mariano 1987. Structural and functional studies of a 39,000-Mr Immunodominant Protein of vaccinia virus. *Journal of Virology*. **61** (12): 3910-3919.

Maeda-Machang and Kasonta, J.S. 1996. A study of the epidemiological factors and cross immunity of pox diseases in goat pox and orf in domestic ruminants in Tanzania. Proceedings for Sci. and Tech. Tazania 32-36.

Maiga, S. and Sarr, J. 1992. Epidemiological survey of the main respiratory viruses of small ruminants in Mali. *Revue-d' Elevage-et-de Medecine-Veterinaire-des-Pays-Tropicaux*. **45** (1): 15-17.

Maity, S.; Chatterjee, A.; Mitra, K.; Chakraborty, S.; Nag, N.C. and Ganguly, J.L. 1997. Studies on adaptation of goatpox virus (Uttarkashi strain) in vero cells. *Indian Veterinary Journal*. **74**(8): 700-702.

Malik, Y.; Chand, P. and Batra, S.K. 1998. Isolation and identification of a virus from a severe outbreak of pox in sheep. *Indian Journal of Animal Sciences*. **68**(12): 1207-1209.

Malik, Y. 1999. Clinicopathological studies in lamb inoculated with virulent isolate of sheep pox virus. *Indian J. of Animal Research.* **33**(1): 19-22.

Mallick, S.G. 1988. A preliminary study on the epidemiological aspect os sheep pox in some organized farm in India. *Indian Journal of Comperative Microbiology, Immunology and Infectious Disease.* **9**: 186-95.

Martin, W.B.; Ergin, H. and Koyla, A. 1973. Test on sheep of attenuated sheep pox vaccines. *Research in Veterinary Sciences.* **14**: 53.

Mathew, T. 1975. Immunological studies on pox group of viruses with special reference to buffalo pox virus. *Indian Veterinary Journal.* **52**: 517-525.

Mayor, H.D. and Diwan, A.R. 1961. Studies on the acridine orange staining of two purified RNA viruses: polio virus and tobacco masaic virus. *Virology.* **14**: 74-82.

Melanidi, C. and Tzortzaki, N. 1936. Goat pox. *3rd Intgernational Congress of Comparative pathology. . I. Veterinary Section.* 182-193.

Merchant, D.J.; Khan, R.H. and Murphy, W. Jr. 1960. Hand book of cell and organ culture. Burgers and Pub. New York.

Milli, U.H. and Alcigir, G. and Altinssat, M.S. 1991. Light and electron microscope studies on the pulmonary lesions in sheep pox. Veteriner-Fakultesi-Dergisi,- Universitesi-Ankara. **38**: 138-163.

Mirchasmy, H. and Ahourai, P. 1971. Comparative adaptation of some pox viruses in two cell culture system. *Archives de l'Institut Razi.* **23**: 29-105.

Mohamed, K.A.; Hagho, B.E.D.; Taylor, W.P.; Nayil, A.A. and Abu Samra, M.T. 1982. Goat pox in Sudan. *Tropical Animal Health and Production.* **14**: 104-108.

Moss, Bernard 1978. Pox viruses. *In*: The molecular Biology of animal viruses. Vol; 2 (Debi Prosad Nayak ed.) Marcel Dekker INC, New York.

Munz, E. and Dumbell, K. 1994. Sheep and goatpox. *In*: JAW Coetzer, G.R. Thomson and R.C. Tustin (eds). Infetious Diseases of Livestock with special reference to southern Africa, Vol. I (Oxford University Press, Capetown) pp 613-615.

Murray, M.; Martin, W.B. and Koylu, A. 1973. Experimental sheep pox. A histological and ultrastructural study. *Research in Veterinary Science.* **15**: 201-208.

Nagichabe, C.K.; Binepal, Y.S.; Njiru, J.W. and Earn, V.M. 1999. Evaluation of an immunocapture enzyme linked immunosorbent assay for detection of capripox viruses. *Veterinary Record.* **145** (8): 231-232.

Nitzschke, E.; Buckley, L.S. and Ergin, H. 1967. Isolation and titration of sheep pox and goat pox viruses in sheep thyroid cell cultures. *Veterinary Record.* **81**: 216-217.

Noyes, W.F. and Watson, B.K. 1955. Studies on the increase of vaccinia virus in cultured human cells by means of fluorescent antibody technique. *Journal of Experimental Medicine.* **102**: 237-241.

O.I.E. Bulletin 1996. Office International-des-Epizooties. **108**(12): 843-845.

O.I.E. Bulletin 1998. Office International-des-Epizooties. **3**: 171-189.

Obijeski, F. John and Palmer, E.L. 1973. Polyacrylamide gel electrophoresis of fowlpox and vaccinia virus proteins. *Virology.* **51**: 512-516.

Okaiyeto, S.O.; Sackey, A.K.; Lawal, I.A.; Daggana, D. and Alam, L. 1995. Outbreak of goatpox in Zaire, Kaduna state of Nigeria. *Israel Journal of Veterinary Medicine.* **50** (2): 65-66.

Olmsted, J.B. 1981. Affinity purification of antibodies from Diazotized paper blot of heterogeneous protein samples. *The Journal of Biological Chemistry.* **256** (23): 11955-11957.

Onar, B.; Ergin, H. and Napthine, P. 1968. Characteristics of sheep and goat pox viruses from Turkey. Studies of ether and chloroform sensitivity. *Pendik Veteriner Kontrol VE Arastirma Estitusu Dergi Istanbul.* **1**: 76-82.

Pandey, A.K.; Malik, B.S. and Bansal, M.P. 1969. Studies on sheep pox virus. I. Adaptation and propagation of the virus in cell culture. *Indian Veterinary Journal.* **46**: 925-926.

Pandey, R. and Singh, I.P. 1970a. A note on heat, chloroform and ether sensitivity of sheep pox and goat pox viruses. *Acta Virologica.* **14**: 318-319.

Pandey, R. and Singh, I.P. 1970b. Cytopathogenicity and neutralization of goat pox virus in cell culture. *Research in Veterinary Science.* **11**: 195-197.

Pandey, R. and Singh, I.P. 1972. Soluble antigens of sheep pox and goat pox viruses as determined by immunodiffusion in agar gel. *Acta Virologica.* **16**; 41-46.

Peeler, E.J. and Wanyanger, S.W. 1998. Infectious causes of small ruminant mortality in Kenya: a review. *Small Ruminant Research.* **29**: 1-11.

Plowright, W. and Ferris, R.D. 1958. The growth and cytopathogenicity of sheep pox virus in tissue culture. *British Journal of Experimental Pathology.* **39**: 424-435.

Plowright, W. and Ferris, R.D. 1959. Ether sensitivity of some mammalian pox viruses. *Virology.* **7**: 357-358.

Plowright, W.; Macleod, W.G. and Ferris, R.D. 1959. The pathogenesis of sheep pox in skin of sheep. *Journal of Comperative Pathology.* **69**: 400-413.

Prakash, V.; Chandra, R.; Rao, V.D.P. and Garg, S.K. 1994. Cultivation of goatpox virus in an established cell line. *Indian Journal of Virology.* **10**: 60-63.

Prasad, G.; Garg, S.K. and Singh, I.P. 1986. Passive transfer of immunity against sheep pox. *Indian Veterinary Medical Journal.* **10**(2): 78-81.

Rafyi, A. and Ramyar, H. 1959. Goat pox in Iran: serial passage in goats and the developing egg and relationship with sheep pox. *Journal of Comparative Pathology.* **69**: 141-147.

Rai 1985. Methods in cell culture. Allied Publishers, New Delhi.

Ramchandran, S. 1967. Observations on the histopathology of lung lesions in experimental pox infection of sheep and goats. *Ceylon Veterinary Journal.* **15:** 78-82.

Ramyar, H. 1966. Propagation of goat pox virus on monolayer cell culture. *Zentralblatt fur Veterinarmedizin.* **13B:** 334-337.

Rao, B.M. and Rao, M.V.S. 1999. An indirect IgM ELISA for diagnosis of sheep pox virus infection. *Indian Veterinary Journal.* **76** (12): 1050-1054.

Rao, M.V.S.; Malik, B.S. 1982. Behaviour of sheep pox, goat pox and contagious pustular dermatitis viruses in cell culture. *Indian Journal of Comparative Microbiology, Immunololgy and Infectious Disease.* **3**:26-33.

Rao, M.V.S.; Malik, B.S. and Sharma, S.N. 1984. Antigenic relationships among sheep pox, goat, pox and contagious pustular dermatitis viruses. *Acta Virologica.* **28**: 380-387.

Rao, P.B. ; Madubala, K. and Rao, M.R. 1998. Prevalence of viral and bacterial diseases among goats in Andhra Pradesh, *Indian Veterinary Journal* **75**(10): 924-925.

Rao, T.V.S.; Negi, B.S. and Bansal, M.P. 1996. Identification and characterization of differentiating soluble antigens of sheep and goatpox viruses. *Acta Virologica* **40**: 259-262.

Rao, T.V.S.; Negi, B.S. and Bansal, M.P.1997. Isolation and characterization of soluble antigens of sheep pox virus. *Indian Journal of Experimental Biology.* **35**: 597-602.

Rao, T.V.S.; Malik, P.; Asgola, D. and Malik, P. 1999. Evaluation of avidin-biotin ELISA for detection of antibodies to goatpox virus using noninfectious diagnostic reagent. *Acta Virologica.* **43** (5):297-301.

Reddy, M.P. and Choudhari, P.C. 1999. Study of lamb mortality in organised sheep farms in Andhra Pradesh. *Cheiron.* **28**(6): 200-204.

Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty percent end points. *American Journal of Hygiene.* **27**: 493-497.

Renshaw, H.S. and Dodd, A.G. 1978. Serologic and cross immunity studies with contagious ecthyma and goat pox virus isolates from Western United States. *Archives of Viroloty.* **56**: 201-210.

Saha, G.R.; Roy, S.S.; Mukherjee, N. and Mitra, K. 1985. Clinical and epidemiological features of pox in Black Bengal goats of West Bengal (India). *Indian Journal of Animal Health.* **24**; 29-34.

Sambrook, J.; Fridsch, E.F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual (Laboratory Press Cold Spring Harbor, New York.

Sarkar, P.; Singh, S.P. and Pandey, A.K. 1976. A note on studies of sheep pox virus by gel diffusion and immunoelectrophoresis. *Indian Journal of Animal Health.* 19-21.

Sarkar, P.; Singh, S.P.; Pandey, A.K.; Kathuria, B.K. and Kumar, S. 1980. Application of fluorescent antibody test in the diagnosis of sheep pox and study of sheep pox virus multiplication in cell culture. *Indian Journal of Animal Science.* **50**; 428-433.

Sedhukhan, T.; Nag, N.C.; Ray, J.P.; Nandi, S. and Das, R. 1998. Application of dot-ELISA in diagnosis of goatpox outbreak. *Indian Veterinary Journal.* **75** (9): 841-42.

Sen, K.C. 1968. Immunological relationship of goat pox and sheep pox viruses. *Indian Journal of Medical Research.* **56**: 1153-1156.

Sharma, B.; Negi, B.S.; Pandey, A.B.; Bandyopadhyay, S.K.; Shankar, H. and Yadav, M.P. 1988a. Detection of goat pox antigen and antibody by the counter immunoelectrophoresis test. *Tropical Animal Health and Production.* **29**: 109-113.

Sharma, B.; Negi, B.S.; Yadav, M.P.; Shankar, H. and Pandey, A.B. 1988b. Application of ELISA in the detection of goat pox antigen and antibodies. *Acta Virologica.* **32**: 65-69.

Sharma, R.M. and Bhatia, H.M. 1959. Contagious pustular dermatitis in goats. *Indian Journal of Veterinary Science.* **28**: 205-210.

Sharma, S.N. 1966. Studies on sheep and goat pox viruses. Summary of Thesis. Agra University. p.9.

Sharma, S.N.; Nilakantan, P.R. and Dhanda, M.R. 1966. A preliminary note on pathogenicity and antigenicity of sheep and goat pox viruses. *Indian Veterinary Journal.* **43**: 673-678.

Sharma, S.N. and Dhanda, M.R. 1971. Studies on sheep and goat pox viruses. Relationship with contagious pustular dermatitis and vaccinia viruses. *Indian Journal of Animal Sciences.* **41**: 864-867.

Sharma, S.N. and Dhanda, M.R. 1972. Studies on sheep and goat pox viruses: Pathogenicity. *Indian Journal of Animal Health.* **11**: 39-46.

Singh, B. and Rai, A. 1991a Virion polypeptides of sheep pox virus. *Indian Veterinary Medical Journal.* **15**: 239-244.

Singh, B. and Rai, A. 1991b Adaptation and growth cycle of sheeppox virus in vero cell culture. *Indian Veterinary Medical Journal.* **15**: 245-250.

Singh, I.P. and Pandey, R. and Shrivastava, R.N. 1979. Sheep pox: A review. Veterinary Bulletin. **49** : 145-154.

Slagsavold, L. 1938. Goat pox and allied conditions in sheep and goats in Norway. *Skandinavisk veterinartidskrift.* **28**: 471-495.

Soad, M.S.; Wafaa, A.Z.; Micheal, A.; Fayad, A.A. and Taha, M.M. 1996. Studies on sheep and goat pox viruses from naturally infected animals. *Assiut Veterinary Medical Journal.* **70**: 29-38.

Soborg, M. and Bendizen, G. 1967. Human leukocyte migration as a parameter of hypersensitivity. *Acta Medicina Scandinavia.* **181**: 247-252.

Soman, J.P. and Singh, I.P. 1980. Cytopathic and Immunologic studies of sheep pox virus serially cultivated in cell culture. *Journal of Comparative Pathology.* **90**: 99-106.

Soman, J.P.; Sinha, R.P. and Jha, G.I. 1985. Occurrence of malignant goat pox in Bihar and confirmation of the causal virus. *Indian Veterinary Journal.* **62**: 907.

Srivastava, R.N. and Singh, I.P. 1980. Antigenic difference between extra cellular and intra cellular sheep pox virus. *Indian Journal of Animal Sciences.* **50**: 1098-1103.

Symington, Janey; Maurice Green and Brackmann, Karl 1981. Immunoautoradiographic detection of proteins after electrophoretic transfer from gels to diaze-paper: Analysis of adenovirus encoded proteins. *Proc. Natl. Acad. Sci.* **78** (1): 177-181.

Talhouk, R. and El-Zein, A. 1986. Characterization of a cell culture adapted goat pox virus. *Journal of Veterinary Medicine.* **33B**: 543-551.

Tantwai, H.H.; Saban, M.S.; Reda, I.M. and El-Dahaby 1974. Camel pox virus I Isolation and characterization. *Bull. Epizootic disease of Africa.* **34**: 347-51.

Tantawi, H.H. and Falluji, M.M. Al. 1979. Laboratory characteristics of four strains of goat pox virus. *Acta Virologica.* **23**: 455-460.

Tantawi, H.H.; Shony, M.O. and Hassan, F.K. 1979. Isolation and identification of the Sersenk strain of goat pox virus in Iraq. *Tropical Animal Health and Production.* **11**: 208-210.

Tantawi, H.H.; Awad, M.M.; Shony, M.O.; Alwan, A.H. and Hassan, F.K. 1980. Preliminary characterization of the Sersenk strain of goat pox virus. *Tropical Animal Health and Production.* **12**: 30-34.

Tiwari, A.K.; Nandi, S. and Negi, B.S. 1995. Reverse phase passive haemagglutination (RPHA) test for the detection of goat pox virus antigen. *Indian Journal of Virology.* **11**: 35-37.

Tiwari, A.K.; Rao, T.V.S.; Negi, B.S. 1996. Spot agglutination test for the rapid diagnosis of goat pox. *Tropical Animal Health and Production.* **28**: 213-215.

Tiwari, A.K. and Negi, B.S. 1996. Comparative efficacy of Dot-ELISA and single radial haemolysis (SRH) test in detecting goatpox antigen. *Indian Veterinary Journal.* **73**: 1017-1020.

Tripathy, D.N.; Hanson, L.E. and Myres, W.L. 1970. Detection of fowl pox virus antigen in tissue culture by fluorescent antibody technique. *Avian Diseases.* **14**: 810-812.

Uppal, P.K. 1963. Studies on sheep pox virus. M.V.Sc. thesis submitted to Agra University, Agra.

Uppal, P.K. and Nilakantan, P.R. 1970. Studies on serological relationship between avain pox, sheep pox, goat pox and vaccinia viruses. *Journal of Hygiene.* **68**: 349-359.

Vegad, J.L. and Sharma, G.L. 1970. (i) Pathogenesis of sheep pox in the skin of experimentally infected sheep-microscopic observations. (ii) Pathogenesis of sheep pox (*Variola ovina*) in the organs of cardiovascular, urinary, male reproductive and endocrine system of experimentally infected sheep. *JNKVV Research Journal.* **4**: 32-36; 79-85.

Vegad, J.L. and Sharma, G.L. 1971. Pathogenesis of sheep pox (Variola Ovina) in the digestive system of experimentally infected sheep. *JNKVV Research Journal.* **5**: 107-115.

Vegad, J.L. and Sharma, G.L. 1973. Cutaneous and pulmonary lesions of sheep pox. *Indian Journal of Animal Science.* **43**: 1061-1067.

Vigario, J.D. and Ferraz, F.P. 1967. Study of sheep pox virus synthesis by fluorescent antibody technique. *American Journal of Veterinary Research.* **28**: 809-813.

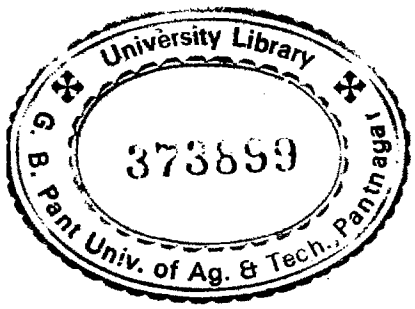
Wang, X.; Ding, M.; Fu, H.; Wang, M. and Zhai, Z. 1987. The fine structure and morphogenesis of the envelope and nucleocapsid of goat pox virus and infectious bovine rhinotracheitis virus.; A freeze fracture study. *Journal of Electron microscopy techniques.* **7**: 97-109.

Warberg, G. and Christian, W. 1941. Spectrophotometeric method of protein estimation. *Biochemistry.* 310-84.

Weber, Klaus and Osbron, Mary 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *The Journal of Biological Chemistry.* **244** : 4406-4412.

Yeruham, I.; Perl, S.; Abraham, A. and Algasi, R. 1998. Simultaneous infections: lamb with contagious ecthyma and sheep pox or contagious ecthyma and papillomatosis. *Revue-de-Medecine-Veterinarie.* **149**(12) : 1115-20.

Zeller, H. 1920. South west African goat pox. *Arbeiten aus dem Reichsgesundheitsamte.* **52**: 501.



APPENDIX

APPENDIX

A. Reagents for cell culture

1. Glassgow minimum essential medium (GMEM), pH 7.2

GMEM (Sigma)	12.6 g
L-Glutamine	0.29 g
Penicillin	1 lakh IU
Streptomycin	100 mg
Distilled water to make	1000 ml

Sterlize the media by filteration through 0.22 μm membrane filter and store at 4^0 C.

2. Minimum Essential Medium (MEM) pH 7.2

MEM (Hyclone)	9.5 g
Tryptose phosphate broth	3.0 g
L-glutamine	0.3 g
Pencillin	1 lakh IU
Streptomycin	100 mg
Distilled water to make	1000 ml

Sterlize the media by filteration through 0.22 μm membrane filter and store at 4^0 C.

3. **Trypsin-Versine solution**

NaCl	10.0 g
KCl	0.25 g
$Na_2HPO_4.2\ H_2O$	1.90 g
KH_2PO_4	0.35 g
Trypsin	1.70 g
Versine (EDTA)	1.40 g
Phenol red (0.5%)	1.00 ml
Distilled water	1000 ml

After addition of Penicillin (100 IU/ml) and streptomycin (100 µg/ml) solution is sterlized by 0.22 µm membrane filter.

B. May-Grunwald stain

1. **Stock solution**

May-Grunwald stain	3.0 g
Methyl alcohol (Acetone free)	1000 ml

Warm upto 50^0 C with constant stirring.

2. **Working solution**

Stock MGG stain	20 ml
Distilled water	10 ml

C. Giemsa stain

1. Stock solution

Giemsa powder	7.5	g
Glycerin	500	ml
Methanol	500	ml

Dissolve stain powder in glycerin at 56^0 C for 1-2 h in water bath and then add methanol and leave atleast for a week.

2. Working solution

Giemsa stock solution	10	ml
Distilled water	90	ml

D. Acridine Orange staining

1. Acridine orange stain

Acridine orange	0.1	g
Distilled water	100	ml

Prepare working solution by mixing one part of stock solution to 9 parts of McIlvaine's citric acid buffer.

2. Mc Ilvaine's citric acid buffer

Disodium hydrogen phosphate	10.08	g
Citric monohydrate	13.55	g
Distilled water	1000	ml

3. Cornoy's fixative

Ethyl alcohol	60	ml
Chloroform	30	ml
Acetic acid	10	ml

Store at room temperature.

E. Reagents for ELISA

1. Coating buffer (Carbonate-bicarbonate buffer, pH 9.6)

Sodium carbonate	0.318	g
Sodium bicarbonate	0.568	g
Sodium azide	0.040	g
Distilled water to make	200	ml

2. Blocking solution

Bovine serum albumin	1.0	g
PBS-tween 20	1000	ml

3. Washing buffer (pH 7.4)

Tween-20	0.5	ml
PBS	1000	ml

4. Phosphate-citrate buffer (pH 5.0)

Solution A	Citric acid	1.921	ml
	Distilled water	100	ml
Solution B	Disodium hydrogen phosphate	2.84	g
	Distilled water	100	ml

Mix 24.3 ml of solution A to 25.7 ml of solution B and make the volume upto 100 ml with distilled water.

5. Substrate for ELISA

Orthophenylene diamine dihydrochloride	10 mg
Phosphate citrate buffer	25 ml
Hydrogen peroxide (30%)	0.5 ml

F. Reagents for Dot-ELISA

1. Phosphate citrate buffer (pH 5.0)

Citric acid	7.3 g
Disodium hydrogen phosphate	11.86 g
Distilled water	1000 ml

2. Substrate solution

Diaminobenzidine tetrahydrochloride	5.0 mg
Phosphate citrate buffer	10 ml
Hydrogen peroxide	5 µl

G. SDS-PAGE Reagents

I. Stock solutions

1. 2x Sample buffer (pH 6.8)

Tris base	1.51g
SDS	8.0 g
Glycerol	30 ml
Bromophenol blue	2 mg
Distilled water	64 ml

Warm the solution to dissolve and allow to cool to room temperature. Adjust the pH to 6.8 and make the total volume of 100 ml. Store at 37^0C.

2. **10% Ammonium persulphate**

Ammonium persulphate	0.1 g
Distilled water	10.0 ml

3. **2x separating gel buffer (pH 8.9)**

Tris base	45.4 g
SDS	1.0 g
Distilled water	460 ml

Add concentrated HCl dropwise until pH falls to 8.88. Make volume upto 500 ml and store in plastic bottles at 4^0 C.

4. **Stock acrylamide for separating gel**

Acrylamide	75 g
Bisacrylamide	0.6 g
Distilled water	181 ml

Make the volume 250 ml and store in dark bottle at 4^0 C.

5. **2 x separating gel buffer (pH 6.8)**

Tris base	6.06 g
SDS	0.4 g
Distilled water	190 ml

Adjust the pH with HCl. Make the volume 200 ml and store at 4^0 C.

6. Stock acrylamide for stacking gel

Acrylamide	15 g
Bisacrylamide	0.4 g
Distilled water	36 ml

Make the total volume upto 500 ml and store in dark bottle at 4^0 C.

7. 10x Electrode buffer (pH 8.3)

Tris base	30.3 g
Glycine	144.2 g
SDS	10 g
Distilled water	885 ml

Make the volume to 1 liter and store at room temperature. For use 1 part of this solution is mixed with 9 parts of water.

8. CBB staining solution

Solution a-	Coomassie Brilliant blue	0.25 g
	Distilled water	25 ml
Solution b-	Trichloroacetic acid	60 g
	Distilled water	720 ml
	Methanol	180 ml
	Glacial acetic acid	60 ml

Mix solution a and b with constant stirring. Make the volume upto 1 litre and store in dark bottle at room temperature.

9. CBB destaining solution

	NaCl	150 g
	Distilled water	5 litres
Or	Glacial acetic acid	50 ml
	Methanol	280 ml
	Distilled water	670 ml

Store at room temperature

II. Working solution

1. Separating gel (60 ml for 2 gels)

	10%	12.5%
2 % separating gel buffer	30 ml	26 ml
Stock acrylamide for separating gel	20 ml	25 ml
Distilled water	10 ml	9 ml
TEMED	100 µl	100 µl
10% Ammonium per sulphate	100 µl	100 µl

2. Stacking gel

	3 %	4 %
2 % stacking gel buffer pH 6.8	2.25 ml	2.25 ml
Distilled water	1.75 ml	1.60 ml
Stock acrylamide for stacking gel	0.5 ml	0.6 ml
TEMED	10 µl	10 µl
10% Ammonium per sulphate	30 µl	30 µl

H. Buffer for protein transfer by semidry system of blotting

1. Anode buffer I pH (10.4)

Tris	3.63	g
Methanol	10	ml
Distilled water to make	100	ml

2. Anode buffer II (pH 10.4)

Tris	0.30	g
Methanol	10	ml
Distilled water to make	100	ml

3. Cathode buffer (pH 9.4)

Tris base	0.30	g
Glycine	0.3	g
Methanol	10	ml
Distilled water to make	100	ml

I. Staining of protein blotted membrane

Coomassie brilliant blue R	0.1	g
Methanol	50	ml
Acetic acid	7	ml
Distilled water to make	100	ml

J. Blocking buffer for Western blotting

Bovine serum albumin	1.0	g
Sodium chloride	0.9	g
Sodium phosphate	0.178	g
PBS	100	ml

VITA

Sanjay Shakya, the author of the manuscript was born on Ist January, 1964 at Farrukhabad (Uttar Pradesh). He passed his B.S.C. Part I examination in the year 1982 from Govt. Science College, Jabalpur. He joined College of Veterinary Science and A.H. Jabalpur in year 1982 and secured B.V.Sc. & A.H. and M.V.Sc. (Veterinary Microbiology) degrees in the year 1987 and 1989, respectively. He served as Veterinary Assistant Surgeon, at Govt. Veterinary Hospital, Bhanupratappur, Bastar, Chattisgarh from 1989 to 1993. Thereafter, he joined as Assistant Professor Department of Veterinary Microbiology, College of Veterinary Science and A.H., Anjora, Durg, Chattisgarh (Indira Gandhi Krishi Vishwa Vidhyalaya). In January, 1999, he joined Ph.D. programme in Veterinary Microbiology and Immunology at College of Post Graduate Studies, G.B.Pant University of Agri. & Tech., Pantnagar as a sponsored candidate of Indira Gandhi Krishi Vishwa Vidhyalaya and completed all the requirements for Ph.D. in September, 2001.

Address-

Sanjay Shakya
Assistant Professor (Senior Scale)
Department of Veterinary Microbiology
College of Veterinary Science & A.H.
Anjora, P.B. NO. 6,
Durg (Chattisgarh State)-491 001.

ABSTRACT

Name: Sanjay Shakya I.D. No. 25871
Semester and year of admission IInd Sem. 1998-99 Degree: Ph.D.
Major: Veterinary Microbiology and Immunology Department: Veterinary Microbiology

Thesis title: "IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF IMMUNOGENIC PROTEINS OF CAPRIPOX VIRUS ".

Advisor: Dr. V.D.P. Rao

Capripox, a severe pox disease of sheep, goats and cattle, is associated with serious economic consequences. The experimental infection of goats with Pantnagar strain of GPV showed development of skin lesions at the site of inoculation that included developmental stages, viz., macules and papules followed by scab formation.

The GPV strains, viz., Pantnagar, Uttarkashi and Sambalpur, were adapted to KTCC at 2nd passage level. The changes observed were rounding and aggregation of cells, which appeared as bunches of grapes. The infected KTCC showed intracytoplasmic inclusion bodies of different shape and size with or without a halo 72 h PI at 3rd passage. The AO staining of GPV infected cells showed viral DNA as small greenish bodies in the cytoplasm. The infectivity titre of $10^{6.57}$ $TCID_{50}$/ml was recorded at 15th passage level. The extracellular and intracellular titres of Pantnagar strain of GPV were $10^{4.49}$ and $10^{6.57}$ $TCID_{50}$/ml, respectively, at 15th passage level. The Pantnagar strain of GPV was adapted to Vero cells after 7th passage.The adapted virus caused rounding and clumping of cells, formation of microplaques and syncytia and intracytoplasmic eosnophilic inclusion bodies. The GPV (Pantnagar strain) infected Vero cells showed infectivity titre of $10^{4.49}$ $TCID_{50}$/ml at 5th passage. The Pantnagar strain of GPV adapted to MDBK cells produced granulation and vacuolation of cytoplasm, formation of syncytia, microplaques and intracytoplasmic inclusions with infectivity titre of $10^{4.63}$ $TCID_{50}$/ml at 15th passage. The KTCC, Vero and MDBK cells, infected with Pantnagar strain of GPV, showed specific areas of fluorescence in the cell cytoplasm 48 h PI at 3rd passage level. The electron microscopic studies of KTCC and Vero cells infected with Pantnagar strain of GPV showed intracytoplasmic virus particles, which were more electron dense than matrix and showed a distinct round, oval or elliptical shape. The serum neutralization of Pantnagar strain of GPV adapted to KTCC, MDBK and Vero cells showed indices of 3.91, 3.3 and 2.66, respectively. The Pantnagar strain of GPV was found sensitive to lipid solvents, heat and pH. It did not agglutinate the erythrocytes of sheep, goat, pig, rabbit, dog, chicken and human type 'O' cells. The maternal antibodies protected the kids born to immunized goats when challenged with GPV. The SPACE test detected GPV specific antigen in skin scab and tissue culture fluids. The test was found to be more sensitive than AGPT. The polypeptides of purified GPV in 10% and 12.5% acrylamide gels yielded 53 and 43 polypeptides, respectively. A total of 55 polypeptides, ranging from 209 KD to 15 KD in molecular weights were resolved. The polypeptides of purified SPV showed 23 polypeptide bands in 10% acrylamide gel with molecular weights ranging between 209 to 13 KD. The purified GPV revealed 14 immunogenic proteins. The MW of 8 major immunogenic polypeptides were 97 KD, 85 KD, 72 KD, 58 KD, 43 KD, 35 KD, 32 KD and 26 KD. The Western blotting of purified SPV showed 5 major immunogenic proteins with MW of 71 KD, 59 KD, 43 KD, 35 KD and 30 KD.

The monospecific sera against immunogenic polypeptides of 58 KD and 35 KD showed highest neutralizing end point titres of 47 and 45, respectively. The envelope of GPV contained major 35 KD protein in association with lower concentrations of 85 KD, 76 KD and 26 KD proteins. The Western blotting with KTCC infected with Pantnagar strain of GPV showed virus specific polypeptides.

(V.D.P.Rao)
Advisor

(Sanjay Shakya)
Author