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Conventional and Molecular Tests of Lumpy Skin Disease

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Abstract: Lumpy Skin Disease (LSD) is a highly infectious disease of cattle caused by a virus belonging to the genus *Capripoxvirus* of the family *Poxviridae*. Members of the *Capripoxvirus* genus are closely related, with genomic identities ranging from 96% between viral species to 99% between isolates of the same species. This study reviews the development and application of available LSD diagnostic methods. These are categorized into several groups and often based on characteristic clinical signs and laboratory diagnosis. Laboratory diagnosis comprises either identification of the virus using electron microscopy, egg inoculation, isolation in cell cultures, fluorescent antibody test or detection of its specific antibody using serological tests. Several Polymerase Chain Reaction (PCR) assays have been developed recently for more accurate and rapid detection of Lumpy skin disease virus in suitable specimens. In most reference laboratories the basic tests for LSD virus diagnosis comprise molecular methods for generic detection of a *Capripoxvirus*. But these methods do not differentiate between LSD virus, sheep pox virus and goat pox virus. The conventional PCR method is not as fast as real-time PCR for *Capripoxvirus* but it is reliable and sensitive. Recent advances in diagnostic technology have dramatically altered laboratory testing of LSD virus and the effort to develop efficient and reliable LSD virus detection methods continues now a days. The availability of a cost-effective diagnostic tool for routine determination of *Capripoxvirus* genotype will assist to clarify the epidemiological picture in the affected regions and effective control of this disease needs sensitive, specific and quick diagnostic tools at each tier of control strategy.

INTRODUCTION

Livestock production constitutes one of the principal means of achieving improved living standards in many regions of the developing world[1]. The livestock sector globally is highly dynamic, contributes 40% of the global value of agricultural output and support the livelihoods and food security of almost a billion

people[2]. However, animal diseases including Lumpy Skin Disease (LSD) became an important cause of reduced productivity of meat, milk as well as draft and hides. LSD is one of the most economically significant viral diseases of cattle characterized by high fever, enlarged lymph nodes, firm and circumscribed nodules[3].

It is an infectious viral disease of cattle caused by LSD virus of the genus *Capripoxvirus*. The LSD virus has double-stranded DNA genome which encodes 30 homologues of poxviral proteins known to be structural or nonstructural and it is antigenically and genetically closely related to Sheep Pox Virus (SPPV) and Goat Pox Virus (GTPV) with nucleotide sequence identities of 96% between species[4].

LSD has a different geographical distribution from that of sheep and goat-pox, suggesting that cattle strains of *Capripoxvirus* do not infect or transmit between sheep and goats. The disease was first observed in 1929 in northern Rhodesia (currently Zambia) and rapidly spread north and south. It now occurs in most of Africa (except Libya, Algeria, Morocco and Tunisia) and much of the Middle East[5]. In Ethiopia LSD was first observed in the Northwestern part of the country (southwest of Lake Tana) in 1981[6]. It is now spread to almost all regions and agro-ecological zones of the country. Major epidemic outbreak of LSD has been documented in different regions of Ethiopia at different time period[1]. LSD can occur in diverse ecological zones from the very dry semi-desert, the wet and dry areas to the high altitude temperate areas[7]. Various strains of *Capripoxvirus* are responsible for the disease and these are antigenically and serologically indistinguishable from strains causing sheep pox and goat pox but distinct at the genetic level[8].

The field diagnosis of LSD is based mainly on the presence of clinical signs. However, sometimes, LSD is difficult to diagnose because only transient fever or few skin lesions are present. In addition, LSD could be confused with other diseases causing skin lesions such as pseudo lumpy skin disease (bovine herpesvirus-2 infection), insect bites, demodecosis and dermatophilosis. Moreover, diseases causing mucosal lesions, such as rinderpest, bovine viral diarrhea/mucosal disease and bovine malignant catarrhal fever, also complicate field diagnosis. Laboratory diagnosis is essential for the confirmation of LSD virus infections[9]. Rapid diagnostic confirmation of the tentative field diagnosis is fundamental for the successful control and eradication of LSD in endemic and particularly in non-endemic countries.

Virus isolation, electron microscopy, serological and molecular techniques have been used for LSD virus detection[3]. Molecular techniques such as conventional and real-time PCR are proved to be more accurate, reliable and faster than other methods for LSD virus detection[10]. PCR and restriction fragment length polymorphism (RFLP) has been used for differentiating SPPV and GTPV[11] and also differentiating virulent LSD virus from vaccine strain[12]. In addition laboratory test of LSD can be made by identification of the agent, routine histopathological examination and immune histological staining[13].

Diagnosis is done by isolation of the virus into embryonated chicken eggs or different tissue culture cells, in addition to using serological tests such as Serum Neutralization Test (SNT), Agar Gel Immunodiffusion (AGID), indirect Enzyme-Linked Immunosorbent Assay (iELISA) and indirect Fluorescence Antibody Test (iFAT), Polymerase Chain Reaction (PCR)-based assays have been developed for the detection of *Capripoxvirus* nucleic acid in a variety of clinical samples[3]. Effective control of LSD needs sensitive, specific and quick diagnostic tools at each tier of control strategy.

Therefore, the objective of this study is to review various diagnostic approaches, access their merit and demerit and recommend the most suitable test for the diagnosis of LSD virus.

LITERATURE REVIEW

Etiology of the disease: LSD virus belongs to the family Poxviridae which is divided into two subfamilies: Entomopoxvirinae (poxviruses of insects) and Chordopoxvirinae (poxviruses of vertebrates) and several genera (Table 1). LSD is one of a serious poxvirus disease of cattle caused by Lumpy Skin Disease Virus (LSDV), a DNA virus of the genus *Capripoxvirus* and of the family *Poxviridae*. The prototype strain is Neethling virus prolonged, since, viral DNA has been found in the semen[8, 14, 15].

Physicochemical properties of the virus: LSD virus is susceptible to sun light and detergents containing lipid solvents. The virus could be inactivated after heating for

Table 1: Genera within the *poxviridae* family

Genus	Viruses
Capripoxvirus	Sheeppox, goatpox, lumpy skin disease virus
Orthopoxvirus	Buffalopox, camelpox, cowpox, vaccinia, ectromelia, monkeypox, rabbitpox, raccoonpox, taterapox, vareola and volepox viruses
Parapoxvirus	Pseudocowpox, bovine popular stomatitis, contagious pustular dermatitis (orf), squirrel parapox viruses and parapox virus of red deer
Suipoxvirus	Swinepox virus
Avipoxvirus	Fowlpox, canarypox, juncopox, pigeonpox, quilpox, sparrowpox, starlingpox, turkeypox, mynahpox and pcittacinepox viruses
Molluscipoxvirus	Mulluscum contagiosum virus
Yatapoxvirus	Yaba and tanapox viruses

Magori-Cohen *et al*.[15]

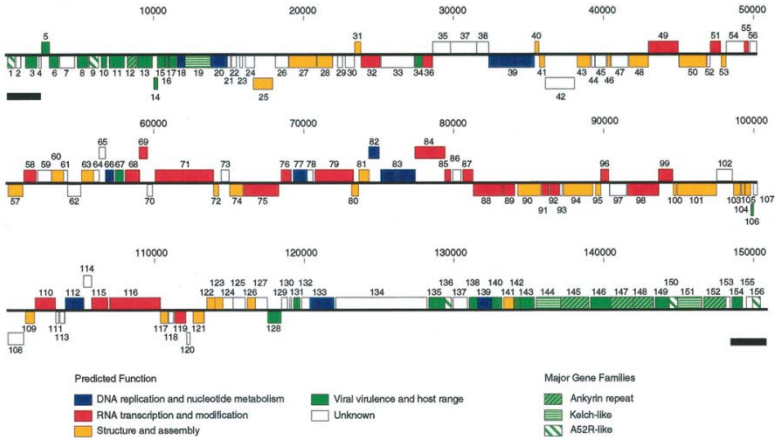

Fig. 1: Linear map of the LSD virus genome[4]

1 h at 55°C. However, it withstands drying, pH changes if not an extreme pH and can remain viable for months in dark room such as infected animal shade off its host. LSD virus can persist in skin plugs for about 42 days. It is likely that the viral A type inclusion body protein in infected cells may protect the virion after the scab has disintegrated, although, this has not yet been proven[8].

Genome of LSD virus: LSD virus shares the genus with Sheep Pox Virus (SPPV) and Goat Pox Virus (GTPV) which are closely related but phylogenetically distinct. There is only one serological type of LSD virus and LSD, SPP and GTP viruses cross-react serologically. The large, double-stranded DNA virus is very stable and very little genetic variability occurs. Therefore, for LSD virus, farm to farm spread cannot be followed by sequencing the virus isolates, as is done with other transboundary animal diseases, e.g., foot-and-mouth disease[16].

The 151 kbp LSD virus genome consists of a central coding region bounded by identical 2.4 kbp-inverted terminal repeats and contains 156 putative genes. Comparison of LSD virus with chordopoxviruses of other genera reveals 146 conserved genes which encode proteins involved in transcription and mRNA biogenesis, nucleotide metabolism, DNA replication, protein processing, virion structure and assembly and viral virulence and host range. In the central genomic region, LSD virus genes share a high degree of collinearity and amino acid identity (average of 65%) with genes of other known mammalian poxviruses, particularly *Suipoxvirus*, *Yatapoxvirus* and leporipoxviruses. In the terminal regions, collinearity is disrupted and poxvirus homologues are either absent or share a lower percentage of amino acid identity. Most of these differences involve genes and gene families with likely functions involving viral virulence and host range. Although, LSD virusresembles leporipoxviruses in gene content and organization, it also contains homologues of interleukin-10 (IL-10), IL-1 binding proteins, G protein-coupled CC chemokine receptor and epidermal growth factor-like protein which are found in other poxvirus genera. The LSD virus genome sequence has been deposited in GenBank accession no. AF325528. Figure 1 shows linear map of the LSD virus genome. Open Reading Frame (ORFs) are numbered from left to right based on the position of the methionine initiation codon. ORFs transcribed to the right are located above the horizontal line ORFs transcribed to the left are below. Genes with similar functions and members of gene families are colored according to the figure key. Inverted Terminal Repeats (ITRs) are represented as black bars below the Open reading framemap[4].

Viral replication: Replication of poxvirus occurs in the cytoplasm. After fusion of the virion with the plasma membrane or via endocytosis, the viral core is released into the cytoplasm. Transcription is initiated by viral transcriptase and functional capped and polyadenylated messenger Ribonucleic Acid (mRNAs) are produced within minutes after infection. The polypeptides produced by translation of these mRNAs complete the uncoating of the core and about half of the viral genome is transcribed prior to replication, comprising genes encoding proteins involved in host interactions, viral DNA synthesis and intermediate gene expression. With the onset of DNA replication 1.5-6 h after infection, there is a dramatic shift in the gene expression and almost the entire genome is transcribed but transcripts from the early genes (i.e., those transcribed before DNA replication begins) are not translated. Two forms of virions are released from the infected cells (virions with one membrane and virions with two membranes) and both types are infectious[17, 18].

Fig. 2: Geographical presence of lumpy skin disease[19]

Epidemiology and transmission: LSD is widespread throughout Africa, causing particularly severe outbreaks in the Horn of Africa. LSD distribution has extended from sub-Saharan countries to Egypt and Western Africa. Outside the African continent Israel has reported LSD outbreaks and sporadically some Middle East countries which showed that there is a real potential risk of the disease to establish endemically there[20].

LSD has been one of the newly emerging diseases of cattle in Ethiopia. LSD was introduced in Ethiopia, for the first time, through north-west (Gojjam and Gondar) in 1981. A major epidemic outbreak of LSD occurred in 2000/2001 in Amhara and Western part of Oromia region, in 2003/2004 again in Oromia and SNNP regions and in 2006/2007 in Tigray, Amhara and Benishangul regions. In terms of the size and magnitude of its occurrence, an epidemic of LSD covering a number of pastoralist areas is reported to have occurred in some districts (Adola and Yabello districts) in the years 2003-2005. In Somali regional state, the first case of an epidemic of Lumpy Skin Disease in cattle was reported in Somali Region in 2005[6, 14].

Prior to 2012, only sporadic LSD virus outbreaks were reported in the Middle East region. In the summer of 2012, LSD was reported by the Israeli veterinary authorities in beef herds in the northern parts of the Golan Heights, adjacent to the borders of Syria. The primary source of infection was inconclusive, although, the outbreak locations indicated that LSD virus was likely to be circulating in the cattle populations in Syria. Between 2012 and 2013, the disease spread throughout the Northern half of Israel, infecting both beef and dairy herds[21].

In late 2012, LSD was detected in Lebanon where 34 outbreaks were reported, followed by outbreaks in Jordan[22]. Between 2013 and 2015, LSD virus spread throughout Turkey to the extent where LSD may now become endemic in the country. Incursion of the virus was subsequently reported in Iraq[23].

In Fig. 2, area colored with red shows global distribution of LSD virus including the spread to Middle East and recently to Caucasus and Balkan countries posing emerging risk to Europe and other countries.

Animal to animal transmission by close contact is minimal. Arthropod vectors play major role in transmission and spread of LSD virus. *Aedes aegypti* mosquito for LSD virus and stomoxys calcitrans transmission for SPPV have been reported[24]. Horn flies, horse flies, midges also reported to transmit the virus. Novel evidence on the role of hard ticks has been found[25].

Virus can be transmitted by intradermal and intravenous injections. Therefore, iatrogenic transmission through injections and other applications have been occurring as well as human playing important role in spreading the virus[16]. The outbreaks depend on cattle movements and immune status, wind and rainfall patterns affecting vector populations[20].

Pathogenesis: Subcutaneous or intradermal inoculation of cattle with LSDV results in the development of a localized swelling at the site of inoculation after four to seven days and enlargement of the regional lymph nodes while generalized eruption of skin nodules usually occurs seven to nineteen days after inoculation. Viremia occurred after the initial febrile reaction and persisted for two weeks. Viral replication in pericytes, endothelial cells and probably other cells in blood vessel and lymph vessel walls causes vasculitis and lymphangitis in some vessels in affected areas. In severe cases infarction may result. Immunity after recovery from natural infection is life-long in most cattle; calves of immune cows acquire maternal antibody and are resistant to clinical disease for about 6 months[5, 25].

Economic importance of the disease: LSD is an economically important disease of cattle, serious

economic losses can follow outbreaks that have a high morbidity and can produce a chronic debility in infected animals. Even though, the mortality rates of LSD is usually low it is an economically important disease of cattle in Africa because of the prolonged loss of productivity of dairy and beef cattle, use of the animals for traction, decrease in body weight, mastitis, severe orchitis which may result in temporary infertility and sometimes permanent sterility. The valuation of the draft power loss depended on the point in the crop season that an ox fell sick and on the corresponding demand for power during that specific season. Thus, the draft work output loss in terms of days is taken into account on two levels: when demand for draft power is high and when it is low with demand determined by the crop calendar prevailing at the onset of the disease[27, 28].

Apart from quality degradation of skin and hides skin diseases including, LSD induce associated economic losses due to reduction of wool quality, meat, losses as a result of culling and occasional mortalities and related with cost of treatment and prevention of the diseases. The disease is more severe in cows in the peak of lactation and causes a sharp drop in milk yield because of high fever caused by the viral infection itself and secondary bacterial mastitis[29].

Treatment cost represents the expenses incurred by farmers for medication at the local public veterinary clinics when farmers bring their clinically sick animals for treatment. Restrictions to the global trade of live animals and animal products, costly control and eradication measures such as vaccination campaigns as well as the indirect costs because of the compulsory limitations in animal movements cause significant financial losses on a national level[28, 5].

DIAGNOSTIC METHODS: CLINICAL AND LABORATORY DIAGNOSIS OF LUMPY SKIN DISEASE

Clinical diagnosis of lumpy skin disease: In experimentally infected cattle, the incubation period varies between four and seven days but in naturally infected cattle it may be up to 5 weeks[30]. Clinical signs include lachrymation and nasal discharge which is usually observed first. Subscapular and prefemoral lymph nodes become enlarged and are easily palpable. High fever (>40.5°C) may persist for approximately a week and sharp drop in milk yield. Appearance of highly characteristic, nodular skin lesions of 10-50 mm in diameter is also observed in sick animal. The number of lesions varies from a few in mild cases to multiple lesions in severely infected animals. Necrotic nodules and deep scabs may be observed all over the body (Fig. 3a and b)[31, 16].

The Lumpy Skin Disease is range from acute and severe to subclinical and is characterized by pyrexia, lymphadenopathy, skin nodules and subsequent sit fasts. Pox lesions can affect internal organs such as the stomach[32]. The severity of clinical signs of LSD (Neethling virus infection or knopvelsiekte), depends on the strain of *Capripoxvirus* and the breed of host. Bos Taurus is more susceptible to clinical disease than Bos indicus; the Asian buffalo has also been reported to be susceptible. Within Bos taurus, the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death. There may be failure of the virus to infect the whole group, depending on vector prevalence[33].

Sample collection, submission and preparation: Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin nodules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralizing antibodies[3, 7, 13].

Samples for genome detection by Polymerase Chain Reaction (PCR) may be collected when neutralizing antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated by PCR for up to 3 months[32].

Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalization of lesions or within 4 days of generalization) can also be used for virus isolation. Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin. Tissues in formalin have no special transportation requirements. Blood samples with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice and processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection should be kept at 4°C, on ice or at -20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy which should be used for virus

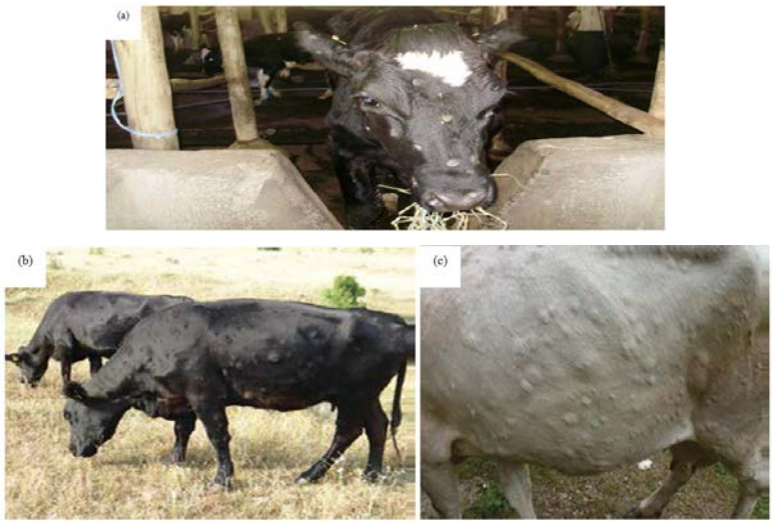

Fig. 3(a-c): (a) Clinical signs of lumpy skin disease: necrotic nodules and deep scabs (a)[31] and (b, c) Severely affected cow with skin lesions covering the entire body (b and c)[19]

isolation. Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin[3].

Lesion material for virus isolation and antigen detection is minced using sterile scissors and forceps and then ground in a sterile pestle and mortar with sterile sand and an equal volume of sterile Phosphate Buffered Saline (PBS) containing sodium penicillin (1000 international units [IU] mL^{-1}), streptomycin sulphate (1 mg mL^{-1}), mycostatin (100 IU mL^{-1}) or fungizone (2.5 µg mL^{-1}) and neomycin (200 IU mL^{-1}). The suspension is freeze-thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 g for 10 min. Buffy coats may be prepared from unclotted blood by centrifugation at 600 g for 15 min and the buffy coat carefully removed into 5 mL of cold double-distilled water using a sterile Pasteur pipette. After 30 sec, 5 mL of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 g for 15 min, the supernatant is discarded and the cell pellet is suspended in 5 mL growth medium such as Glasgow's Modified Eagle's Medium (GMEM). After centrifugation at 600 g for a further 15 min, the resulting pellet is suspended in 5 mL of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample by using a Ficoll gradient[3].

Histopathology: Histopathological examination of acute-stage skin lesions typically reveals a large cellular infiltration, vasculitis, edema and the presence of eosinophilic intracytoplasmic inclusions in cells in the dermis[34]. The skin lesions are characterized by multifocal necrosis and inflammatory infiltration in the epidermis and/or dermis. In some lesions, the necrosis and inflammatory responses were limited to the dermis while the overlying epidermis is largely intact. These lesions are observed near sweat glands, hair follicles or sebaceous glands. The inflammatory cells observed are mainly lymphocytes with low numbers of macrophages and occasionally eosinophils. These cells are particularly prominent around blood vessels adjoining the necrotic lesions (Fig. 3a). Intracytoplasmic inclusions were observed in mono nucleated cells (Fig. 3b). The vascular changes are very prominent and included vasculitis, perivasculitis and perivascular necrosis with concomitant thrombosis. Some arteries depicted thickening of tunica media associated with narrowing of the lumen (Fig. 4a). In Fig. 5b, electron micrograph images of negatively stained preparations showed high densities of typical poxvirus particles[35] (Fig. 5).

The H and E stained section from different parts of the skin lesion showed ballooning degeneration of epithelial cells and presence of eosinophilic intracytoplasmic inclusion bodies due to infection with LSD virus[36].

Electron microscopy: If available, this provides a rapid means for the diagnosis of LSD. The method should be used with at least three different sets of biopsy material from different affected animals. This is due to the possible misdiagnosis, often made other herpes viruses are seen in large numbers in the lesion material. Simple negative staining with phosphotungstic acid may be carried out. Taking a needle, insert it into the lesion material and wash this into a drop of distilled water. A 400 mesh carbon-coated grid is then placed on the drop, blotted and stained with PTA in 0.4% glucose at pH 7.0 for 1 min. Prepare several grids from each sample and examine at

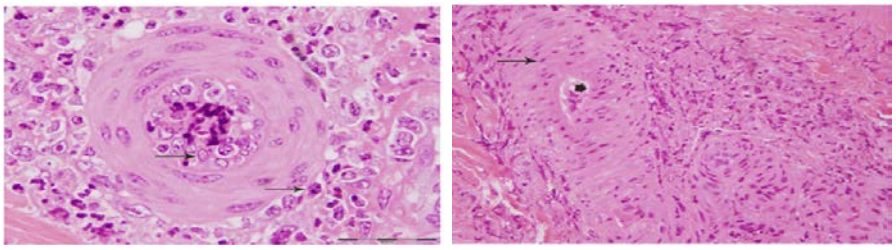

Fig. 4(a, b): Small artery in the vicinity of a skin nodule. Note the vasculitis indicated by the presence of inflammatory cells inside and around blood vessels (a). Small artery showing marked thickening of the tunica media (arrow) as well as a narrow lumen (b)

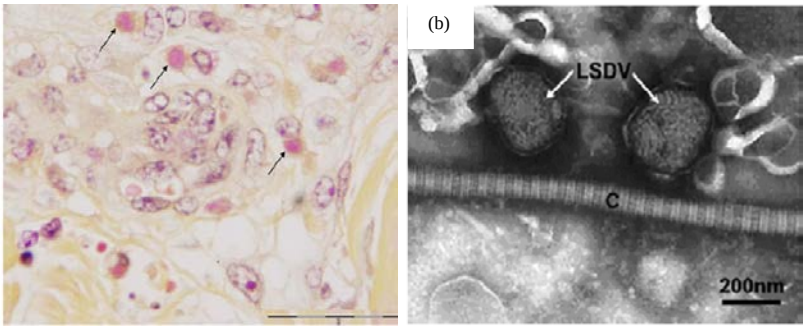

Fig. 5(a, b): Mononuclear cells displaying intracytoplasmic inclusions (a). Transmission electron micrograph of two negatively Phosphotungstic Acid (PTA) stained LSD virus particles indicated (arrows) in close association with a collagen fibre (b). The particles show a typical thread-like structure on their surface and typical "brick-shaped" morphology[35]

6000-18,000 magnification. The large brick-shaped *Capripoxvirus* particles are readily seen in most lesion material[7].

Viral isolation/cultivation of LSD virus: Virus isolation is considered the gold standard method for the diagnosis of viral diseases but its application in the detection of *Capripoxvirus* is limited due to the long incubation times it needs to obtain results[37]. Virus isolation is the method used to investigate the viability of the virus in the samples. LSD virus can be propagated in a variety of primary cells or cell lines of bovine, ovine or caprine origin. It grows slowly on cell cultures and the first cytopathic effect changes can usually be detected four to six days after inoculation. Because the cytopathic effect may take up to two weeks to appear and may require several passages, Polymerase Chain Reaction (PCR) assays have replaced virus isolation as a primary diagnostic assay[16].

Sample material prepared as above, i.e., 1 mL of clarified supernatant or buffy coat, is inoculated on to a 25 cm^2 culture flask at 37°C and allowed to absorb for 1 h. The culture is then washed with warm Phosphate Buffered Saline (PBS) and covered with 10 mL of a suitable medium, such as Glasgow's Modified Eagle's Medium (GMEM),containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing Lamb Testis (LT) cells and a flying cover-slip or tissue culture microscope slides, are also infected. The flasks are examined daily for 14 days for evidence of Cytopathic Effect (CPE). Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4-6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 14, the culture should be freeze-thawed three times and clarified supernatant inoculated on to fresh LT culture. At the first sign of CPE in the flasks or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies which are variable in size but up to half the size of the nucleus and surrounded by a clear halo are diagnostic for poxvirus infection. The CPE can be prevented or delayed by inclusion of specific anti-LSD serum in the medium. Formation of syncytia is not a

feature of *Capripoxvirus* infection, unlike the herpesvirus causing pseudo-LSD. Strains of Capripoxvirus that cause LSD have been adapted to grow on the Chorioallantoic Membrane (CAM) of Embryonated Chicken Eggs (ECE) and African green monkey kidney (Vero) cells. This is not recommended for primary isolation[3].

Cytopathic effects began on incubation days 5-11 and it's characterized by rounding of single cells, aggregation of dead cells and destruction of monolayers. None of the negative control cultures showed any CPE[31]. Isolation of LSD virus revealed the characteristic pock lesion on CAM of ECE and prominent CPE on Madin Darby Bovine Kidney (MDBK) cells starts from third day of post inoculation until complete destruction of cell sheet (Fig. 6 and 7)[38].

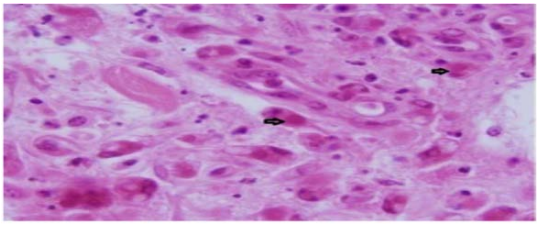

Fig. 6: Photomicrograph of skin showing granuloma with intracytoplasmic inclusions due to lumpy skin disease virus. H&E; X 400[36]

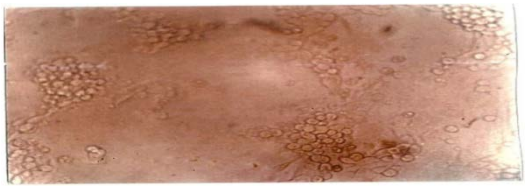

Fig. 7: Characteristic CPE of LSD virus by cell round in, cell aggregation, coalesce together to form clusters within 72 h post inoculation[38]

Serological tests of lumpy skin disease

Virus Neutralization Test (VNT): VNT is the most widely used serological test for Capripox antibody detection[3, 8]. It has high specificity to rule-out false positives due to cross-reaction with cowpox and *Parapoxvirus* antibodies but its sensitivity is lower to trace small antibody titration[8]. It is an accurate gold standard assay but it requires live virus and cell cultures and hence cannot be used in national reference laboratories operating in low-level biocontainment facilities. To increase the number of samples tested on a single plate and to reduce the time needed to interpret the results, the serum/virus neutralization test can be modified to use only the two lowest dilutions of the test serum and a fluorescent marked LSD virus[39].

A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID50 [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 neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus and the consequent difficulty of ensuring the use of 100 TCID50, the neutralisation 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 neutralisation test has been reported to give more consistent results[3].

Fluorescent antibody tests: Davies and Otema[40] usedindirect Fluorescence Antibody Test (iFAT) using the *Capripoxvirus* antigen fixed in the tissue culture plate to detect antibodies against LSD in the serum and in their study, they reported iFAT to have a good sensitivity but cross reacting Parapox and Orthopox viruses might affect its specificity at lower serum dilution rates. IFAT demonstrated to be suitable for use in serological surveys in a study carried out in Ethiopia and it was evaluated test for accuracy[41].

Capripoxvirus antigen can also be identified on the infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 min. The indirect test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from Capripox) or from rabbits hyperimmunised with purified *Capripoxvirus*. Uninfected tissue culture should be included as a negative control as cross-reactions, due to antibodies to cell culture, can cause problems[3].

Enzyme-Linked Immunosorbent Assay (ELISA): ELISA came into use as diagnostic methods for many infectious diseases around the year 1975; till then it has been used as one of the most accepted serological techniques. Currently no sufficiently validated ELISA is commercially available for *Capripox* diagnostics and therefore, virus-serum neutralization tests are still considered as "gold standard" serological assays for *Capripoxviruses*. Various antibody ELISAs have been developed in the past with limited success. The earliest ELISA developed for Capripoxviruses utilized a protein encoded by P32 (vaccinia H3L homologue) as an antigen[42, 43].

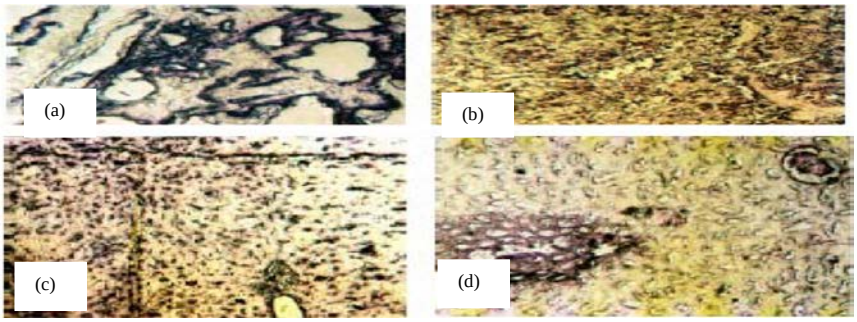

Fig. 8(a-d): Deeply stained dark brown areas in lung, lymph node, liver and kidney sections, respectively after indirect immunoperoxidase staining[44]

More recently, an indirect ELISA was developed based on whole heat-inactivated sheep pox virus as an antigen[45]. When 276 cattle serum samples were tested the diagnostic sensitivity and specificity of this assay were 88 and 97%, respectively. Unfortunately, due to difficulties in producing the inactivated antigen in sufficient quantities, this assay is currently not available for use in the open market. In another study, 42 Open Reading Frames (ORF) of the *Capripoxvirus* genome were evaluated for their antigenic potential and 2 ORFs encoding virion core proteins were selected as the best candidate antigens for use in ELISA. These proteins were then expressed in *Escherichia coli* and used as antigens for an indirect ELISA[37]. However, only 9 serum samples collected from 2 experimentally infected calves were available for evaluating the performance of the test. Recently, an ELISA based on a synthetic peptide targeting the major antigen P32 has been described for the detection of sheep pox and goat pox antibodies[46].

Agar Gel Immunodiffusion test (AGID): Agar Gel Immunodiffusion test (AGID) has been used for detecting the precipitating antigen of *Capripoxvirus* but has the disadvantage that this antigen is shared with *Parapoxvirus* and has also less sensitivity[3].

Indirect immunoperoxidase/IP/technique: Results of the study conducted by El-Kenawy and El-Tholoth[44] indicate that the immunoperoxidase techniques used for detection of LSD virus in collected samples serve as a rapid, effective and economic method for laboratory confirmation of disease. The use of these techniques for direct detection of virus reduce the dependence on tissue culture and the time required to isolate the virus which may delay disease control[44].

Sarma[47] reported that immunoperoxidase is preferable than immunofluorescent test due to results can be read with a light microscope rather than a fluorescent microscope, sensitivity of the assay can be enhanced by increasing the incubation period, endogenous enzyme activity can be blocked where as auto-fluorescence of cells interferes in some immunofluorescent assays, enzyme-antibody conjugates are more stable than fluorescent conjugates and fewer nonspecific reactions with enzyme antibody conjugates than with fluorescein-conjugated antibodies[47] (Fig. 8).

Molecular diagnostics techniques: Molecular-based assays are powerful and precise diagnostic tools for the detection of clinically relevant infectious agents. These novel technologies have demonstrated a number of advantages over traditional culture-based approaches, including increased sensitivity and specificity, rapid turnaround time, multiplexing, reproducibility and the ability to detect fastidious and unculturable organisms[48].

Molecular diagnosis that involves the amplification of the nucleic acid that is DNA or RNA has become a gold standard for rapid detection and diagnosis of viral diseases including *Capripoxviruss*. These diagnostic techniques include LAMP assay, real time PCR and conventional PCR. They are rapid sensitive and specific in detecting *Capripoxvirus* genome compared to immunological and viral isolation methods. The strength of the real time PCR has been its speed, sensitivity, its quantitative nature, detection of results in real time and the ability to include controls for detection of reaction inhibitors. However, it requires expensive high-precision instruments and specialized training for operation and data analysis[49].

In most reference laboratories the basic tests for LSD virus diagnosis comprise molecular methods for generic detection of a *Capripoxvirus*[37, 10, 13, 50, 51]. However, these methods do not differentiate between LSD virus, sheep pox virus and goat pox virus.

Polymerase Chain Reaction (PCR): A PCR technique to detect *Capripoxvirus* antigen from cell culture and biopsy specimens has been developed and the reagents are available commercially[37]. Primers for the viral attachment protein gene and the viral fusion protein gene are specific for all the strains within the genus *Capripoxvirus*[52]. DNA was extracted using a DNeasy kit (Qiagen, USA), according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) assay was used to detect the virus with capripoxvirus-specific primers: forward primer (5-TCTATGTCTTGATA TGTGGTG GTAG-3), reverse primer (5-AGTGATT AGGTGGTGTATTATTTTCC-3). DNA was amplified in a final volume of 50 μL containing the following: 5 μL PCR buffer (10 mM), 1.5 μL MgCl2 (25 mM), 1 μL dNTP mixture (10 mM), 1 μL forward primer (50 mM), 1 μL reverse primer (50 mM), 5 μL DNA template, 0.5 μL Taq DNA polymerase (5 U μL^{-1}) (Invitrogen) and 35 μL of RNAse-free water. The PCR was run in a thermocycler (Applied Biosystems® 2720, USA) using the following amplification programme: initial denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. Additional elongation was at 72°C for 5 min. Amplified products were analysed using 1.5% gel electrophoresis and positive results were confirmed based on the size (172 base pairs [bp]) of the bands[31].

Several highly sensitive, well-validated, real-time and gel-based PCR methods are available and widely used to detect the presence of *Capripoxvirus* DNA[10, 13, 37, 51-53].

These molecular assays cannot differentiate between LSD virus, SPPV and GTPV, nor do indicate whether or not the virus is the cause. The conventional *Capripoxvirus* PCR method is not as fast as real-time PCR but it is reliable and sensitive[16]. Figure 9 shows that polymerase chain reaction-based detection of lumpy skin disease virus from different study sites.

Quantitative real time Polymerase Chain Reaction (qPCR): Quantitative real time Polymerase Chain Reaction (qPCR) assays are routinely used for rapid detection of *Capripoxviruss* in surveillance and outbreak management programs. The modified qPCR assays were multiplexed for detection of beta-actin as an indicator for potential false-negative results. The multiplex modified qPCR assays exhibited the same diagnostic sensitivities as the singleplex assays suggesting their utility in the detection of *Capripoxviruss*[53] (Fig. 10).

One study conducted in southern Azerbaijan showed a total of 269 samples were tested by real-time PCR for the presence of LSD virus from 176 animals, including 130 skin samples, 106 blood samples and 33internal organ

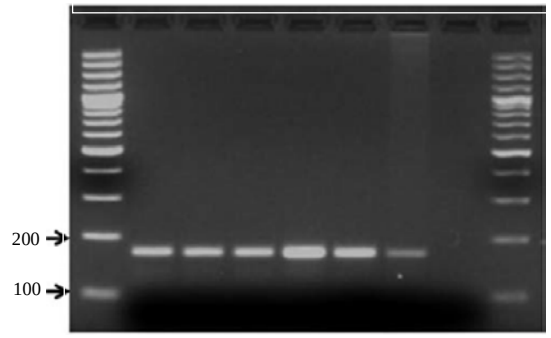

Fig. 9: Polymerase chain reaction-based detection of lumpy skin disease virus. Lane M: 100 bp DNA ladder; Lane NTC: negative template control; Lanes 1, 2: positive samples from Adama; Lanes 3, 4: positive samples from Wenji; Lane 5: positive sample from Mojo; Lane 6: positive sample from Welenchiti[31]

pools. A total of 199 (74%) samples were positive by PCR. All skin lesions tested were positive and had lower CT values than blood or organ samples, suggesting higher concentrations of virus. Blood had the highest average CT value and was least likely to be positive, suggesting lower concentrations of virus[54] (Fig. 11).

Loop-mediated isothermal Amplification (LAMP) assay: LAMP is a novel method of nucleic acid amplification that is catalyzed by a DNA polymerase with strand displacement activity and occurs under isothermal conditions at temperatures between 60 and 65°C. LAMP employs a minimum of four specially designed primers, including a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (FIP, comprised of two binding domains, F1c and F2) and a backward inner primer (BIP, comprised of two binding domains, B1 and B2c) that in combination, recognize six specific regions within the target genetic locus[55].

Additional primers including a Forward Loop Primer (FLP) and a Backward Loop Primer (BLP), are typically optional and may be used to accelerate or enhance the sensitivity of the LAMP assay. Predictably, LAMP assays tend to have high specificity, as the amplification occurs only when six specific regions of the target amplicon are recognized by the primers[56].

The LAMP assay for rapid detection of *Capripoxvirus* was reported by Das *et al*.[49]. LAMP primers were designed to target a conserved gene encoding the poly (A) polymerase small subunit (VP39) of *Capripoxvirus*. Hydroxynaphthol Blue (HNB) was incorporated to monitor assay progress by color change from violet when negative to sky blue when positive and

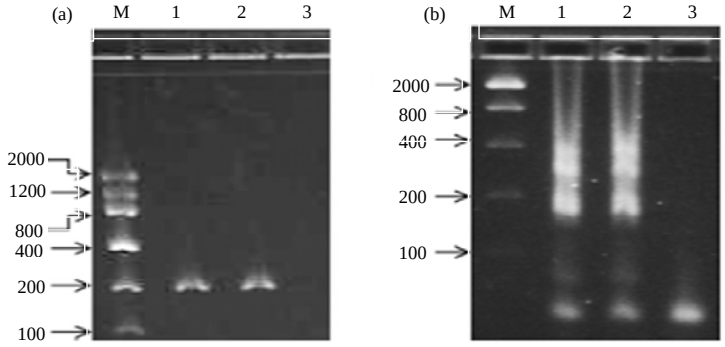

Fig. 10(a, b): Agarose gel electrophoresis of DNA fragments amplified by PCR (a) and LAMP (b) using different templates. (A) PCR amplification of SPV-HELD DNA (lane 1) and control plasmid pORF068 (lane 2) with ORF068 LAMP outer primers F3 and B3. (B) LAMP amplification using SPV-Held DNA as the template (lane 1) and the ORF068 amplified PCR product as the template (lane 2). Water was used as a no-template control for both PCR and LAMP (lanes 3). The molecular weight standards are shown (lanes M)[49]

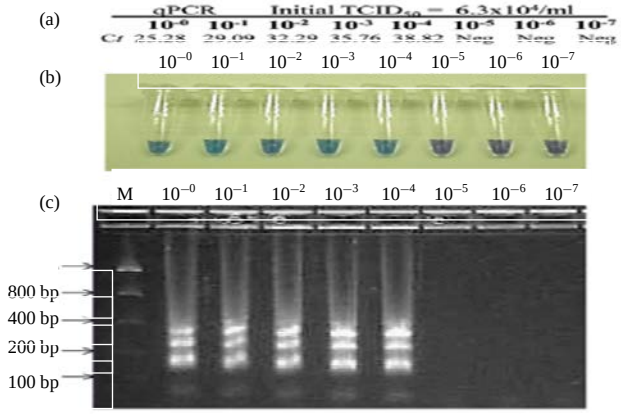

Fig. 11(a-c): Limit of detection (LOD) of SPV-HELD by LAMP and qPCR. SPV-Held (TCID50 = 6.3×10^4 mL^{-1}) and used as the template for amplification by qPCR (a) and LAMP (b and c).Threshold cycle crossing values for qPCR are indicated (CT). LAMP was analyzed by color change using HNB (b) as well as by agarose gel electrophoresis (c)[49]

results were verified by agarose gel electrophoresis. The LAMP assay was shown to be highly specific for *Capripoxvirus* with no apparent cross-reactivity to other related viruses (near neighbors) or viruses that cause similar clinical signs (look-a-like viruses). The performance of LAMP was compared to that of a highly sensitive quantitative real-time PCR (qPCR) assay. LAMP and qPCR exhibited similar analytical sensitivities with limits of detection of 3 and 8 viral genome copies, respectively. The analytical sensitivity of LAMP was shown to be 10-100 fold higher than that of virus isolation. On the other hand, both the analytical and diagnostic sensitivities of LAMP were in close agreement with those of qPCR. LAMP was shown to be specific for *Capripoxvirus* only with no cross-reactivity against other viruses[49].

LAMP assay is more rapid method than real time PCR consisting of ramp time and temperature with cycle number and costly thermal cycler. For any real-time PCR, it requires 75 min to generate a cycle threshold and determining real-time amplification whilst for a gel based it is >120 min. Batra *et al.*[57] in their study reported that LAMP reaction can provide positive results in 30 min after incubation at 65°C and negative result defined not yielding any LAMP product after 60 min. The test described is quick (30 min), sensitive and specific as well as did not show any cross-reactivity to other related viruses that cause apparently similar clinical signs. It was found to be ten times more sensitive than conventional PCR however, 100 times less sensitive than quantitative PCR (qPCR). LAMP assay results were monitored by

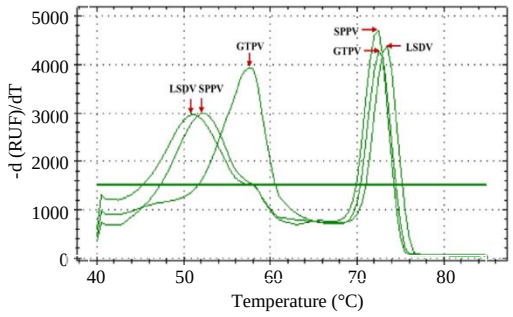

Fig. 12: Snapback primer genotyping of *Capripoxvirus*. The fluorescence melting curve analysis of the PCR products shows two melting peaks for each of the *Capripoxvirus* three genotypes (GTPV, SPPV and LSDV) corresponding to the snapback stem melting peak at lower temperature and the full length PCR amplicon melting peak at higher temperature (see arrows)[58]

color change method using picogreen dye and agarose gel electrophoresis[57]. Das *et al*.[49] determined the Limit of Detection (LOD) of *Capripoxvirus* LAMP using viral DNA extracted from 10-fold serial dilutions of cell culture-grown SPV-HELD as a template. LAMP was monitored by color change using HNB in addition to evaluation by agarose gel electrophoresis. For performance evaluation, the LOD was also determined by qPCR. Based on the initial titer of the virus (6.3×10^4 mL^{-1}) used for serial dilutions and DNA extractions, the LOD was determined to be 6.3 TCID50 mL^{-1} by both qPCR and LAMP as shown in Fig. 12[49].

Genotyping using snapback primer and dsDNA intercalating dye: Cost-effective, cross-platform compatible and easy-to-perform real time PCR assay was developed by Gelaye *et al*.[58] for *Capripoxvirus* genotyping. They described the design and analytical performances of a new molecular assay for *Capripoxvirus* genotyping using unlabeled snapback primers in the presence of dsDNA intercalating Eva Green dye. This assay was able to simultaneously detect and genotype *Capripoxvirus* in 63 samples with a sensitivity and specificity of 100%. A snapback probe element added to the 59 end of the forward primer allowed the formation of a second melting peak during the melting of the PCR products, corresponding to the melting of the snapback stems. Using a combination of the melting of the snapback stems and those of the amplicons, they were able to develop a new approach for *Capripoxvirus* genotyping. The genotyping was achieved by using information from both snapback and amplicons melting. The melting of the amplicons was used to differentiate LSD virus (Tm = 73.5°C) from GTPV/SPPV (Tm = 72.5°C) because the melting peaks separation can be more accurately determined in this region due to height of the peaks as compared to the snapback melting peaks of SPPV (52.0°C) and LSD virus (51.0°C) which are more flat. Furthermore, this 1°C difference between LSD virus amplicons Tm and those of GTPV/SPPV was maintained in all real time PCR machines that were used. This confirmed that the amplicons melting is the best option to differentiate LSD virus from GTPV/SPPV. In contrast, the snapback stem Tm difference between LSDV and SPPV varied according to the real time PCR machine (from 1°C with the CFX and Mini Opticon of Bio Rad to 0.4 uC with the Rotor Gene of Qiagen). The genotyping was achieved by observing the melting temperature of snapback stems of the hairpins and those of the full-length amplicons, respectively. In their report, they described that, the method is highly pathogen specific and cross platform compatible. It is also cost effective as it does not use fluorescently labelled probes, nor require high-resolution melting curve analysis software. Thus, it can be easily performed in diagnostic and research laboratories with limited resources[58] (Fig. 13).

The main weakness of this assay is its low analytical sensitivity as compared to the dual hybridization assay developed by Lamien *et al*.[59]. This is due to the fact that the best conditions of the amplification were not selected because an improved amplification is offset by lower snapback signal. This is probably due to the lower level of ssDNA production and the difference in the ratio between the amplicon melting peak and that of the probe element in the snapback primer which then tend to become flat as the amplicons melting peak increases[59] (Fig. 14 and 15).

A novel HRM assay: This assay is based on High-Resolution Melting Curve Analysis (HRMCA) of PCR amplicons produced using genus specific primer pairs and dsDNA binding dye. Novel multiplex PCR method for the simultaneous detection and differentiation of eight poxviruses which belongs to three different genera of poxviruses (*Orthopoxvirus*, *Capripoxvirus* and *Parapoxvirus*) were developed by Gelaye *et al*.[60]. Differences in fragment size and GC content were used as discriminating power. The assay generated three well separated melting regions for each genus and provided additional intra-genus genotyping allowing the differentiation of the eight poxviruses based on amplicon melting temperature. The assay provides a rapid, sensitive, specific and cost-effective method for the detection of pox diseases in a broad range of animal species and humans. The assay was highly specific with no inter-species cross-reactivity among the different poxviruses and no reactivity to other ruminant viral and

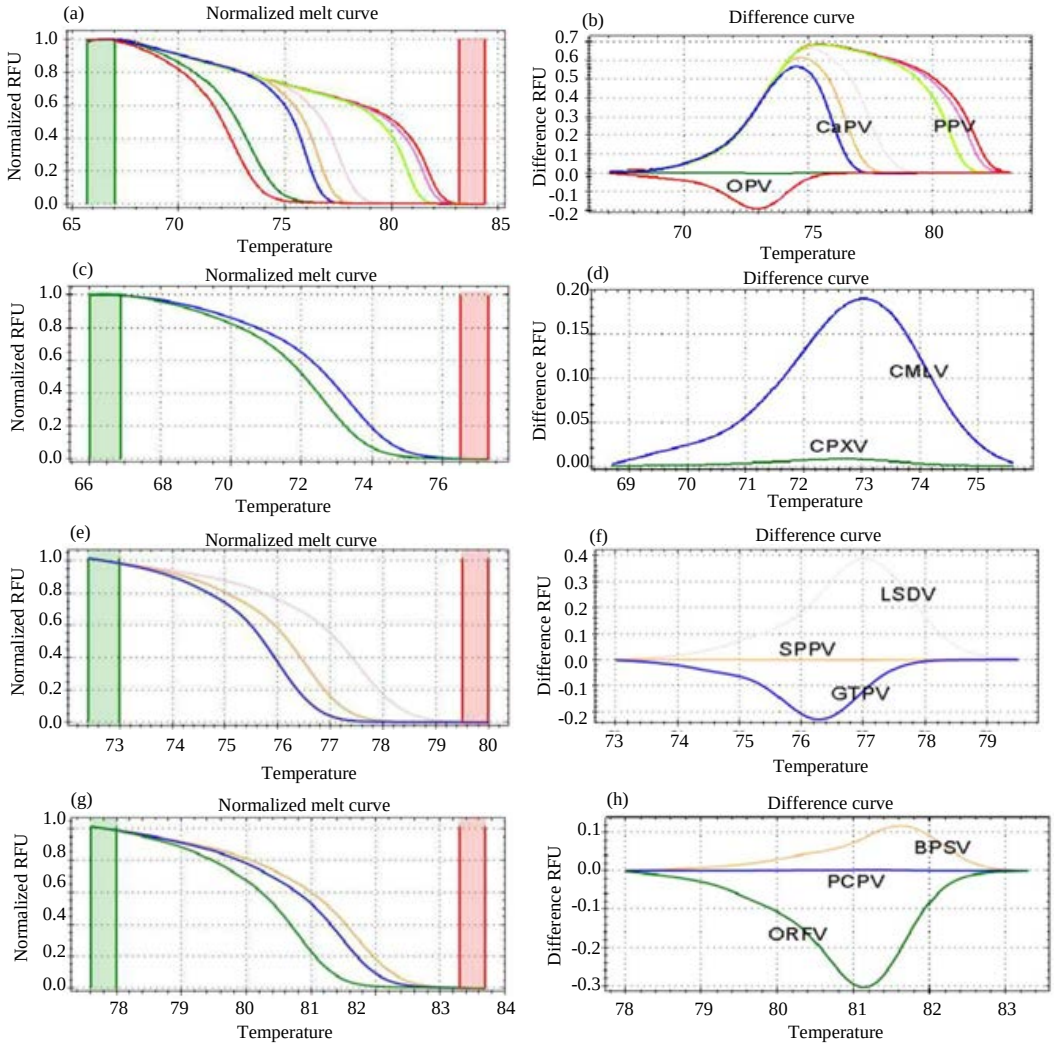

Fig. 13(a-h): Normalized HRM plots of the PCR products of eight poxviruses. Three primer pairs were used for the amplification. Each virus genotype clustered separately within the genus. The normalized melt curve and difference curve plots are presented separately with different line color for each genotype within the genus: for the eight poxviruses (a, b), *Orthopoxviruses* (c, d), Capripoxviruses (e, f) and *Parapoxviruses* (g, h), respectively. Green and red columns in the normalized melt curve plot represent pre- and post-melt normalization regions[60]

bacterial pathogens tested in this study. Additionally, the assay displayed good sensitivity making it suitable as a screening tool during pox disease outbreak investigations. As this assay does not require the use of a probe or labelled primers, is easy to set-up and interpret with a straight-forward analysis of the melting data; it can easily be implemented in laboratories with moderate resources. Another advantage is that the method is very fast, since the complete PCR protocol needs only 85 min or less depending on the PCR platform used[60].

Gelaye *et al.*[60] indicated in his work that the HRM analysis results were in agreement with the classical melting curve analysis, although, a clearer view of the separation between the species was observed by clustering and assigning different colors for each genotype (Fig. 3).

STATUS OF LUMPY SKIN DISEASE IN ETHIOPIA

Lumpy Skin Disease (LSD) is an infectious viral disease of cattle caused by a virus of the genus *Capripoxvirus*. LSD was reported for the first time in Ethiopia in 1981 and subsequently became endemic[61]. LSD was introduced in Ethiopia, for the

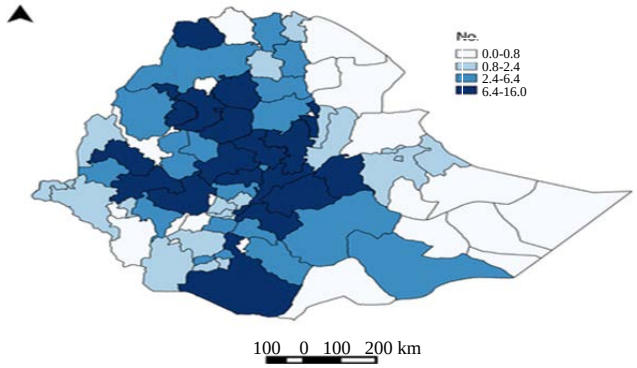

Fig. 14: Zonal distribution of LSD outbreaks per 16 district years in Ethiopia over the period 2000-2015[61]

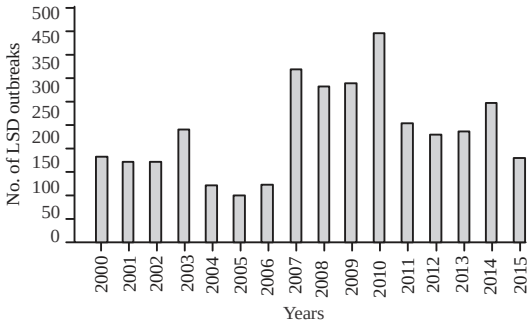

Fig. 15: LSD outbreaks reported over the period 2000-2015 in Ethiopia[61]

first time, through North-West (Gojjam and Gondar) in 1981 with subsequent introductions in the West (Wollega) in 1982 from Sudan and in the central part (Shewa) in 1983. After the introduction, the disease initially spread East wards, later to all directions and currently it has affected all regions and agro-climatic zones of the country[6]. Seasonal variation in the incidence of LSD outbreaks is common in Ethiopia in which it occurs most frequently between September and December[31].

During the period 2000-2015, LSD has been reported from all regional states (n = 9) and city administrations (n = 2) of Ethiopia. About 82% of the districts (n = 683) and 88% of the administrative zones (n = 77) in the country reported at least one LSD outbreak in this time period. In total 3811 LSD out-breaks were reported in Ethiopia in the study conducted by Molla *et al.*[61]. Most of the outbreaks reported in different region of Ethiopia were from Oromia (54.5%), Amhara (27.9%), SNNP (10.1%) and Tigray (3.6%)[61].

Targeted sampling from outbreak areas around Southern range land, Wolliso town and Sero-prevalence of lumpy skin disease in selected districts of West Wollega zone reported prevalence of 11.6 and 6.43%, respectively[41, 62]. As reported by Gari *et al.*[14] LSD has been extensively circulating across diverse agro-climatic zones of Ethiopia with large variations between districts that could be attributed to their respective agro-ecological zones and farming practices.

Analysis of retrospective data between January 2007 and December 2011 reported by Ayelet *et al.*[31] indicated that a total of 1,675 outbreaks with 62,176 cases and 4,372 deaths which were reported to the Ethiopian MOA. The highest number of outbreaks was recorded in 2010 (447) followed by 2009 (339). The frequency of reported outbreaks was higher between September and December with the highest numbers in October (266) and November (287) the lowest number was reported in May. The high number of outbreaks from September to December is evident[31].

CONCLUSION

LSD virus, a member of the genus *Capripoxvirus*, belongs to family *Poxviridae* with typical poxvirus geomorphology and closely related to the viruses of sheep and goat pox. Lumpy skin disease causes serious economic losses in most African countries including Ethiopia. The diagnosis of LSD may be tentatively made after appearance of the typical skin lesions. Virus isolation is the method used to investigate the viability of the virus in the samples and LSD virus can be propagated in a variety of primary cells or cell lines of bovine, ovine or caprine origin. It grows slowly on cell cultures which results in first cytopathic effect changes that can usually detected four to six days after inoculation. However, the cytopathic effect may take up to two weeks to appear and may require several passages. Due to this, Polymerase Chain Reaction (PCR) assays have replaced virus isolation as a primary diagnostic assay. Development of molecular assays such as loop-mediated isothermal amplification, novel HRM assays and the use of snapback primer and dsDNA intercalating dye were reported by different researchers and the specificity and sensitivity of each assays were reviewed. For instance, molecular tests using loop-mediated isothermal amplification are reported

to provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost. Rapid, sensitive, specific and robust tool for diagnosis of the causative agent is an essential prerequisite for controlling any outbreaks and effective disease surveillance and monitoring programs. Although, numerous research efforts have been made for the development of sero-diagnostic assays for LSD virus detection, it needs further improvement.

RECOMMENDATIONS

Accordingly, the following recommendations are forwarded:

- Molecular diagnostic tools are the best and reliable diagnostic tests for LSD detection
- Serological tests like ELISA that can be used for screening of cattle against the disease needs further improvements and existing ones need to be tested in the country
- Cost-effective sero-diagnostic tests that can potentially discriminate between *Capripoxviruses* of cattle, sheep and goats need to be developed as Ethiopia is endemic to the disease

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