THE USE OF POLYMERASE CHAIN REACTION IN DIAGNOSIS OF SHEEP POX AND GOAT POX OUTBREAKS

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Preface

This work has been carried out in the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum under supervision of Dr. Abdelmelik Ibrahim Khalafalla.
DEDICATION

To

My Dear Mother
My Dear Father
My Brother, Sisters and my friends.
For their perpetual encouragement
I dedicate this work
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<td>Agar gel immunodiffusion test</td>
</tr>
<tr>
<td>BHK₂₁</td>
<td>Baby hamster kidney cell lines</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxy nucleic acid</td>
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<td>CEF</td>
<td>Chicken embryo fibroblast</td>
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<tr>
<td>CIE</td>
<td>Counter immunoelectrophoresis</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<td>CVRL</td>
<td>Central veterinary research laboratories</td>
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<td>DNA</td>
<td>Deoxy nucleic acid</td>
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<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>GMEM</td>
<td>Glasgow modified eagle’s medium</td>
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<td>GPV</td>
<td>Goat poxvirus</td>
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<td>HIS</td>
<td>Hyperimmune serum</td>
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<td>ICTV</td>
<td>International committee taxonomy of viruses</td>
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<tr>
<td>IEF</td>
<td>Immunoelectro focusing</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose 50</td>
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<tr>
<td>LSD</td>
<td>Lumpy skin disease</td>
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<tr>
<td>LTC</td>
<td>Lamb testis cell culture</td>
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<tr>
<td>MA104</td>
<td>Rhesus monkey fetal kidney cell lines</td>
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<tr>
<td>MAR</td>
<td>Ministry of animal resources</td>
</tr>
<tr>
<td>MDa</td>
<td>Mega dalton</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin darby bovine kidney cell lines</td>
</tr>
<tr>
<td>NI</td>
<td>Neutralisation index</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<td>OIE</td>
<td>Office internationale des epizootic</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>PAGE</td>
<td>Poly acrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PD</td>
<td>Phosphate diluent</td>
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<tr>
<td>PI</td>
<td>Post inoculation</td>
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<tr>
<td>RNA</td>
<td>Ribo nucleic acid</td>
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<td>SGP</td>
<td>Saanen goat pox</td>
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<td>SPV</td>
<td>Sheep poxvirus</td>
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<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Tissue culture infective dose&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>VAP</td>
<td>Viral attachment protein</td>
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**ABSTRACT**

Two outbreaks of sheeppox that occurred in Gedarif State in June 2003 and one outbreak of goatpox that occurred in Khartoum State in March 2005 were investigated. Clinical signs were reported, morbidity rates were estimated and the total mortalities were recorded. Clinically, the disease was characterized by fever, depression and eruption of generalized pox lesions. Mortality rate ranged between 5.2 and 6.7 with a mean of 6.1. All age, sex and breed groups were affected. However, more than 50% of deaths were reported in young animals in comparison to adult sheep.

Skin Scabs were collected from sick animals, homogenized and inoculated in cell culture and embryonated eggs. Two virus isolates were obtained when 4 skin samples were inoculated onto Lamb testes (LT) cell culture. These viruses as well as one previous isolate of sheeppox virus (SP4) and the vaccine strain 0240 induced no lesions on the chorioallantoic membrane (CAM) of embryonated chicken eggs.

On the other hand, sheeppox and goatpox isolates grew well in lamb testes (LT) cell culture with no difference in virus yield or type of CPE produced by the two viruses. In Vero cells, both viruses produced more than 90% CPE within 7 days PI. In MDBK however, both viruses induced slight CPE that reached 60% in 9 days. On the other hand, both viruses induced no CPE in chick embryo fibroblast (CEF) cells.

Titration of an isolate of sheeppox virus (SP1O) was performed in Vero cells and resulted in a titer of $10^{5.2}$/ml. The same virus was identified as sheeppox in virus neutralization test with 1.4 neutralization index.

Polymerase chain reaction (PCR) was used in this study to determine the feasibility of improving diagnosis of sheep pox and goat pox in scabs collected from the field as well as in supernatant of infected cell cultures. Three methods of DNA extraction were tried. The first method (PCR 1) employed no DNA extraction step with scab homogenate or cell culture supernatant added directly to the PCR mix. This method failed to detect pox virus DNA in all scab homogenates tested but three out of six cell culture supernatants gave positive results. In the second method (PCR 2), viral DNA was extracted by phenol-chloroform from skin scabs; three samples out of six gave positive
results. In the third method (PCR 3), viral DNA was extracted using a commercial DNA extraction Kit; two out of three gave positive result. Accordingly, the best PCR method for sheeppox and goatpox diagnosis should include an initial step of DNA extraction.
Introduction

Capripoxviruses are the most important poxviruses of animals, causing diseases in sheep, goats, or cattle respectively. These viruses are responsible for some of the most economically significant diseases of domestic ruminants in Africa and Asia. Sheeppox and goatpox exhibit similar clinical signs that are typical generalized pox viral disease, including pyrexia, cutaneous lesions, and notably the development of lung lesions (Esposito and Fenner, 2001; Munz and Dumbell, 1994).

In the Sudan the presence of sheeppox had been confirmed since 1940 (Bennet et al., 1944), and it has remained enzootic as well as in most part of Africa. Sheeppox in the Sudan was generally known to be a seasonal disease associated with the cold winter (Muzichin and Ali, 1979), however, in last few years outbreaks of the disease were observed at different times of the year. Goatpox has also been confirmed in the Sudan and caused severe diseases in both goats and sheep.

Efforts for prophylaxis against sheeppox has begun in the Sudan since the eighties of the last century when the 0240 vaccine strain of Kenya sheep and goatpox virus was brought in the country (Ali & Hajer, 1989). However, the occurrence of sheeppox in some vaccinated animals has also been reported by some stock owners (Sheikh Ali, 1997). It is therefore, important to determine the immunogenicity of the vaccine in use vis the pathogenicity of the local isolates of the virus. As a prerequisite for this approach field outbreaks should be investigated to determine the epidemiology of the disease and the causative agent should be isolated and characterized.

All strains of sheeppox virus and goatpox virus examined so far are antigenically similar and share a common neutralizing site irrespective of their geographical origin.

The diagnosis of goatpox and sheeppox is usually based on clinical signs followed by laboratory confirmation. Initially, the laboratory test was mainly confined to agar gel precipitation, for which conventionally produced hyperimmune sera against GPV and SPV were used, subsequently, a soluble antigen fraction, which is not infectious, has efficiently replaced the infectious virus in various serological tests. This soluble antigen
is commonly used in diagnostic tests and can avoid the risk of spread of virus from the laboratory.

Diagnosis of SPV and GPV is made by identification of Capripoxvirus by virus isolation on cell culture and confirmation by virus neutralization or immunoflourescence using hyperimmune anti Capripoxvirus serum. Capripoxvirus isolation is a lengthy procedure due to the slow growth of the virus in cell culture, in which cytopathic effects may take up to 10 days to develop (Plowright and Ferris, 1958). Serology is limited in its application due to often low antibody response following infection. Another problem, which occurs when serological diagnosis is undertaken, is that Capripoxvirus and Parapoxvirus are related closely serologically. Both viruses cause very similar diseases in sheep and goats and it is difficult to differentiate them clinically. Many serodiagnostic assays, such as, agar gel immunodiffusion, complement fixation, counter immunoelectrophoreses or ELISA, because they detect total antibody response including that from cross-reactive antibodies, are not reliable for differentiation between these infections.

The most specific classical technique, virus isolation on susceptible cell cultures followed by confirmation (virus neutralization or immunoflourescence using hyperimmune anti Capripox serum) is a lengthy procedure and may take up to 2 weeks.

These diagnostic problems can be overcome by the use of PCR, which is specific, rapid, sensitive and accurate assay for the detection of Capripoxviruses.

Recently, various PCR methods for detection and diagnosis of sheep and goatpox virus infection were developed (Ireland and Binepal, 1998; Heine et al., 1999; Mangana-Vougiouka et al., 1999; Parthiban et al., 2005).

The purpose of the study is to introduce polymerase chain reaction (PCR) for rapid diagnostic technique of sheep and goatpox virus infections in the Sudan.
1.1. Sheep and goats production in Sudan

Sheep and goats are considered as an important group of animals in Sudan, the total population of sheep was estimated to reach more than 30 million head goat was more than 20 million head (Ministry of Animal resources), distributed all over the Sudan specially in the eastern, middle and western states of Sudan. The most important breeds of sheep and goats in Sudan can be mentioned as follow: Desert sheep, Gezera and buttana breeds (Ashgar, Abbrag, Dubasi and Garagi) Watteish breed, Kabbashi breed, Elmedobe beerd, Beeja breed, West Africa breed (Zagawa and Faullani or Fallata sub-breeds), Nilotic breed and Fat tailed breed (equatorial region). Goat breeds in Sudan include the Sudanese Nubian goat breed and the Sudanese desert goat breed (Elazaiem. 1993).

1.2. Importance of sheep and goat pox diseases

Sheep and goatpox diseases are a weighty problem in sheep and goats production. In fact they are most serious of all pox diseases of domestic animals. Regarding sheep and goats production and economy, the importance of the diseases lies in the high morbidity, mortality in young animals, partial loss of reproduction function and damage to wool and hair.

1.3. Definition

1.3.1. Sheeppox

Sheeppox is a contagious disease of sheep caused by sheeppoxvirus (SPV). It is considered one of the most dangerous poxvirus infections among animals. The disease is characterized by a rise of body temperature and skin lesions in the areas denied of wool; the disease may also affect the mucous membranes of the respiratory and gastrointestinal tracts. Sheep are the natural host of the virus; affection is determined by age and breed (Jubb and Kennedy, 1970; Buxton and Fraser, 1977).
1.3.2. Goatpox

Goatpox is caused by goatpoxvirus (GPV) which is closely related to sheeppox virus; clinically it resembles sheeppox but the disease in goats takes a mild course (Merza and Mushi, 1990).

1.4. History and distribution of SPV and GPV

Hansen first reported goatpox in 1879 from Norway (Rafyi and Ramyar, 1959). Later, it was observed during the period of the First World War in Macedonia, where it became enzootic during the year 1926, with a mortality rate of 15% (Blanc et al., 1928).

The disease is present mainly in Asia, Africa, the Middle East and parts of Europe, but it has also been reported from many other parts of the world, e.g. southwest Africa (Zeller, 1920), France (Besnoit and Robin, 1923), Malaysia (Kuppuswamy, 1936), Norway (Slagsvold, 1938), Morocco (Grimpret, 1938), Tajikistan (Vannovski, 1955), Sweden (Bakos and Brag, 1957), Sudan (Mohamed et al., 1982), Iraq (Karim, 1983), Nigeria (Okaiyeto et al., 1995), the western USA (Renshaw and Dodd, 1978), the Republic of China, Bangladesh, the Yemen Arab Republic and Oman (Kitching et al., 1986a). In India, an outbreak of goatpox was reported for the first time in 1936 (Imperial Institute of Veterinary Research, 1936–37). Later it was reported from the states of Haryana (Lall et al., 1947), Uttar Pradesh (Das et al., 1978), Orissa (Bandyopadhyay et al., 1984) and West Bengal (Saha et al., 1985), and recently from Madhya Pradesh (Joshi et al., 1999).

Sheeppox has a documented history almost as long as that of smallpox. The disease was apparently present in Asia and Europe as early as the second century AD (Hutyra et al., 1946), and its infectious nature was recognized in the mid-eighteenth century. Eradication was achieved in Britain in 1866, but in other areas of Europe eradication was more difficult, probably because of the extensive livestock trade between countries (Fenner et al., 1993).

The disease has also been reported from Niger (Mariner et al., 1991). In India, the first report of the prevalence of sheeppox came from Tamil Nadu, Bombay and United Provinces (Sathe, 1931–32) and from Mysore (now Karnataka) in 1936 (Srikantaiah, 1936). Minnet (1949) described the occurrence of the disease, which caused considerable economic loss throughout the country through mortality and permanent damage to the
skin in affected sheep. Subsequently, there have been several reports of pox in sheep from different parts of the country (Murthy and Singh, 1971; Sharma et al., 1986; Rao et al., 1994). The disease is still reported (Malik et al., 1998) despite the availability of an efficient vaccine in India, which raises doubt about the effectiveness of the vaccination regimen being followed.

1.5. Classification of SPV and GPV

GPV and SPV classified by the International Committee of Taxonomy of Viruses (ICTV) as belong to the genus *Capripoxvirus*, classified in the subfamily Chordopoxvirinae of the Poxviridae family (Matthews, 1982) (Table 2). According to records of Office Internationale des Epizootic (OIE), sheep and goatpox are classified as list (A) infectious diseases, which requires notification, restriction of animal movement and vaccination.

Table 1: Taxonomic Structure of the Family Poxviridae

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
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<tr>
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<td></td>
<td>Orthopoxivirus</td>
<td>00.058.1.01.</td>
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<tr>
<td></td>
<td>Parapoxivirus</td>
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<td>Entomopoxvirinae</td>
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<td>B.Entomopoxvirus</td>
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</tr>
<tr>
<td></td>
<td>C.Entomopoxvirus</td>
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</table>
1.6. Physiochemical properties of SPV and GPV

The virions are brick-shaped, enveloped with rounded ends and of complex symmetry (Dales and Pogo, 1981). The genome is a double stranded DNA of 73–91 MDa, with a central, less variable region of about 145 kilobase pairs and more variable termini with cross-links. There is extensive DNA cross-hybridization between species (Black et al., 1986; Gershon et al., 1989a; Murphy et al., 1995). The virion is about 300 X 270 X 200 nm in size and resembles other viruses of the orthopox group. Two types of particles are observed in negatively stained preparations, namely the mulberry or ‘m’ form, which is electron-transparent, and the electron-dense capsule or ‘e’ form (Kitching and Smale, 1986). The poxviruses are generally resistant to drying, survive freezing and thawing, and remain viable for months in the lyophilized state. Sensitivity to heat differs among strains.

The Iranian and Egyptian strains of GPV are more resistant than the Dushmbe strain as heating at 56°C for 1 h fails to reduce their titer significantly (Tantawi and Al-Falluji, 1979), whereas the Sambalpur isolate of GPV is inactivated within 30 min at 60°C (Sharma et al., 1988a). Moreover, the thermal inactivation of a Turkish strain of SPV leads to the loss of infectivity when it is heated to 55°C for 1 h (Koylu and Nitzschke, 1968). In contrast, the infectivity of the Jaipur isolate follows a bimodal curve, with residual infectivity titratable even after 1 h at 50°C (Pandey and Singh, 1970). The infectivity is sensitive to ether and chloroform (Murphy et al., 1995) but a few strains are resistant to ether (Bennet et al., 1944). Replication of viruses is inhibited by actinomycin D and bromodeoxyuridine (Tantawi and Al-Falluji, 1979).

1.7. Epidemiology and transmission

The diseases (GPV, SPV) are endemic in southwest Asia, the Middle East and most parts of the Indian subcontinent (Carn, 1993). The virus commonly enters through the host’s respiratory tract, and therefore transmission of disease frequently occurs via an aerosol during direct or close contact between infected and susceptible animals.

Transmission also occurs by skin abrasion or mechanically by the action of insects. The disease may be transmitted by Stomoxys calcitrans and the tsetse fly (Webbs et al., 1980). Experimentally, the diseases can be transmitted by intradermal, intravenous
and subcutaneous inoculation, and by artificially produced virus aerosols. Under natural conditions, GPV and SPV are highly host-specific but the host specificity varies from isolate to isolate. Kenyan and Yemen isolates and an Oman sheep isolate infect sheep and goats equally readily. However, isolates from the Middle East and India are host-specific—GPV does not infect sheep and SPV does not infect goats (Bakos and Brag, 1957; Kitching, 1983; Soman et al., 1985).

It is believed that the virulence of capripoxviruses is so variable that some strains are pathogenic in goats only, and thus are considered goatpoxviruses. Other strains are pathogenic in sheep only, and thus are considered sheeppoxviruses. The remaining strains can produce disease in both sheep and goats. The host preference shown by the different strains is probably due to their adaptation to either sheep or goats in a restricted geographical area (Kitching and Taylor, 1985). Therefore, it is arguable that the malignant pox diseases of sheep and goats should be designated by the single term ‘capripox’, as proposed earlier (Black, 1986).

Goatpox and sheeppox affect goats and sheep of all ages, both sexes and all breeds, but are more common and more severe in young and old animals and in lactating females. In a susceptible flock, morbidity is 75–100% and mortality 10–58% depending on the virulence of the isolate. The virus is present in the vesicular fluid, scab and milk of infected goats and sheep, and blood and spleen emulsions are also infective. Human infections during the handling of infected animals are rarely seen (Rao and Bandyopadhyay, 2000).

1.8. Zoonosis of Capripoxvirus

Mild lesions of small red papules followed by vesicles on the hands and arms have been reported in humans working with Capripoxvirus in Sweden and India. No generalization occurred. These are isolated incidents and humans are generally regarded as been non susceptible. No pathogenicity for humans has been recorded for most sheeppox strains (Ausvet plan, 1996).
1.9. Pathogenesis

Following intradermal inoculation, SPV and GPV, replicates locally in the tissue (Plowright et al., 1959). Peak virus titer in the skin was attained on day 7, when pox lesions were detected. The virus spreads to the regional lymph nodes before invading the blood stream to produce primary viremia after 3-4 days (Singh et al., 1979). Internal organs such as spleen, liver and lungs are affected after viremia; lung lesions, however, may also develop directly from inhaled virus. The peak virus titers in skin nodules persist from day 7 to 14 and decline thereafter, concomitantly with the development of serum antibodies. Excretion of SPV and GPV from sheep and goats is not documented; most likely skin scabs are the main source of the virus shedding, but also ocular and nasal secretions might be infectious (Merza and Mushi, 1990).

1.10. Clinical signs

Goat pox is clinically similar to sheep pox (Kitching and Taylor, 1985). In natural cases, the diseases have an incubation period of 1–2 weeks. Thereafter, the animal starts showing pyrexia, labored breathing, depression and loss of appetite. Body temperature may rise as high as 108°F. Cutaneous lesions develop after 1 or 2 days, and they progress through the macular, papular, vesicular and pustular stages until scabs are formed.

1.11. Pathology

The lesions are distributed over the hairless parts of the skin and mammary glands, and on the mucous membranes of the digestive and respiratory tracts. The animal may recover in 3–4 weeks, with permanent depressed scars. Scabs that have been shed by the animals remain infective for several months.

Generalization of the disease is often noticed in kids and lambs aged 4–5 months, whereas in older animals mild infections are seen. In sucking animals the disease is more severe, with lesions on the buccal mucosa and in the anterior nares with mucopurulent and lachrymal discharges. Pneumonia, enlargement of the udder and abortion may occur in severe cases. Ulcers are also seen on the tongue and gums. Such lesions constitute an important means of virus dissemination.
Post-mortem lesions include tracheal congestion, lentil-sized, bullet-shaped nodules and white patches on the lungs, an inflamed spleen and lymph nodes with graying white necrotic lesions, and an increased quantity of blood-tinged pleural fluid. In some animals, lesions develop in the lungs as multiple consolidated areas.

Microscopically, the affected lung tissue is characterized by congestion, red hepatization and exudation, coagulative necrosis surrounded by a marked zone of inflammatory reaction, and thickening of the interlobular septae. Depletion of the lymphocyte population in the paracortical regions and absence of germinal centers in the spleen and lymph nodes are also observed (Buller and Palumbo, 1991; Saha et al., 1991).

**1.12. Replication of poxvirus**

The poxviruses replicate entirely in the cytoplasm. The assembly of progeny virions is located in specific cytoplasmic places called viroplasm or viral factories, which can be separated from other cellular structures in the form of virosomes. The cytoplasmic mode of replication implies that the machinery necessary for RNA synthesis and modification has to be carried by the incoming particles in order for the early viral genes to be expressed.

Consequently among the active enzymes found in proteins included in the capsid, there are an RNA polymerase, capping and methylating enzymes, and a ploy A polymerase. These enzymes upon uncoating of the virus, start the transcription of a series of early genes, among which are those responsible for DNA synthesis, such as DNA polymerase, ligase, thymedine kinase and thymedine sythatase. Once the DNA synthesis has taken place, the mode of transcription changes to a discontinuous one to transcribe the so-called late genes (e.g. structural genes).

The life cycle is divided into several steps. Penetration and uncoating (30 min), early transcription (1-2 hr), DNA synthesis (2-4 hr), late transcription and assembly (4-6 hr), and release (budding) of the virions. During the late transcription period many early genes cease to be transcribed.

It is during this phase of the life cycle that the structural viral proteins are expressed. An early effect of poxvirus infection on the host cell is the inhibition of cellular protein and DNA synthesis (Talavera and Rodriguez, 1991b).
1.13. Isolation of Capripoxvirus

Capripoxvirus isolation is a lengthy procedure due to slow growth of the virus in cell culture, in which cytopathic effects may take up to 10 days to develop (Plowright and Ferris, 1958).

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin papules, Lung lesions or lymphnodes. Samples for virus isolation and antigen detection enzyme-linked immunosorbant assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralizing antibodies.

Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralizing antibody is present. Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetracitic acid) during the viraemic stage of Capripox (before generalization of lesions or within 4 days of generalization), can also be used for virus isolation. Samples for histopathology should include tissues from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin.

1.14. Virus purification and characterization

Capripoxviruses are purified by different methods, sucrose gradient (440-60%) and potassium tartrate gradient (density range 1.05-1.35 g/ml) centrifugation of partially purified virus through a sucrose gradient (36%) (Kitching et al., 1986b). Viruses of scab origin are purified in a similar fashion (Rao et al., 1997a).

The main structural polypeptides of Capripoxviruses are identical on analysis by polyacrylamide gel electrophoresis (PAGE), and more than 20 polypeptide bands can be distinguished in a purified virus preparation (Kitching et al., 1986b). The molecular weight of the polypeptides ranges from 14 to 130 kDa, but only a few of them are immunogenic (Roy. 1994; Jaikumer et al., 1996). The major common precipitating antigen, of about 67 kDa, induces serum-neutralizing antibodies (Singh and Rai. 1991b).

Immunization with poxviruses evokes both humeral and cell-mediated immune responses (Pandey et al., 1969; Negi et al., 1988). The relative importance of circulating antibodies and cytotoxic T lymphocytes in the suppression of the infection is not fully
understood. However it is quite clear that infection subsides in the host with the appearance of circulating antiviral antibodies (Cho and Wenner, 1973). The serum of the immune host contains a spectrum of antibodies reactive with virus-induced antigen (Ved et al., 1969; Weinraub and Dales, 1974).

1.15. Propagation and cultivation of SPV and GPV

Natural hosts are used successfully for cultivation of the Capripoxviruses. Growth of the virus in heterologous hosts, i.e. GPV in sheep and SPV in goats, is achieved with varying success. The viruses do not grow in any species of laboratory animal. The reports of cultivation of SPV in embryonating chick eggs are conflicting. Ortitenz and Teicco (1954), Abdulla Khan (1960), Sharma et al. (1966), Adlakha et al. (1971), Onar (1971-72), Sen and Uppal (1972), Sharma and Dhanda (1972) and Bhatnager and Gupta (1974) failed to propagate SPV in chick embryos. Gins and Kunert (1937) and Aygun (1955), however, observed oedema, leucocytic infiltration and necrosis in the infected CAM. Rao (1938), in addition, reported cytoplasmic inclusions in the ectoderm of the CAM. Sabban (1957), adapted a virulent strain of SPV (Cairo strain) on the CAM of 12 days chick embryos. No loss of virulence of sheep was observed after 21 alternate passages through eggs and sheep. A Roumanian strain of SPV could not be adapted to embryonating chick eggs. Yuan et al. (1957) reported attenuation of SPV after 90 successive passages on the CAM and used it as vaccine. Likhachev et al. (1961), stated that the chick embryo adapted Chinese strain was harmless for sheep and induced strong immunity at a dose of 0.2 ml intradermally or 0.5 ml subcutaneously. Borisovich (1962) and Borisovich et al. (1966) reported that this chick embryo adapted strain was strongly immunogenic. Arik and Kurtal (1974) successfully passaged SPV on CAM 25 times without any change in viral characteristics. GPV isolates differ significantly in their growth behavior in embryonated chick eggs (Adlakha et al., 1971; Tantawi and Al-Falluji, 1979). Iranian, Kenyan and Indian strains grow well on chorioallantoic membranes (Joshi et al., 1996), whereas Russian strains do not. The Iranian Gorgon strain and Egyptian strains grow poorly on chorioallantoic membranes.

SPV produces CPE in various cell culture systems. Edlinger and Iftimovici (1973) in their studies on SPV in lamb kidney and testicle cell cultures found that the CPE was
non-specific and consisted of diffused areas of rounded cells without plaque formation and no detachment of the cell layer. Mateva and Stoichev (1975) also could not find any characteristic CPE with SPV in LT cells. Studies on the replication of SPV in LT cells have shown that most of the progeny virus remained intracellular, as only approximately 3% of the total yield was released into the medium, the rest of the virus remaining cell-associated (Srivastava 1975). GPV and SPV are adapted to and are able to grow in a number of host systems in vitro, most of which are derived from sheep, goats and cattle. Of these, lamb testis (LT) cells and sheep or goat kidney cells are very sensitive systems in which the viruses produce characteristic cytopathic effects, such as rounding, tract formation, retraction and ballooning of cells, nuclear vacuolation, chromatin fragmentation, loss of continuity of the cell sheet, and intracytoplasmic inclusion bodies (Adlakha et al., 1971; Joshi et al., 1994). Other cell culture systems, e.g. sheep thyroid cell monolayers and embryonic goat cell culture, are also quite sensitive. Some isolates will grow in bovine fetal muscle cells and calf kidney cells (Davies and Mbugwa, 1985).

The viruses (SPV, GPV) can also be grown in some established cell lines. GPV will grow in the Vero (Ved Prakash et al., 1994) and MDBK cell lines (Joshi et al., 1995). Similarly, most SPV isolates will grow in MDBK (Pandey et al., 1985) and Vero (Singh and Rai, 1991a) as well as BHK21 cells (Kirubaharan et al., 1993).

### 1.16. Soluble antigens

Cragie (1932) used the term ‘filterable precipitable substances’ to describe the material present in virus-free extracts of vaccinia-infected tissues that reacted with anti-vaccinia sera. Since then, the term ‘soluble antigen’ has been applied to virus-free, serologically reactive extracts obtained from virus-infected tissues after the viral particles have been pelleted by high-speed centrifugation (Anthony et al., 1971).

These antigens, which are released or are separable from the infectious virions, are presumed to represent structural subunits of the virus and virus-induced enzymes. Furthermore, immunological analysis of preparations of soluble antigens of capripoxvirus reveals a number of immunoprecipitinogens. Hence, soluble antigen is considered a valuable diagnostic tool. Soluble antigens are abundantly available in the scab lesions of poxviruses compared with infected cell cultures.
A soluble antigen fraction is obtained by high-speed centrifugation of clarified scab suspensions (Rao et al., 1996a). The clear supernatant is saved and concentrated, if necessary, by reverse dialysis with polyethylene glycol. The soluble antigens are also extracted by treating the crude antigen with fluorocarbon followed by centrifugation (Pandey and Singh, 1972; Isloor and Negi, 1995). All the methods use centrifugation to precipitate the virus particles physically, leaving the soluble antigens in the supernatant fluid; this fluid can be stored indefinitely at −20°C in small aliquots. Soluble antigens are necessarily virus-induced antigens that are produced perhaps in excess quantities during viral replication. The antisera raised against them can neutralize the infectivity of the virus effectively because some of the soluble antigens are structural components of the virus (Sambyal and Singh, 1978; Tiwari and Negi, 1994; Rao et al., 1997a).

The soluble antigens of GPV or SPV also contain a major polypeptide of about 67 kDa, which may be similar to that found in the intact virion. These properties, together with its noninfectious nature, make the soluble antigen fraction an important diagnostic tool for poxvirus diseases. The soluble antigens differ markedly in their molecular weight, with five to seven precipitinogens detectable.

Among the soluble GPV antigens, at least one precipitinogen is stable at boiling temperature for 10 min, and another is resistant to trypsin. Ether and chloroform have no effect on the immunoprecipitation pattern of the soluble GPV antigens (Isloor and Negi, 1995). The soluble SPV antigens show a varied electrophoretic mobility, with sharing of antigenic determinants among a few proteins (Rao et al., 1997a).

Moreover, their physicochemical properties differ slightly from those of GPV, as all the soluble SPV antigens are sensitive to trypsin and ether as well as to boiling temperature. Chloroform, however, has no effect on their immunoprecipitation pattern.

1.17. Antigenic relationship

Capripoxviruses are antigenically related, and GPV and SPV share one or more precipitin band depending upon the antiserum used (Sharma and Dhanda, 1969; Subbarao et al., 1984). The viruses cross-react in the complement fixation test (Sharma and Dhanda, 1971). The sharing of antigens between the viruses can be further demonstrated
by ELISA, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the leukocyte migration inhibition test, immunoelectrofocusing (IEF) and IEF-gel blotting.

The techniques of IEF, IEF-gel blotting and dot-immunobinding are useful in the differential diagnosis of goat pox and sheep pox (Indian Veterinary Research Institute, 1986; Datta and Soman, 1990). Though the major portion of the antigens of GPV and SPV are similar, there may be some differentiating antigenic components in each virus (Rao et al., 1996b).

Moreover, within isolates or strains of SPV (and possibly GPV), the antigenic make-up is largely similar (Bhat, 1993) but may not be identical (Puranchand et al., 1987). Furthermore, it seems that neither GPV nor SPV is serologically related to contagious pustular dermatitis virus, a parapoxvirus (Rao et al., 1999; Ngichabe et al., 1999); however, the results of cross-reactions often vary, which is probably due to the different methods used by different workers employing different isolates of capripoxvirus (Sharma and Dhanda, 1971; Subbarao et al., 1984; Kitching et al., 1986b).

1.18. Diagnostic tests

Pox diseases are usually identified according to their clinical signs and gross pathology and the host species affected. The infections caused by GPV and SPV cannot be distinguished either serologically or on the basis of the clinical signs as the viruses are very closely related.

It appears that the host preference shown by these viruses with respect to either sheep or goats, accompanied by the case history, may be regarded as partially affirmative for either sheeppox or goatpox, but accurate identification requires laboratory studies. It is also known that homologous diagnostic reagents tend to be more efficient than heterologous reagents in the accurate diagnosis of these diseases (Pandey and Singh, 1972).

Therefore, separate diagnostic procedures have evolved for the diseases. The non-infectious soluble antigen fraction alone can efficiently replace the use of infected scab suspensions or infectious virus, which pose a considerable disease security problem as the virus is highly infectious (Rao and Negi, 1997; Rao et al., 1997b; Singh et al., 1998).
In addition, the diagnosis of goatpox and sheeppox by classical virological or serological techniques that depend on live viruses is not suitable in countries where the viruses are exotic and live viruses are not available. There is an extremely useful diagnostic method based on the polymerase chain reaction (PCR) for the detection of the viral nucleic acid of GPV and SPV in those countries that are free of these viruses.

Besides animal inoculation tests in natural hosts, the following tests can be employed routinely for the diagnosis of goatpox and sheeppox in field samples.

1.18.1. Serological diagnosis

1.18.1.1. Virus neutralization

A test serum can either be titrated against a constant titre of Capripoxvirus (100 TCID$_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralization index (Kitching and Carn, 1996). Because of the variable sensitivity of cell culture to Capripoxvirus, and to consequent difficulty of ensuring the use of 100TCID$_{50}$, the neutralization index is the preferred method. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralization test has been reported to give more consistent results (OIE Manual, 2004).

1.18.1.2. Agar gel precipitation test (AGID)

A gel diffusion technique for the diagnosis of sheeppox and goatpox was introduced as early as the 1960s, using either homologous (Bhambani and Krishnamurthy, 1963) or heterologous (Uppal and Nilakantan, 1967) antiserum. Currently, a more efficient agar gel precipitation test (AGPT) using better diagnostic reagents, such as the soluble antigens, is available (Rao and Negi, 1997; Rao et al., 1997b). In addition, the use of $[^{35}S]$methioninelabeled antigen preparations considerably improves the sensitivity of the AGPT in the detection of capripoxvirus antibody (Kitching et al., 1986b).
1.18.1.3. Counter-immunoelectrophoresis (CIE)

Immuno-electrophoretic precipitation is commonly used for the detection of various antigens and antibodies. The counter-immunoelectrophoresis test (CIE) test is more sensitive and rapid than the AGPT in the diagnosis of goatpox (Sharma et al., 1988a). Normal saline solution may be used as an alternative to barbitone buffer to carry out the CIE test to make it more efficient (Rao et al., 1999). The test can also detect antigens in organs of sheep infected with sheeppoxvirus (Puranchand et al., 1985), and it is also used for the rapid diagnosis of sheeppox (Rao et al., 1997b).

1.18.1.4. Latex agglutination test

Latex beads provide a convenient carrier for antigens in agglutination tests, and coating the beads with antigen is not difficult (Hudson and Hay, 1989). Latex agglutination assays have been used successfully in the detection of various antigen–antibody systems and have proved to be rapid and simple to perform, and they need no expensive equipment. The test is more efficient than the CIE test in the diagnosis of goatpox in field samples (Rao et al., 1996a) and can also be used to diagnose sheeppox (Rao et al., 1997c).

1.18.1.5. Reverse-phase passive hemagglutination test

Capripoxviruses are non-hemagglutinating (Matthews, 1982). Therefore, an indirect hemagglutination test, such as the reverse-phase passive hemagglutination test using sensitized sheep erythrocytes, is useful in the laboratory diagnosis of goatpox and is more sensitive than the AGPT and the CIE test. Among the various types of sensitized sheep erythrocytes, glutaraldehyde- and tannic acid-treated erythrocytes give the best results for the diagnosis of goatpox and sheeppox (Rao and Negi, 1997; Rao et al., 1997b). Several agglutination tests, viz. coagglutination (Joshi et al., 1989), passive hemagglutination (Joshi et al., 1991) and spot agglutination (Tiwari et al., 1996), are also available for the simple and rapid diagnosis of goatpox. Though all the hemagglutination tests consist basically of an indirect hemagglutination test that has been modified slightly, the coagglutination test is based on the ß-globulin-binding ability of protein A, which is found frequently on the cell wall of certain strains of Staphylococcus aureus.
1.18.1.6. Indirect fluorescent antibody test

Capripoxvirus-infected cell culture grown on flying cover-slips or cell culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected cell culture control, and positive and negative control sera, should be included in the test.

The infected and control cultures are fixed in acetone at -20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein thiocyanate (Davies and Atema, 1978). Cross reaction can occur with Orf, bovine popular stomatitis virus and perhaps other poxviruses.

1.18.1.7. Western blot analysis

Western blotting of test sera against Capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to Capripox structural proteins, although the test is expensive and difficult to carry out (Chand and Black, 1994).

1.18.1.8. Single radial hemolysis test

This test depends on the fact that complement lyses erythrocytes when antigen–antibody complexes use it. The test gives a simple and quantitative estimate of antigen and antibody and is used successfully to diagnose goatpox and sheeppox (Rao and Chandra, 1986; Tiwari and Negi, 1996a).

1.18.1.9. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is now used widely to detect antibodies and antigens in a variety of test systems, and is more sensitive than virus-neutralization tests (Carn et al., 1994a). Various methods of ELISA are available to diagnose goatpox and sheeppox, but problems such as a considerable background reaction (Sharma et al., 1988b) and the requirement for special reagents, such as recombinant proteins (Carn, 1995), often limit their use as routine screening tests.

Hence, an immunocapture ELISA has been developed as a relatively simple assay for the detection of GPV and SPV antigens in scab suspensions (Rao et al., 1997d).
However, this assay also has a limitation in that it is best used only in combination with the CIE test for accurate and confirmative diagnosis.

A dot ELISA, carried out on nitrocellulose strips or paper, is a valuable addition to the battery of diagnostic methods for goatpox and is about three times more sensitive than the single radial hemolysis test (Tiwari and Negi, 1996b). Recently, an avidin–biotin ELISA was introduced for the detection of antibodies to GPV in goat sera; it uses an isolated fraction of the soluble antigens that substantially reduces the background reaction in the assay (Rao et al., 1999).

This assay is a reliable test for capripoxvirus antibody that may be used to assess immunity to goatpox and possibly sheeppox, and can also be used in epidemiological studies.

1.18.2. Molecular Methods

There have been few studies of the molecular biology of GPV and SPV. Restriction analysis of genomes of the viruses isolated from different countries revealed that sheeppox and goatpox are caused by very closely related isolates of capripoxvirus (Black, 1986). Such analyses not only demonstrated the genetic relatedness (Black et al., 1986; Bhat et al. 1993) but were also used as molecular epidemiological tools for the characterization of the viral genomes of capripoxviruses (Bhat and Mishra, 1989).

The extent of nucleotide divergence values within the genomes suggest that the typical sheep and cattle isolates are more closely related to each other than to goat isolates (Gershon and Black, 1988).

There is, however, less divergence between Capripoxvirus genomes than that seen in orthopoxvirus genomes (Gershon et al., 1989b). Furthermore, the Capripoxviruses can be used successfully as viral vectors in the preparation of recombinant vaccines by incorporating foreign viral genes, such as those of the rinderpest and bluetongue viruses (Romero et al., 1993; Wade-Evans et al., 1996).
1.18.2.1. Polymerase chain reaction (PCR)

PCR was discovered by Kary B. Mullis in 1983 (Mullis, 1990). PCR can be defined as an in vitro (cell free) method for enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. Repetitive cycles involving template denaturation, primers annealing and the extension of annealed primers by DNA polymerase results in the exponential accumulation of specific fragment whose termini are defined by 5’ ends of the primers (Reubel and Studdert, 1998).

Although they are sensitive, methods such as ELISA and virus isolation in cell culture fail to detect virus particles that are bound to neutralizing antibody (Ireland and Binepal, 1998), and the sensitivity of precipitation and agglutination tests is relatively low. Hence, highly sensitive molecular biological techniques based on the PCR are now employed for the identification of many viruses, including GPV and SPV in skin biopsies and cell cultures (Ireland and Binepal, 1998; Heine et al., 1999; Mangana-Vougiouka et al., 1999; Parthiban et al., 2005).

The oligonucleotide primer pairs used for PCR amplification of genomes of either GPV or SPV are listed in Table 1. PCR-based diagnostic methods are exceptionally effective for the diagnosis of goatpox and sheeppox in suspected skin samples obtained from the field (Rao et al., 2001).

However, differential diagnosis of goat pox and sheep pox based solely on the PCR technique is not yet feasible, but may be possible by restriction analysis of PCR-amplified products.

Sheep and goatpox should be differentiated from: Bluetongue, peste des petits ruminants, contagious ecthyma, photosensitization, dermatophilosis, insect bites, parasitic pneumonia, caseous lymphadenitis and mange (scabies) (OIE Manual 2004).

1.19. Clinical management

All infected sheep or goats should be placed in a clean, well-ventilated enclosure and fed a balanced diet at a high level. Animals reluctant to feed should be given 10% glucose saline parenterally. All diseased animals should be treated with antibiotic to restrict secondary bacterial infections.
To relieve respiratory-related signs, the nostrils should be cleaned and washed with a weak solution of potassium permanganate (1:10 000). Respiration should be stimulated with eucalyptus oil inhalations or coramine. Antibiotic ointment or powder should be applied topically to the skin lesions (Nandi et al., 1999).

1.20. Immunity of Capripoxvirus

Maternal immunity provides protection from sheep and goatpoxvirus up to 3 months (Kitching, 1986). Animals that have recorded from Capripoxvirus infection do not remain carriers of the virus and have lifelong immunity.

1.21. Control of diseases (SPV, GPV)

In Sudan, a live attenuated vaccine developed against Capripox, from strain 0240 by serial culturing in LT cells was used to protect sheep and goats throughout the Capripox enzootic areas. The vaccine is stable and safe to use, and provided substantial protection for at least a year (Kitching et al., 1986). In enzootic areas, both live attenuated and inactivated vaccines may be useful in the prevention and control of goatpox and sheeppox. However, the inactivated vaccines that are available for immunization against GPV and SPV infections give only short-term immunity (Prasad and Datt, 1973; Yadav et al., 1986).

The live attenuated vaccines are highly immunogenic but their usefulness is limited because they stimulate a pock reaction and/or lead to the death of some of the vaccinated animals because of generalization of the disease. Usually, homologous vaccination incorporating the locally prevalent strains of GPV or SPV is successful in protecting goats and sheep against goatpox and sheeppox.

Therefore, in different countries and sometimes within a country, various live attenuated vaccines for goatpox have been available from time to time, with varying degrees of protective efficacy (Ramyar et al., 1974; El-Zein et al., 1983; Davies and Mbugwa, 1985; Guo et al., 1986; Wang and Jiang, 1988; Mahmood et al., 1993).

Similarly, live attenuated vaccines using different isolates of SPV serially passaged in various cell culture systems have been available for the control of sheeppox (Singh et al., 1984; Mahmood et al., 1988; Chandra et al., 1990). In India, a live
attenuated vaccine prepared from the Romanian strain of SPV is currently used to confer immunity in all types of sheep against sheeppox and is quite safe and effective (Jadhav et al., 1989).

Although it may not always be successful, a single vaccine prepared from a strain of capripoxvirus that infects sheep and goats equally can be effective in controlling both sheeppox and goatpox (Kitching et al., 1987). A subunit vaccine has proved effective in protecting goats against capripox (Carn et al., 1994b).

On the whole, reports of cross-protection of sheep and goats against goatpox and sheeppox and other related diseases, such as contagious pustular dermatitis, are often contradictory and inconclusive because attempts to protect goats with SPV vaccines are usually unsuccessful (Prasad and Datt, 1973). Hence, it is recommended that homologous vaccines should be used to protect goats and sheep against goatpox and sheeppox.

Table 2: Oligonucleotide primers for PCR amplification

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5´Æ 3´)</th>
<th>Length (bp)</th>
<th>PCR product size (bp)</th>
<th>Virus detected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Left</td>
<td>TTTCTGATTTTTCTTTACTAT AAATTATATACGTAAATAAC</td>
<td>21 20</td>
<td>192</td>
<td>SGPV</td>
<td>Ireland and Binepal, 1998</td>
</tr>
<tr>
<td>2. Left</td>
<td>ATGGACAGAGCTTTATCA TCATAGTGTTGTACTTCG</td>
<td>18 18</td>
<td>472</td>
<td>GPV</td>
<td>Ireland and Binepal, 1998</td>
</tr>
<tr>
<td>3. Left</td>
<td>GTCTGACTTTTCCCTGGCGAAT TCTATTTTATTTCCGTATATC</td>
<td>20 20</td>
<td>149</td>
<td>SPV</td>
<td>Mangana-Vougiouka et al., 1999</td>
</tr>
<tr>
<td>4. Left</td>
<td>AGAAACGAGGTCTCGAAGCA GGAGGTTGGCTGGAAATGTGT</td>
<td>20 20</td>
<td>289</td>
<td>SPV</td>
<td>Mangana-Vougiouka et al., 1999</td>
</tr>
<tr>
<td>5. Left</td>
<td>CTAAAATTAGAGAGCTTACTAC TCTT CGATTCCATAAAACTAAAGTG</td>
<td>25 21</td>
<td>390</td>
<td>SPV</td>
<td>Heine et al., 1999</td>
</tr>
</tbody>
</table>
CHAPTER II
MATERIALS AND METHODS

2. Field work
2.1. Sheep and goatpox outbreaks

Two outbreaks of sheeppox that occurred in June 2003 in Gedarif State, east of the Sudan were investigated. The first outbreak occurred at Shott, east river Atbara and the second outbreak occurred at Abu Garra, south of Showak city. No infections in goats were recorded. Another outbreak of goatpox occurred at Hillat Kuku in Khartoum State, in March 2005. The disease affected Saanen goats imported from Holland and raised for breeding by Ministry of Animal Resources (MAR). These goats were previously vaccinated against sheep and goatpox with 0240 vaccine strain.

During each investigation the total number of animals at risk, number affected, number dead as well as other species of animals affected were recorded. Clinical signs and course of the disease were noted. Table 3 shows total number of animals at risk and epidemiology of sheep and goatpox infections in Gedarif and Khartoum states.

2.2. Collection of samples

Two samples of skin scabs (SP1O) from Shott region and skin nodule (SP2) from Abu Garra region were collected from sick sheep. Two samples of skin scabs (GP1 and GP2) were collected from sick goats (Saanen breed). These samples were collected into 50% buffered –glycerol solution (Appendix 6.1) and stored at + 0\textdegree C until brought to Virology Research Laboratory, Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum on ice.

2.3. Laboratory work
2.3.1. Viruses
2.3.1.1. Laboratory strains of sheeppox

Sheeppox isolate (SP4) obtained from the virus stock of Virology Research Laboratory Department of Microbiology, Faculty of Veterinary Medicine, University of
Khartoum, SP4 was isolated in LT cell culture from skin scabs collected from sick sheep in an outbreak that occurred at Gezira State in 1997.

2.3.1.2. Lumpy skin disease virus (LSD)

This strain of LSD was isolated from cattle during an outbreak of the disease that occurred in Gezira State in June 2003 (Khalafalla, unpublished data).

2.3.1.3. Virus samples for Kenya sheep and goatpox vaccine strain 0240

Samples of the vaccine strain 0240 were obtained from the vaccines seed bank, at the viral vaccines production unit of the Central Veterinary Research Laboratories (CVRL), Khartoum, Sudan.

2.3.1.4. Hyper immune serum

This was obtained from the vaccines seed bank, at the Viral Vaccines Production Unit of the Central Veterinary Research Laboratories (CVRL), Khartoum, Sudan.

2.3.2. Preparation and sterilization of glassware

Glassware like flasks, beakers and measuring cylinders were rinsed in running tap water, brushed with special tissue culture flasks soap, and left overnight in 1% HCL. They were then washed thoroughly by rinsing several times in tap and distilled water. After that, they were wrapped with aluminum foil and sterilized in hot air oven at 160 °C for 2 hours. The volumetric pipettes were left overnight in 3% HCL. Then they were washed thoroughly by rinsing several times in tap and distilled water. The clean dry pipettes were cotton plugged, placed in canisters and sterilized in hot air oven at 160 °C for 1 hour.

2.3.3. Preparation and sterilization of plastic wares

Rubber liners for bottles and plastic cylinders were cleaned with detergent, washed with tap water followed by distilled water, left to dry and sterilized by autoclaving at 121°C for 15 minutes.
2.3.4. Preparation and sterilization of filters and papers

Millipore filters (0.22µ) were assembled in their appropriate holders, wrapped in aluminum foil and sterilized by autoclaving at 121°C for 15 minutes.

2.4. Virus isolation

2.4.1. Preparation of samples

Skin specimens were homogenized using sterile mortars and pestles with the aid of sterile sand and physiological saline (PS) (Appendix 1.1). 20% suspensions were made and then centrifuged at 1000 rpm for 10 minutes. Supernatant fluids were collected into sterile bottles and treated with antibiotics (1000 IU penicillin- 250 mg of streptomycin (Appendix 9.5.1) and 5.000 IU of mycostatin (Appendix 9.5.4). The supernatant fluids were left for ½ -1 hour at 4°C and then stored at -20°C till used. In vesicle swabs samples 2ml of medium, Glassgow Modified Eagle, s Medium (GMEM) with antibiotics, were added, mixed and left overnight at 4°C, then centrifuged at 1000 rpm for 10 minutes and supernatant fluids were collected.

2.4.2. Fertile eggs

Nine to eleven days-old chicken eggs were obtained from the poultry farm of department of Microbiology, Faculty of Veterinary medicine, University of Khartoum. The eggs were candled for viability and then the shell surface was cleaned by immersing in alcohol for 5 minutes or by swabbing with tincture of iodine. The eggs were used to grow the virus on the chorioallantoic membrane (CAM).

2.4.3. Inoculation of the chorioallantoic membrane (CAM)

A hole was made through the shell of the air sac by the pointed punch and another hole on the top of the egg just to penetrate the shell and shell membrane. The dropping of the membrane was verified in dark room by candling, and then a rubber bulb was placed over the hole in the air sac to slowly aspirate air from the sac by releasing pressure on the deflated bulb. The suction caused false air sac in the area of the second hole. To deliver the inoculum on the chorioallantoic membrane (CAM), the tip of one ml needle was inserted just within the shell and 0.2ml was inoculated into embryonated eggs. Four
samples (SP1O, SP4, and GP1) and vaccine strain 0240 were inoculated on CAM. The inoculated eggs were incubated at 37°C, and were examined daily for 5 days.

2.4.4. Harvest of the chorioallantoic membrane (CAM)

After 5 days incubation the shell was disinfected on the small end of the egg by 70% alcohol, and the end of the egg was cracked off by sterile forceps. The CAM was removed from the shell by sterile forceps. The CAM was placed in a petri dish and examined for pock lesion formation and or any other lesions.

2.4.5. Preparation of cell culture

2.4.5.1. Primary lamb testis cell culture (LT)

Primary lamb testis (LT) cell cultures were prepared from prepubertal lamb as described by Plowright and Ferris (1958). The testis were aseptically removed from 2-6 weeks old lamb immediately after slaughter and placed in sterile Petri dishes and all tunica vaginalis, epididymes and testicular bursa were removed. Testis were then transferred to other Petri dishes then fragmented with scissors into small pieces. The tissue fragments were then transferred to a trypsinization flask containing a magnetic bar and washed several times with PD till the supernatant fluid become clear. A freshly prepared prewarmed 0.25% trypsin in HPSS (10% trypsin in Hank,s) was added to the washed tissue fragments in amounts sufficient to cover them. The contents were stirred for 30 minutes at room temperature and the supernatant fluid was discarded, fresh prewarmed 0.25% trypsin solution was added at a volume that was three times the volume of tissue fragments. The tissue fragments were then digested for 1 hour at 37°C in 10% trypsin in HPSS. At the end of the trypsinization period, large fragments of tissue were removed by filtration through sterile gauze. The dispersed cells in the filtrate were deposited by the centrifugation at 600 rpm for 5 minutes.

The cells were then washed once with PD containing 2% calf serum and centrifuged. The packed cells were resuspended in 10 ml of PD and vigorously pipetted. The cells suspension was finally diluted in growth medium and then distributed in the tissue culture flasks. Confluent monolayers were established within 3-5 days.
2.4.5.2. African Green Monkey Kidney cell culture (Vero cell culture)

Confluent monolayer cell culture of Vero cell was purchased from Veterinary Laboratories Agency, United Kingdom. The growth medium was removed and the cell briefly washed with sterile PD. Three ml of trypsin and versin solutions were added and the bottle incubated at 37°C until cells flew freely when the bottle was tilted. Few drops of calf serum were added to stop the action of trypsin and versin and the suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was pour out and the pelleted cells were resuspended in 10 ml of growth medium and mixed well by pipetting. The suspension was diluted in growth medium and distributed in tissue culture flasks or tubes and then incubated at 37°C.

2.4.5.3. Madin Darby Bovine Kidney cell lines

Confluent monolayer of Madin Darby Bovine Kidney cell line (MDBK) was purchased from Veterinary Laboratories Agency (VLA), United Kingdom and was prepared as described for preparation of Vero cell line.

2.4.5.4. Preparation of chicken embryo fibroblast (CEF)

Embryonated chicken eggs 9-11 day old were obtained from the poultry farm of Department of Microbiology Faculty of Veterinary Medicine, University of Khartoum. Eggs were candled for viability and then the shell surface was cleaned by immersing in 70% alcohol for 5 minutes or by swabbing with tincture iodine. Round shell just below the air sac was cut, the embryo lifted out. The head was cut, viscera were removed and the remains were fragmented with scissor into small fragments. Then the fragments were washed several times with PD and transferred to a trysinization flask.

The trysinization was carried out in discontinuous manner; 5 ml of 0.25% prewarmed trypsin solution per embryo were added and stirred slowly for 15 minutes at 25°C or 37°C. After the tissue had settled out, the supernatant cells (containing single cells and small clusters of cells) were collected in a container with 1-2 ml of calf serum and kept on ice; this was repeated 3-5 times until only the white fibrous tissue were left and no more cells disperse. The procedure for preparation of monolayer was similar to
that described for lamb testis cell culture. A confluent monolayer was established within 1-2 days.

2.4.6. Virus propagation and adaptation

Four samples (SP1O, SP2, SP4 and GP1) were inoculated into semi confluent lamb testis cell culture in 25cm$^3$ tissue culture flask. Each flask received 0.5 ml of virus suspension and left for 1 hour at 37°C for adsorption. Then the inoculum was removed and the semi confluent monolayer were washed twice with PD and refed with maintenance medium (GMEM) and kept at 37°C. At day 7 post inoculation, the monolayer was splitted using trypsin-versin and the infected cells reseeded again. This procedure was repeated for 3 passages in (LT), one passage in MDBK cells and 2 passages in Vero cell until clear CPE was seen. The cultures were harvested by freezing and thawing 3 times, and then centrifuged at 1000 rpm for 5 minutes. The supernatant was collected and labeled as stock virus.

2.4.7. Cell culture spectrum

Prepared LT cells, Vero cells, MDBK cells and CEF cells were inoculated, splitted, reseeded again and harvested as described for propagation and adaptation of the virus.

2.5. Virus titration

Determination of tissue culture infective dose 50/ml (TCID$_{50}$/ml) for SPV and GPV was performed following the procedure described by Villegas and Purchase (1983). Micro plates containing 96-wells were used. Ten-fold dilution of virus ($10^{-1}$ _ $10^{-8}$) was prepared in GMEM. 100µl of each dilution was added to 5-wells then 100µl of 2X Vero cells suspension was added to each well. The micro plate was incubated at 37°C and examined for the presence of CPE 9 days post inoculation. Titres were expressed as TCID$_{50}$/ml calculated according to the method of Reed and Muench (1938) as described by Villegas and Purchase (1983).
2.6. Virus neutralization

The alpha neutralization procedure (constant-serum, diluted virus) as described by Beared (1983) was followed. In this method, micro plate containing 96-wells were used, 10 fold serial dilutions (10^{-1} - 10^{-8}) of virus under test were prepared in GMEM and each 5-wells received 100µl of virus dilution. Each virus dilution was mixed with an equal volume of 1/5 dilution of hyper immune serum (HIS) against SPV and GPV (100µl). The mixtures were shaken vigorously and incubated at 37°C for ½ hour. After that 100µl of Vero cells suspension were added to each well and incubated at 37°C. The end point titre of each serum-virus mixture was calculated. Determination of the neutralization index (NI) was made by calculating the difference between the log titre of the virus titration and the log of HIS-virus mixture.

2.7. Polymerase chain reaction (PCR)

2.7.1. DNA extraction

Three methods of DNA release were used. The first method of DNA extraction (PCR1) was the simplest method: by adding (20-15-10-5 µl) of cell culture or biopsy supernatant to the PCR minus the polymerase and heat the reaction at 99°C for 15 minutes. The second method of DNA extraction (PCR2) was done according to Tripathy (2003) with phenol-chloroform extraction .2 ml of cell lysis solution (10mM Tris-Hcl,ph7.4),100mM Nacl,10mM EDTA, 0.5% SDS, 2% β mercaptoethanol) were homogenized with scabs in morter and pestle ,and then transferred to eppendorf tube.Then 15µl of protinase K solution (10mg/ml) was added and the sample was vortixed for few seconds, incubated at 55°C for 4 hours or at 37°C for overnight ,then the sample was vortixed after every 15 minutes during incubation.10 µl of RNase were added and inverted 25 times, then incubated at 37°C for 15 minutes .DNA was extracted from the digest in the presence of equal volumes of phenol-chloroform-isoamyalchohole (50:2:48).The extraction lasted for 30 second with mixing by inverting the eppendorf tube followed by centrifugation at 10,000 rpm for 1 minute. The tube will be divided to three layers, phenoic phase, interphase (protein), aqueous phase.

The aqueous phase was removed to another eppendorf tube with phenol-chloroform and this was repeated for three times. The last aqueous phase was transferred
to another tube (1.5ml) and 3Mna acetate were added in a volume equivalent to 1:10 of aqueous phase and an equal volumes of Isopropanol alcohol was mixed and stored at -20°C overnight. The DNA was pelleted by centrifugation at 13,000 rpm for at least 45 minutes at +4°C. Then the DNA pellet was washed with 300µl 70% alcohol with centrifugation at 13,000 rpm for 15 min at +4°C, and the ethanol was poured off. The tubes were then allowed to dry for 10-15 minutes. .50µl DNA hydration solution was added and DNA was dissolved by incubating at 65°C for 1 hours and then the DNA was stored at 4°C until used.

The third method of DNA extraction (PCR3): when 90% CPE appeared, cell culture in plastic flasks were harvested by freezing and thawing three times, then pelleted by ultracentrifugation (Suprafuge 22, fixed angle, rotors HFA 22.50, 8X50ml, Heraeus Sepatech), for 1hour at 20000 rpm to pellet the virus. The supernatant was discarded, the pellet drained, covered with 0.5 ml of PBS and kept at 4°C overnight. The pellet was dissolved and transferred to an eppendorf tube (1.5ml).

DNA was extracted by using PUREGENE® DNA isolation kit (Gentra System, Minneapolis, USA) with some modifications. Briefly, 300µl cell lysis solution was added to the cell lysate in 1.5ml micro tube, and the mixture was homogenized in a vortex. Then 2µl of proteinase K solution (20mg/ml) were added to the lysate and the suspension was mixed by inverting 25 times, incubated at 55°C overnight, followed by the addition of 1.5µl RNase A solution (4mg/ml) to the cell lysate. The samples were then mixed by inverting the tube 25 times and the tubes were incubated at 37°C for 15-60 minutes, cooled to room temperature and 100µl protein precipitation solution was added to the cell lysate. The tubes were vortexed at high speed for 20 seconds and then centrifuged at 16.000xg for 3 minutes. Then 300µl of 100% Isopropanol was added to the supernate fluid into a clean 1.5 microfuge tube, mixed by inverting gently 50 times, and centrifuging at 16.000xg for 1 minute to pellet the DNA. The supernatant was poured off and the tubes were drained briefly, after that DNA was washed with 300µl of 75% ethanol, centrifuged at16.000xg for 1 minute and the ethanol was poured off. The tubes were then allowed to dry for 10-15 minutes. 50µl DNA Hydration Solution was added and DNA was dissolved by incubating overnight at room temperature and then stored at -20°C until used.
2.7.2. Oligonucleotide Primers

Primers were purchased as freeze dried oligonucleotides (INVITROGEN). Reconstituted with 1ml DDW and left overnight at 4°C. Working solutions of each primer were prepared so as to contain 10Pmol/µl of oligonucleotides pair of primers of viral attachment protein (VAP) gene were described by Ireland and Binepal (1998), the VAP F sequence is (5´ to 3´) TCC GAG CTC TTT CCT GAT TTT TCT TAC TAT with molecular weight of 9056.0 and the sequence of VAP R is (5´ to 3´) TAT GGT ACC TAA ATT ATA TAC GTA AAT AAC with molecular weight 9197.0.

2.7.3. PCR Condition

The PCR condition was described by Ireland and Binepal (1998), amplification was achieved by 35 cycles each including a denaturation step at 94°C for 1 min, annealing step at 50°C for 30 seconds and extension step at 72°C for 1 min. The final step was prolonged to 5 min to ensure complete extension of amplification products. The PCR reaction was carried out in a Biometra T3 Thermal cycler (BIOMETRA, Germany).

2.7.4. Detection of PCR product

The PCR products were separated electrophoretically in 1.5% agarose gel (SIGM) (1gm of agarose was dissolved in100 ml TAE buffer) containing Ethidium bromide 1µl/40ml agarose (PROMEGA, Madison, USA, 10mg/ml). 10µl of 100bp DNA Ladder (INVETROGEN) which was prepared by adding 20µl of ladder to 80µl of bromophenol blue dye (bromophenol blue 11% + Glycerol 40µl +DDW 50µl) was loaded in the first slot of the gel. The 10µl of the PCR products were mixed with 5µl of dye and loaded on the rest of the wells. Electrophoresis was performed in a minigel electrophoresis (BIOMETRA) using 75 volt for 45 min after the gel was covered with TAE buffer (40 ml of 40 m M Tris-HCL pH 8.0+ 20 ml of 20 m M Na acetate) using standard Power Pak P25 (BIOMETRA). DNA bands were visualized by fluorescence in UV light using the BIODOC ANALYZE gel documentation system BIOMETYA.
CHAPTER III
RESULTS

3.1. Epidemiology of sheep and goatpox diseases

Sheeppox disease originated in early June 2003 in Shutt, east river Atbara and Abu Garra region, south of Showak, Gedarif State. Goatpox was reported in early March 2005 in Hillat Kuku, Khartoum State, Sudan. Clinically the two diseases were characterized by hyperthermia 40°C, labored breathing, depression and loss of appetite, eruptions on cheeks, nostrils, lips (Fig. 1) and wool free skin like udder (Fig. 2). The vesicular stage which was hemorrhagic with a tendency to generalize was followed by the development of pustules with later turn into scabs (Fig. 3). Mortality rates vary in accordance with the area as shown in Table 3.

Table 3: Epidemiology of sheep and goatpox infections in Gedarif and Khartoum states (2003, 2005).

<table>
<thead>
<tr>
<th>Area</th>
<th>Animal species</th>
<th>Total animals</th>
<th>No. affected</th>
<th>No. dead</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Shott, east river Atbara</td>
<td>Sheep</td>
<td>400</td>
<td>30</td>
<td>25</td>
<td>6.3%</td>
</tr>
<tr>
<td>2. Abu Garra, south of Showak</td>
<td>Sheep</td>
<td>550</td>
<td>45</td>
<td>37</td>
<td>6.7%</td>
</tr>
<tr>
<td>3. Hillat Kuku</td>
<td>Goats</td>
<td>370</td>
<td>35</td>
<td>19</td>
<td>5.1%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1320</td>
<td>110</td>
<td>81</td>
<td>6.1%</td>
</tr>
</tbody>
</table>
All age, sex and breed groups were affected. Age distribution of dead animals is shown in Table 4. More than 50% of deaths were reported in young animals in comparison to adult sheep.

Table 4: Age distribution of mortalities due to sheeppox virus infection in Sudan.

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. affected</th>
<th>No. dead</th>
<th>Case fatality rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult sheep (1-4 years)</td>
<td>32</td>
<td>25</td>
<td>78.1</td>
</tr>
<tr>
<td>Young sheep (3-11 months)</td>
<td>45</td>
<td>40</td>
<td>88.8</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>65</td>
<td>84.4</td>
</tr>
</tbody>
</table>

3.2. Virus isolation

Two samples yielded virus when 4 tissue homogenates were inoculated onto lamb testes cells. The isolates were identified as sheeppox virus and goatpox virus, respectively by virus isolation and PCR. The viruses were named SP10 and GP1, respectively. The first cytopathic effect (CPE) appeared in passage 4 at 7 days post inoculation (PI). The CPE reached 90% at day 7 of the 6th passage.

3.3. Growth of SPV and GPV on the chorioallantoic membrane (CAM)

No pock lesions or thickness were observed when goatpox sample (GP1) and sheeppox samples (SP1O, SP4) and vaccine strain 0240 were inoculated on embryonated chick chorioallantoic membrane (CAM) and harvested 5 days post inoculation.

3.4. Growth of SPV and GPV in cell cultures

Sheeppox isolate (SP1O) replicated in Vero cells and producing 90% CPE at day 7 post inoculation was characterized by cells rounding (Fig. 4) and destruction of the whole cell sheet. Clear cytopathic effect (CPE) was observed when the SP4 (sheeppox isolate) was inoculated on Vero cells starting on the 6th day post inoculation (PI). The CPE consisted of round cell formation, retraction and ballooning of cells, nuclear
vaculation, chromatin fragmentation and loss of continuity of the cells sheet and gave 80% CPE 7 day post inoculation. GP1 isolate of goatpox produced round cell formation and detachment (Fig.5). Three samples of sheeppox (SP1O, SP2 and SP4) and one of goatpox isolate (GP1) were inoculated onto MDbK cells. Infected cells showed a few round cell formation and 60% CPE 9 days post inoculation. No CPE was observed when SP1O and GP1 isolates were inoculated onto CEF cells for 2 passages.

3.5. Titration of SPV

SP1O isolate of sheeppox was selected for virus titration in Vero cells. The tissue culture infective dose 50% (TCID50/ml) was found to be 10^{5.2}/ml.

Table 5: Results of titration of sheeppox virus according to Reed and Muench (1938)

<table>
<thead>
<tr>
<th>Dilution</th>
<th>positive</th>
<th>Negative</th>
<th>Positive accumulation</th>
<th>Negative accumulation</th>
<th>Rate</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-1}</td>
<td>5</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>18/18</td>
<td>100</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>5</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>13/13</td>
<td>100</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>8/8</td>
<td>100</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3/5</td>
<td>60</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>7</td>
<td>0/7</td>
<td>0</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>12</td>
<td>0/12</td>
<td>0</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>17</td>
<td>0/17</td>
<td>0</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>22</td>
<td>0/22</td>
<td>0</td>
</tr>
</tbody>
</table>

50% end point 10^{4.2}

Proportional distance (P.D) = (Mortality next above 50% - 50%)

(Mortality next above 50%) – Mortality next below 50%

P.D = \frac{60-50}{60-0} = 0.2

ED_{50} = \text{Log lower dilution in which}% mortality next above 50% + P.D \times \text{dilution factor}

factor = -5 + (0.2 \times -1) = 5.2
3.6. Neutralization of SPV

Isolate of SP1O was selected to perform neutralization test as confirmatory test for diagnosis. The titer Virus-HIS mixture was $10^{3.8}$/ml. accordingly, the neutralization index was calculated to be 1.4.

Table 6: Results of virus neutralization test for sheeppox virus.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive accumulation</th>
<th>Negative accumulation</th>
<th>Rate</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>5</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>12/12</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>5</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>13</td>
<td>0/13</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>18</td>
<td>0/18</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>23</td>
<td>0/23</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>28</td>
<td>0/28</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ P.D = \frac{100-50\%}{100-40} = 0.83 \]
\[ \text{ED}_{50} = -3 + (0.83 \times -1) = 3.83 \ (\log_{10}) \]
\[ \text{The ED}_{50} (NI) = \log_{10} 5.2 - \log_{10} 3.83 = 1.4 \]

3.7. PCR Amplification

3.7.1. Direct PCR (PCR1)

3.7.1.1. Direct PCR from skin scabs

All the three samples (GP2, LSD and SP4) gave negative results (Fig. 6). No amplification product was detected when control negative (DDW) was used.

3.7.1.2. Direct PCR from cell culture harvest

The three samples out of six gave positive results (Vaccine strain 0240, SP1O and GP1) for viral attachment protein (VAP) gene. Strong bands were detected in the
Ethidium bromide stained gel that corresponded exactly to the expected DNA band size to the (VAP) 192 bp (Fig. 6). No amplification product was detected when control negative (DDW) was used as template.

3.7.1. PCR with DNA extracted by phenol-chloroform from skin scabs (PCR2)

Five samples were tested (GP2, SP1O, GP1, SP4 and LSD). Three samples out of five gave positive results (SP1O, GP1 and GP2) for viral attachment protein (VAP) gene. Strong bands were detected in the Ethidium bromide stained gel that correspond exactly to the expected DNA band size to the (VAP) 192 bp (Fig. 7). No amplification product was detected when control negative (DDW) was used as a template.

3.7.2. PCR with DNA extracted by DNA extraction kit from cell culture harvest (PCR3)

Three samples were tested (SP1O, GP1, and vaccine strain 0240). Two out of three gave positive result (SP1O, GP1) for viral attachment protein (VAP) gene while vaccine strain 0240 gave negative result. Strong bands were detected in the Ethidium bromide stained gel that correspond exactly to the expected DNA band size to the (VAP) 192 bp (Fig. 8). No amplification product was detected when control negative (DDW) was used as a template.
Fig. 1 Adult sheep affected by Sheeppox showing pox pustules on the Neck
Fig. 2 Adult goat affected by goatpox showing pox scabs and ulcerations on the Udder
Fig. 3 Adult Saanen goat affected by goatpox showing pox ulcers, pustules and scabs on the tail region
Fig. 4 Vero cells inoculated with SP1O showing rounding of cells at day 7 post inoculation
Fig. 5 Vero cells inoculated with GP1 showing rounding of cells
Fig. 6 Ethidium bromide stained agarose gel (1.5%). PCR was carried out on DNA samples extracted from vaccine strain 0240 and skin scabs of infected sheep and goats by boiling using primers (VAP). Lane M: 100bp ladder, lane 1 (SP1O) from cell culture, lane 2 (GP1) from cell culture, lane 3 (vaccine strain 0240) from cell culture. Lane 4 (GP2) from scabs, lane 5 (LSD) from scabs of cattle, lane 6 (SP4) from cell culture, lane 7 control negative (DDW).
Fig. 7 Ethidium bromide stained agarose gel (1.5%). PCR was carried out on DNA samples extracted from skin scabs of infected sheep and goats by phenol-chloroform using primers (VAP). Lane M: 100bp ladder, lane 1 (GP2) scabs from goat, lane 2 (SP1O) scab from sheep, lane 3 (GP1) scab from goat. Lane 4 (LSD)cabs from cattle, lane 5 (SP4) from sheep lane 6 control negative (DDW).
Fig. 8 Ethidium bromide stained agarose gel (1.5%). PCR was carried out on DNA samples extracted from skin scabs of infected sheep and goats by Kit using primers (VAP). Lane M: 100bp ladder, lane 1 (SP1O) cell culture harvest from sheep, lane 2 (GP1) cell culture harvest from goat. Lane 3 (vaccine strain 0240) cell culture harvest, lane 4 control negative (DDW)
CHAPTER IV
DISCUSSION

Sheep and goatpox diseases are a weighty problem in sheep and goats production. In fact they are the most serious of all pox diseases of domestic animals. Sheep and goatpox, as highly contagious diseases, needs an urgent accurate laboratory diagnosis, once suspected in a herd.

In the present study three outbreaks of sheep and goatpox that occurred in Al Shut and Abu Garra areas of Gedarif State and Khartoum State in June 2003 and March 2005 respectively were investigated. The clinical diagnosis was confirmed in the laboratory by virus isolation and identification or PCR. In the two outbreaks of Gedarif State the disease affected sheep population of all ages but in contact goats were not affected. The third outbreak occurred in a Saanen herd of imported goats that were previously vaccinated against sheeppox. Clinical signs were reported, morbidity rates were estimated and the total mortalities were recorded. Clinically, the disease was characterized by fever, labored breathing, loss of appetite, depression and eruption of generalized pox lesions namely on cheeks, nostrils, lips and wool free skin. The vesicular stage which was hemorrhagic with a tendency to generalize followed by the development of pustules which later turned into scabs. These clinical signs are similar to those described by Kitching and Taylor (1985). Mortality rate ranged between 5.2 and 6.7 with a mean of 6.1%. These mortality rates are lower than that described by Rao and Bandyopadhyay (2000) who reported a 16% mortality rate in sheep in India. In the third outbreak goatpox affected a Saanen herd imported from Holland where sheep and goatpox are eradicated. The affected goats were previously vaccinated against sheep and goatpox with 0240 vaccine strain locally produced. Infection of this vaccinated goat herd points to a vaccination failure. Whether this vaccination failure is due to poor quality vaccine or poor application procedure is not clear. Similar observations of vaccination failure were previously reported (Sheikh Ali, 1997).
In the investigated outbreaks all age, sex and breed groups were affected. However, more than 50% of deaths were reported in young animals in comparison to adult sheep. These findings are in line with previous observations of sheep and goat pox outbreaks (Rao and Bandyopadhyay, 2000).

Skin Scabs were collected from sick animals, homogenized and inoculated in cell culture and embryonated eggs. Two virus isolates were obtained when 4 skin samples were inoculated onto Lamb testes (LT) cell culture. These two viruses as well as one previous isolate of sheeppox virus (SP 4) and the vaccine strain 0240 induced no lesions on the chorioallantoic membrane (CAM) of embryonated chicken eggs. This result agrees with Ortitenz and Teieco (1954), Abdulla Khan (1960), Sharma et al. (1966), Adlakha et al. (1971), Onar (1971-72), Sen and Uppal (1972), Sharma and Dhanda (1972) and Bhatnagar and Gupta, (1974), who failed to propagate sheep poxvirus in chick embryos. Goat poxvirus (GPV) isolates differ significantly in their growth behavior in embryonated chick eggs (Adlakha et al., 1971; Tantawi and Al-Falluji, 1979).

Sheeppox virus isolation is difficult; it grows slowly or requires additional passages, even if cultured in most sensitive lamb testis (LT) cells. When passaged two to three times in these cells it grows easier and faster (Mangana-Vougiouka et al., 1999). In the present study skin Scabs were collected from sick animals, homogenized and inoculated on cell culture and embryonated eggs. Two virus isolates (one sheeppox virus [SP1O] and one goatpox virus [GP1]) were obtained when 4 skin samples were inoculated onto Lamb testes (LT) cell culture. Sheeppox and goatpox isolates grew well in lamb testes (LT) cell culture with no difference in virus yield or type of CPE produced by the two viruses. The two isolates of the viruses (SP1O, GP1) and the vaccine strain (0240) as well as one previous isolate of sheeppox virus (SP 4) produced characteristic pox cytopathic effects (CPE), such as rounding of cells, retraction and ballooning of cells, nuclear vaculation, chromatin fragmentation and loss of continuity of the cells sheet, after 7 days PI. These findings agreed with Adlakha et al (1971); Joshi et al (1994). In Vero cells, both viruses produced more than 90% CPE within 7 days PI. In MDBK cells however, both viruses induced slight CPE that reached 60% in 9 days. On the other hand, both viruses induced no CPE in chick embryo fibroblast (CEF) cells. The growth of goatpox strain (GP1) in MDBK cells in our results, agreed with Ved Parkash et al.,
(1994), who reviewed that GPV will grow in the Vero cells and MDBK cell lines. Growth of sheeppox strain (SP1O) in Vero cells, agreed with Joshi et al (1995), who reviewed that most SPV isolates will grow in MDBK and Vero cells. Our results showed that isolation of sheeppox and goatpox viruses require at least three blind passages to be possible. This agreed with the report of OIE Manual (2004). SP1O and GP1 isolates produced rapid and clear 90% CPE in comparison to the previous isolates (SP4), so that SP1O and GP1 were passaged 6 times and this confirmed that additional passages of the virus field isolates shows rapid and clear cytopathic effects.

Titration of an isolate of sheeppox virus (SP1O) was performed in Vero cells and resulted in a titre of $10^{5.2}$/ml. The same virus was identified as sheeppox in virus neutralization test with 1.4 neutralization index. The neutralization test was used in this study as a confirmatory test for diagnosis of sheeppox and for the purpose of comparing the test with polymerase chain reaction (PCR). Vero cells infected with SP1O were used, and their results were taken 9 days PI. The results confirmed the identity of sheeppoxvirus isolates used in the present study since the test gave a neutralization index of 1.4.

At present the laboratory diagnosis of sheeppoxvirus in the Sudan is based on virus isolation and its subsequent identification. These assays are costly, time consuming, laborious and some times the interpretation of the results is difficult. Virus isolation on susceptible cell culture (LT) cells or Vero cells followed by virus neutralization (VN) or immunoflourscence using hyperimmune anti Capripox serum is a lengthy procedure and may take two to three weeks.

These diagnostic problems can be overcome by the use of polymerase chain reaction test (PCR) which has considerable potential for detection of Capripoxvirus and differentiation from Orf (contagious pustular dermatitis) of sheep (Ireland and Binepal, 1998; Markoulatos et al., 2000). Highly sensitive molecular biological technique based on the PCR are now employed for the identification of many viruses, including GPV and SPV in skin biopsies and cell culture (Ireland and Binepal, 1998; Heine et al., 1999; Mangana-Vougiouka et al., 1999). Recently, a PCR assay was reported on cell culture or skin biopsy samples (Ireland and Binepal,1998), which compared to an antigen trapping enzyme-linked immunosorbent assay (Carn, 1995) and has greater sensitivity and
specificity. Antigen detection by ELISA may give false-positive results due to interference by virus-specific antibodies in the test samples. These antibodies are removed from samples for PCR when nucleic acid is released by boiling, phenol-chloroform extraction or used a polymeric material such as Gene Releaser. The oligonucleotide primers pair used for PCR amplification of genomes of either SPV or GPV were obtained from the viral envelope protein viral attachment protein (VAP) gene.

PCR-diagnostic methods are exceptionally effective for the diagnosis of sheep pox and goat pox in suspected skin samples obtained from the field (Rao et al., 2001). However, differential diagnosis of goat pox and sheep pox based solely on the PCR technique is not yet feasible, but may be possible by restriction enzyme analysis of PCR-amplified products (Rao and Pandyopadhyay, 2000).

Polymerase chain reaction (PCR) was used in this study to determine the feasibility of improving diagnosis of sheep pox and goat pox in scabs collected from the field as well as in supernatant of infected cell cultures. The test was performed according to the method described by Ireland and Binepal (1998) using a single primer-pair based on sequences coding for the viral attachment protein (VAP) gene. Three methods of DNA extraction were tried. The first method (PCR1) employed no DNA extraction step with scab homogenate or cell culture supernatant added directly to the PCR mix and heated the reaction at 99°C for 15 minutes to release the DNA and then followed by ordinary PCR. This method failed to detect pox virus DNA in all scab homogenates tested but three out of six cell culture supernatants gave positive results. In the second method (PCR2), viral DNA was extracted by phenol-chloroform from skin scabs; three samples out of six gave positive results. In the third method (PCR3), viral DNA was extracted using a commercial DNA extraction Kit; two out of three gave positive result. Accordingly, the best PCR method for sheep pox and goat pox diagnosis should include an initial step of DNA extraction.

In the second method (PCR2) DNA was extracted by using phenol-chloroform. A total number of five samples from skin scabs were tested. Three out of five samples gave positive results (SP1O, GP1, GP2), while two samples (LSD and SP4) gave negative results. The DNA band sizes corresponded to the expected size of 192 bp. This method seems to be more sensitive than method 1 (PCR1). It is therefore, recommended to use
this method for routine diagnosis of sheep pox and goatpox in the Sudan. This method requires only biopsy materials which are easy to obtain. Cell culture facilities are not needed and results can be obtained within 24 hours.

In the third method (PCR3) viral DNA was extracted by a commercial DNA isolation kit from cell culture supernatants and used as templates to conduct PCR. A total numbers of three samples (SP10, GP1 and vaccine strain 0240) were tested. Two out of three gave positive results (SP10 and GP1) while no DNA band appeared when the vaccine strain 0240 was tested. This method was less sensitive than the second method (PCR2). This method is a valuable tool to virus detection. It can replace the lengthy method of virus neutralization that requires at least 2 weeks.

All results of PCR in this study corresponded with Ireland and Binepal (1998) who used PCR to detect Capripoxivirus DNA in tissue culture supernatants and biopsy samples.

The PCR-based test is preferable to the neutralization test (VNT) for additional reasons than its greater sensitivity. It does not require any reagents that cannot be obtained commercially. Many firms sell custom primers for PCR, and all the other reagents are common to all PCR reactions. The neutralization test reagents, on the other hand, include hyperimmune serum (HIS) which is commercially not available. The PCR test for Capripoxivirus in biopsy samples described in this study is a valuable addition to the current methods for virus detection.

Our results suggest that the Capripoxvirus PCR has a diagnostic specificity comparable to that of the virus isolation although a much greater number of biopsy samples are required to confirm the findings. Although not as robust as the virus neutralization (VN), the PCR has additional advantages. The diagnostic sensitivity of the PCR is greater than that of virus neutralization test (VNT) and the test can be used later in the course of the disease when virus-specific antibodies are present.

This to our best knowledge is the first investigation using a polymerase chain reaction (PCR) for diagnosis of sheep and goatpox virus in Sudan.
CONCLUSION AND RECOMMENDATIONS

Sheep and goatpox are serious diseases that cause heavy economic losses in the Sudan. Isolation and identification of sheep and goatpox is expected to open new windows in the field of epidemiology and effective control of the disease and investigation aiming at comparing local isolates with the 0240 vaccine strain which is currently used in the Sudan for the control. Polymerase chain reaction is able to detect Capripoxvirus genome in scab materials reducing time required for diagnosis. Further work to be done in future should include PCR followed by sequencing, restriction enzyme analysis and a hybridization method for sheep and goatpox isolates (SP1O and GP1).
REFERENCES


Australian veterinary emergency plan (Ausvet plan) version 0.2, 1996. Sheep pox Zoonosis.


Imperial Institute of Veterinary Research (1936–37). Annual Report. Imperial Institute of Veterinary Research, Mukteswar (UP).

Indian Veterinary Research Institute (1986). Annual Scientific Report. Division of Virology, Indian Veterinary Research Institute, Mukteswar (UP).


APPENDIX

1. Preparation of reagents Solution:

1.1. Normal Saline (NS):

   Stock solution of 0.85% (W/V) NaCl was prepared in DDW and autoclaved at 121°C for 20 minutes.

1.2 Preparation of phosphate diluent (PD)

   Solution A of PBS was completed to 2 litres with DDW, then autoclaved and cooled before antibiotics were added.

1.3. Sodium Hydroxide Solution:

   Na OH 45 gm
   DW 300 ml

2. culture media

2.1. Glasgow Minimum Essential Medium (GMEM) 5x

   Dissolve the content of one bottle (125.78 gm in two liters of DDW). Avoid over stirring and aeration. Filter through Millipore. Filter (0.22µ). Test sterility using Thioglycolate medium. Store at -20°C.

2.1. GMEM 1X:

   To prepare 1 litre:

   G MEM 5X 200 ml
   Yeast extract (1%) 25 ml
   Lactalbumin hydrolysate 25 ml
   Penicillin Strptomycin (10^5 I.U/ml) 1 ml
Neomycin (10 mg/ml) 1 ml
Mycostatin 1 ml
Na HCO₃ (7.5%) 7.5 ml
DDW to complete 1000 ml
Add 50 ml Tryptose phosphate buffer store at 4°C.

3. Preparation of Stock Trypsin (2.5%)
   2.5 grams of trypsin (Gibcol td UK1: 250 Usp Grade) were dissolved in 100 ml of PD, filtered through What Mann filter and stored at -20°C.

4. Preparation of Stock Versin (5%)
   Five grams of versin powder were dissolved in 100 ml PD, autoclaved at 121°C for 15 minutes.

5. Trypsin Versin Solution (T.V)
   To prepare 100 ml
   Trypsin (2.5%) 6.0 ml
   Versin (5%) 4.0 ml
   Phosphate Diluent (PD) 90.0 ml
   Several drops of phenol red (0.2%) were added and the pH adjusted by IM NaOH until the colour is faint pink (slightly alkaline).

6. Preparation of Buffers

6.1. Glycerol (50%)
   Glycerol 50 ml
   PBS 50 ml

6.2. Formalin (10%)
   Formalin 100 ml
   Na H₂PO₄ 4 gm
   K₂ HPO₄ 6 gm
Complete to one litre with DDW.

6.3. Phosphate Buffer Saline (PBS):

To prepare 2 litres

6.3.1. Solution A

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>KCl</td>
<td>0.4 gm</td>
</tr>
<tr>
<td>Na Cl</td>
<td>16.0 gm</td>
</tr>
<tr>
<td>Na₂ HPO₄ (anhydrous)</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>KH₂ PO₄</td>
<td>0.4 gm</td>
</tr>
<tr>
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6.3.2. Solution B

<p>| | |</p>
<table>
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<tr>
<td>Mg Cl₂</td>
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<tr>
<td>DDW</td>
<td>200 ml</td>
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</table>

6.3.3. Solution C

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<tr>
<td>Ca Cl₂</td>
<td>0.26 gm</td>
</tr>
<tr>
<td>DDW</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Autoclave cool and add A to B then C. Complete to 2000 ml with DDW.

7. Phosphate Buffer, pH 8.5

7.1. Solution A:

KH₂ PO₄ 0.5 M (anhydrous, 68.04 gm/litre).

7.2. Solution B:

K₂HPO₄ 0.5 M (anhydrous, 87.09 gm/litre) take 33 ml/litre from solution A, mix with 967 ml/litre from solution B.

8. Tris-EDTA (TE)
Tris-HCl pH 8 10 mM
EDTA 1Mm

9. Media Additives

9.1. Lacalbumin Hydrolysate 25%:
Dissolve 25gm of Lacalbumin enzymatic Hydrolysate in 100 ml DDW and keep at 4ºC after sterilization.

9.2. Yeast Extract 1%
Dissolve 1 gm of Yeast Extract in 100 ml DDW and keep at 4ºC after sterilization.

9.3. Sodium bicarbonate 7.5%
Dissolve 0.75 gm of Na HCO3 in 9.5 ml DDW. Add 0.5 ml of 0.2% Phenol red. Sterilized by autoclaving at 121ºC for 15 minutes and store at 4ºC.

9.4. Tryptose phosphate broth 29.5 %
Dissolve 29.5 gm of powder in 100 ml DDW. Autoclaved at 121ºC for 15 minutes and stored at 4ºC.

9.5. Antibiotics:

9.5.1. Penicillin-Streptomycin Solution:
One gram of streptomycin powder and the contents of 2 vials of penicillin (1000000 IU/vial) were dissolved in 10 ml of sterile deionized distill water (DDW) to give solution contained 100 mg streptomycin and 200000 IU penicillin per ml. The solution was kept at -20ºC.

9.5.2. Kanamycin Solution:
One gram of kanamycin powder (equivalent to 700000 IU) was dissolved in 13.3 ml of sterile DDW to give a concentration of 52500 U/ml and kept at -20ºC.
9.5.3. **Fungizon Solution:**

The content of one vial of fungizon (50000 µg) was dissolved in 10 ml of sterile DDW and kept at 4°C.

9.5.4. **Mycostatin Solution:**

The content of one vial of mycostatin (50000 µg) was dissolved in 10 ml of sterile DDW and kept at 4°C.

9.6. **Bovine serum:**

Calves were bled from the jugular vein. The whole blood was left overnight at room temperature. The separated serum was centrifuged at 2000 rpm for 10 minutes, then filtered through a Seitz filter under negative pressure. The filtrate was passed through a Millipore filter (0.22 µ) under positive pressure and then tested for sterility by culturing on Thioglycolate medium in two vials, one of them was incubated at 37°C and another one at room temperature for two days. Then the sterile serum was kept at -20°C.

10. **Thioglycolate medium:**

29.5 gm of Thioglycolate medium were dissolved in 1000 ml of DDW. The solution was heated in steamer to ensure complete dissolving and then dispensed in bijoux bottles. The medium was sterilized by autoclaving at 121°C.

11. **PCR Master mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Mg Cl₂ 50 mM</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>dNTPs 10-11 mM</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Mg SO₄ 5mM</td>
<td>15.0 µl</td>
</tr>
<tr>
<td>DDW</td>
<td>16.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>40.0 µl</td>
</tr>
</tbody>
</table>

12. **Preparation of materials for gel electrophoresis:**

12.1. **TAE buffer**
40 mM Tris HCl (pH 8.0) 40 ml
20 mM Na acetate 20 ml
EDTA powder (292.25 MW) 5845 gm
Complete to one litre

12.2. Loading Dye:
   Bromophenol Blue (11%) 10 μl
   Glycerol 40 μl
   DDW 50 μl

12.3. 1.5% agarose gel:

   1.5 gram of agarose (SIGMA) was dissolved in 100 ml TAE buffer. The mixture was melted in a microwave oven for 60 seconds, cooled down. 1.5 μl Ethidium bromide was added at a final dilution of 0.2 μg/μl and swirled. Poured on a plate. The comb was placed and the gel was left to solidify.

12.4. 100 bp DNA Ladder (INVITRGEN):

   Ladder (1.0 ug/ul) 20 μl
   Blue dye 80 μl

12.5. Ethidium bromide:

   Stock Solution (PROMEGA, Madison) 10 mg/ml
   And protected from light.
ملخص الطرقية

يعتبر كل من مرضى جدري حيوان (Sheepox) وجدري الماعز (Goatpox) من مجموعات الفيروسات والتي تسببها فيروسات (Poxviridae) من هم الأمراض الجلدية في الأغنام والماعز والتي تحدث خسائر اقتصادية كبيرة وقيلة في الإنتاج.

أجريت هذه الدراسة لمعرفة تشخيص مرضى جدري الحيوان وجدري الماعز عن طريق ادخال تفاعل البلمرة المستقلة خلية بديلة من الوسائل التقليدية المستخدمة.

تم اخذ عينات من منطقتي أبو قرع جنوب الشوك ومنطقة شوت شرق نهر عطرة، شرق السودان ولاية القضارف من أجرا لوحظ عليها علامات المرض وتتم تقدير معدل المرضة وتتم رصد معدل النفوق الكلي طوال فترة المرض وذلك لتحديد نسبة النفوق. أظهرت النتائج أن حوالي أكثر من 50% نسبة من النفوق لوحظ في الأعمار الصغرى مقارنة بالاعمار الكبيرة في الأغنام.

كما تم اخذ عينات من مرضى مشروع الساعين من منطقة حلة كوكو ولاية الخرطوم بعد ظهور تفشي للمرض علماً بأن الحيوانات حلقت بلجف جدري الماعز والإغنام عتة 400، وتتم تحديد معدل النفوق ومعدل المراضة. تم اخذ عينات قشر لجذع وكشفات وعقد لجذع ومسحات أفقية من الحيوانات المصابة وتتم إعداد العينات بمسحها ومعاملتها بالمضادات الحيوية وتتم تزريعها في اجة البيض النامية وخلايا خصى الحمالة البدنية وخلايا المستمرة وتم تمريرها لعدد سته تمريرات. أظهرت العينات تأثيرات مرضية Vero المستمرة وتم تمريرها لعدد سته تمريرات. أظهرت العينات تأثيرات مرضية Vero المستمرة في خلايا Vero المستمرة في درجة الخلوي 90% عبارة عن استدارة الخلايا وانفاذها وتحطيمها وذلك بعد أسبوعاً اياً من الحقن. بمثابة هذه النتائج لوحظ ظهور تغيرات مرضية في خلايا Vero ولكن بعد التمرير في خلايا خصى الحمالة كما ان في تحت اى تغيرات مرضية في اجة البيض المحفوة نفس العينات المرضية واللقاح.

تم تشخيص العينات عن طريق العزل في الزرع الخلوي وتأكيدها عن طريق اختبار التعامل الفيروسي ومعاعبة الفيروس. اختبار التعامل الفيروسي أذى أن الفيروس موجباً باستخدام مصل مصنع لجردري الإغنام وعطي رم تعادل 1.4 كما كان معيار الفيروس المحدث للتغيرات المرضية في الزرع الخلوي 1.4/105.2/ml.
تفاعل البلمرة التمتلسل المستخدم في هذه الدراسة ادخل كوسيلة حديثة لتشخيص جدري القنان والمعز من القسر الجلدي للحيوانات المصابة، وكذلك من حصاد الزرع الخلوي. تم استخلاص الحمض النووي منقوص الاو克斯جين

الللفيروس بواسطة ثلاث طرق مختلفة:

1. مباشرة من الملعقة المتجنس للفيروس من كورونا الجلدي وحصاد زرع خلوي. هذه الطرق فشلت في اكتشاف الفيروس مباشرة من الفطر الخلوي للملعقة المتجنس للعينة بينما تم اكتشاف ثلاثة عينات موجبة للفيروس من حصاد الزرع الخلوي.

2. استخلاص الحمض النووي منقوص الاوكسجين عن طريق الكلوروروم والفينول من القشرة (DNA) 

3. بواسطة DNA extraction kit. 

اختيار تفاعل البلمرة التمتلسل اظهر نتائج موجبة للعينات كما اثبت أنه أكثر حساسية من التعادل الفيروسي

واسرع منه.

وفقاً لهذه النتائج وجد أنه أفضل طريقة لأجراء اختبار تفاعل البلمرة التمتلسل لتشخيص جدري القنان والمعز.

يجب أن تضم خطوات الاستخلاص الحمض النووي منقوص الاوكسجين (DNA).