Validation of Loop-Mediated Isothermal Amplification method for Specific Detection of Camelpox Virus Genome

By

Rasha Awad Ibrahim

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Under the supervision of:

Prof. ABDELMALIK. I. KHALFALLA

University of Khartoum

Faculty of Veterinary Medicine

Department of Microbiology

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Dedication

To my great family

To my lovely kids

And of course to my husband

I dedicate this work
ACKNOWLEDGMENTS

First and foremost, I would like to thank my merciful Allah for giving me strength and health to do this work.

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ABSTRACT

Camelpox (CP) is a highly contagious viral disease affecting mostly young animals and characterized by papular eruption on the skin and mucus membrane. The causative agent is an orthopox virus of the family poxviridae. The disease causes high mortality and morbidity in its single host species. Camelpox is widespread in the arid zone of Africa, the Middle East and central Asia.

Loop-mediated isothermal amplification (LAMP) was optimized to amplify the hemagglutinin (HA) gene of camelpox virus (CMPV). LAMP is a novel method of gene amplification that amplifies nucleic acid with high specificity, efficiency, and rapidity under isothermal conditions with a set of six specially designed primers that recognize eight distinct sequences of the target. Primers used for gene amplification were designed from the camelpox Hemagglutinin gene. The whole procedure is very simple and rapid, and amplification could be obtained in less than 1 h by incubating the reaction mix in a single tube at 63°C and visualizing the products by agarose gel electrophoresis. Naked-eye visualization for amplification of LAMP method was also possible through observation of a color change following addition of 1 µl (1:1,000) of SYBR Green I dye to the tube. In positive amplification, the original orange colour of the dye changes to green, which can be seen under natural light as well as under UV light (302 nm).

The sensitivity and specificity of the LAMP were evaluated with PCR using six extracted DNA samples of different poxvirus-species (camelpox, sheeppox, vaccinia and parapox virus). Both LAMP and PCR showed the same specificity as both detected camelpox without false positive result with other pox virus species. The specificity of the LAMP was observed by the absence of any cross-reaction
with other, closely related, members of the pox viruses species. When the sensitivity of the developed LAMP assay was compared to that of PCR, it was found that the LAMP demonstrated 16-fold higher sensitivity compared to PCR with a detection limit of LAMP in serially diluted quantified camelpox DNA of 0.075 ng/µl, compared to that of PCR (1.25 ng/µl). These results demonstrate that the LAMP-based assay is a useful tool for the rapid and sensitive diagnosis of camelpox. It is cost-effective, highly sensitive, and specific. The developed assay is expected to improve laboratory methods for diagnosis of this important disease in the Sudan and in the other countries where camels are raised and subsequently assist in its control.
الاستئصال، وليست بثة، وعندما يكون الجلد في الواقعة يتغير، يكون المنقاد عليه الشديد، وع础 عليه. وعندما يكون الجلد في الواقعة يتغير، يكون المنقاد عليه الشديد، وع础 عليه. 

(Loop-Mediated Isothermal Amplification Technique LAMP) 

ومضارب (نوك) (Hemagglutinin) في الأبل، ثم على تعرفه، بسانتة بمثابة له، وأجل تكنولوجيا. 

وإذا كانت (LAMP) لانست، يحتمل أن تكون هذه الأجزاء. 

وإذا كانت (LAMP) لانست، يحتمل أن تكون هذه الأجزاء.
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<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CMPV</td>
<td>Camelpox virus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DDW</td>
<td>Deionized distilled water</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>GMEM</td>
<td>Glasgow Minimum Essential Media</td>
</tr>
<tr>
<td>OPV</td>
<td>Orthopoxvirus</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Phosphate diluents</td>
</tr>
<tr>
<td>LAMP</td>
<td>Loop mediated isothermal amplification</td>
</tr>
<tr>
<td>HE</td>
<td>Hemagglutinin gene</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolution per minutes</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA</td>
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</table>
INTRODUCTION

Camelpox (CP) is a highly contagious viral disease affecting mostly young animals and is characterized by papular and pustular eruptions on the skin and mucus membranes (Khalafalla, 1998). A typical pox exthyma appears over the entire body and on the head (Murphy et al., 1999). The disease is also characterized by fever and enlargement of lymph nodes (Al-Ztabi et al., 2007). In systemic form, pox lesions can be found in the mucous membrane of mouth, respiratory and digestive systems (Wernery, 1997).

Camelpox virus (CMPV), the etiologic agent of camelpox belongs to the family Poxviridae genus Orthopoxvirus (Sophie et al., 2007). The family Poxviridae contains cytoplasmic DNA viruses that replicate in vertebrate and invertebrate hosts (Clarissa et al., 2000).

Orthopox viruses are morphologically large and antigenically closely related vertebrate viruses that include the now eradicated smallpox variola virus, the smallpox vaccine vaccinia virus and several animal pathogens of veterinary economic and public health zoonotic importance (Ropp et al., 1995).

The disease in nature causes significant economic impact attributable to high morbidity (up to 100%) and mortality (up to 25%), which result in reduction of milk production and loss of body weight (Mayr and Czerny, 1990). Camelids may become infected via small abrasions of the skin, by aerosol infection of the respiratory tract, or by arthropod bites (Wernery and Kaaden, 2002).

Camelpox can be diagnosed by clinical symptoms in combination with electron microscope (Al-Ztabi et al., 2007). Identification and differentiation of orthopox virus species and strains has been achieved by variety of immunologic and biologic
methods (Susan et al., 1995). Confirmatory diagnosis can be accomplished by electron microscope, detection of orthopox viruses in tissue culture cells and in the chorioallantoic membrane (CAM) of embryonated chicken eggs as well by serological tests (Munz, 1992). Conventional serological tests (hemagglutination, hemagglutination inhibition, neutralization, complement fixation and fluorescent antibody test have been used to detect camelpox virus antibodies but have a number of restrictions. The most commonly used test (virus neutralization) for diagnosis of orthopox does not measure antibody to envelope antigens which is important in pathogenesis (Azwai et al., 1995).

Ropp et al (1995) developed a PCR strategy to differentiate between orthopox viruses species including camelpox virus. They have successfully used this strategy to identify virus DNA in clinical materials, infected cell culture and CAM. Due to extreme sensitivity, PCR methods have evolved to be diagnostic method of choice (Andreas et al., 2004). Unfortunately, conventional PCR method are time consuming, require laborious post PCR handling and include high risk of unwanted carry over contamination by processed PCR product particularly when genotyping by restriction fragment length polymorphism analysis used for differentiation (Loperen et al., 2001).

In the year 2000, Notomi and others described a novel nucleic acid amplification method; Loop mediated isothermal amplification of DNA (LAMP), in which reagents react rapidly and efficiently with high specificity under isothermal conditions (Notomi et al., 2000). It relies on autocycling strand-displacement DNA synthesis performed by BST polymerase large fragment. The amplification products are stem loop DNA structure with several inverted repeats of the target and structure with multiple loops. The LAMP reaction can be conducted under isothermal conditions ranging 60-65° C by using only one type of enzyme and four
or six primers recognizing six or eight distinct regions. The most important of this method is that no denaturation of DNA template is required (Nagamine et al., 2001). When the two additional loop primers were added to the LAMP reaction, time can be shortened (Nagamine et al., 2002). With this modification, the LAMP method is able to amplify the target DNA in shorter time than and with extremely high specificity compared to PCR method. Moreover, the LAMP method produces large amount of amplified product resulting in easier detection such as detection by visual judgment based on turbidity or fluorescence of the reaction mixture (Mori et al., 2001).

Although no LAMP method is established for detection of CMPV as yet, but it can be one of the efficient diagnostic methods.

**Objectives of the study:**

1. To establish a LAMP method for the detection of CMPV.
2. To determine the sensitivity and specificity of the developed LAMP.
3. To compare the sensitivity of the LAMP assay to that of conventional PCR.
CHAPTER ONE

REVIEW OF LITERATURE

1.1 Definition

Camelpox (CP) is species specific contagious disease characterized by fever, enlargement of lymph nodes and localized and generalized pox lesions (Kalafalla et al., 1998; Wernery, et al., 1997). Camelpox is caused by a virus which has been classified under the orthopox virus genus of the family Poxviridae (Mahnel, 1974, Meyer et al., 1993., Wernery and Kaaden, 2002).

Camelpox virus (CMPV) has characteristics and properties of a true poxvirus and is closely related to vaccinia-virola group (Mahnel and Bartenbach, 1973).

1.2 structure, genome, replication and Taxonomy:

Orthopox viruses are characterized by large brick –shaped virus particles containing double stranded DNA genome of approximately 200,000 bp. The average size of the virion is 265–295 nm. Orthopox viruses are enveloped, and the outer membrane is covered with irregularly arranged tubular proteins. A virion consists of an envelope, outer membrane, two lateral bodies and a core. The nucleic acid is a double-stranded linear DNA. Among the characterized orthopox genomes, the highest sequences homology of orthopox species is found in the middle of the genome, while the terminal section of the genome can exhibit considerable variability probably reflecting differences in host range, tissue tropism and virulence. The virus replicates in the cytoplasm of the host cell, in so-called inclusion bodies (Tantawi et al., 1974, Anderas et al., 2004).
CMLV genome contain 205,719 bp, 211 putative genes and consist of central region bound by inverted terminal repeats of approximately 7kb and a high degree of similarity in gene order, gene content and amino acid composition in region located between CMLV017 and CMLV184. CMLV contain unique region of approximately 3kb which encode three open reading frames CMLV 185, CMLV 186 and CMLV 187 absent in other OPV. Among orthopox viruses (OPVs), CMPV is most closely related to variola virus (VARV) sharing all genes involved in the basic replicative functions and the majority of genes involved in other host related functions (Afonso et al., 2000).

Poxviridae is a family of large DNA viruses that replicate in the cytoplasm of infected cells. There are two major infective forms of the virus: a single-membrane wrapped virion also known as mature virion (MV) and a double-membrane wrapped virion, also known as enveloped virion (EV) (Moss, 2006). An additional subdivision is used to describe the different intracellular and extracellular forms of the virus. The intracellular progeny is subdivided to a single-membrane wrapped virion also named as intracellular-mature-virus (IMV) and to intracellular-enveloped-virus (IEV) which is wrapped with two additional membranes. The extracellular forms are divided to an extracellular-cell-associated-virus and to the extracellular-enveloped-virus (CEV and EEV respectively) (Smith et al., 2002). Attachment of EV particle to the cell results in the rupture of the outer membrane by glucose-amino glycans (GAGs) revealing single-membrane wrapped particle: the MV. At this stage the mechanism, of entry is identical to that of naked MV particle. During MV entry, the membrane fuses either with the host-cell plasma membrane or with the endosome membrane, releasing the viral core into the cytoplasm (Carter et al., 2005).
Multiplication of pox viruses in the cytoplasm required that the infecting virion carries the enzymes needed for mRNA synthesis, including a DNA-dependant RNA polymerase and enzymes that cap methylate, and polyadenylate resulting mRNAs. Splicing activities are not known to be used by these viruses. Using the enzyme from infecting particles, viral transcription begins in cytoplasmic replication. Only subset of viral genes (the early genes) is transcribed initially. Early gene product includes proteins needed for replication and to activate the intermediate genes, which then activate the late genes. Independence from the cells owns transcription machinery allows the virus the opportunity to shut the cell nucleus down, so that all metabolic recourses of the cell are devoted to the virus. Poxviruses are exclusively cytolytic. Poxviruses make a large array of proteins involved in modulating the host immune response to infection (Flint and Shenk, 1997).

Orthopox viruses are divided into 11 species, 8 Eurasian –African species (variola virus [VAR smallpox virus], monkeypox virus [MPX], vaccinia virus [VAC], cowpox virus [CPV], camelpox virus [CMLV], ecromelia taterapox and Uasin Gishu disease viruses) and 3 North American species (raccoon poxvirus [RCN], volepox virus [VPV] and skunkpox virus [SKN]) (Victoria et al., 2004).

1.3 Physiochemical properties:

Poxviruses are susceptible to various disinfectants including 1% sodium hypochlorite, 1% sodium hydroxide, 1% per acetic acid, formaldehyde, 0.5-1% formalin and 0.5% quaternary ammonium compounds. The virus can be destroyed by autoclaving, boiling for 10 minutes and is killed by ultraviolet rays (245 nm wave length) in a few minutes (Coetzer, 2004).
There are conflicting reports for sensitivity of camelpox to ether or chloroform. Alfalluji et al (1979) stated that the virus (Iraqi isolate) was sensitive for both ether and chloroform. Ramyar and Hessami (1972) found that the virus (Indian isolate) was sensitive to ether and chloroform. Khalafalla and Mohamed (1998) reported that some Sudanese isolates are sensitive to both ether and chloroform. In Kenya and Niger, Davies et al (1975) found that the virus is sensitive to chloroform and resistance to ether.

Effect of temperature on CMLV varies between different isolate in different countries. AlFalluji et al (1979) reported that the CMLV isolates resist heat at 56°C for one hour. Reduction of infectivity and complete inactivation occur at 60 °C for one hour and two hour respectively. Nguyen et al (1989) found that a CMLV isolate in Niger was thermo labile at 56 °C for 10 min. The CMLV isolates lost infectivity to cell culture when heated at 56 °C for 10 min (Khalafalla and Mohamed, 1998).

The virus is readily inactivated by heat, direct sun light, week acid and alkali, Iodine and potassium permanganate (McGrane et al., 1985).

1.4 Transmission:

The camelpox disease spread mainly by direct contact with sick animals particularly at watering places or indirectly via contaminated environment (Khalafalla and Mohamed, 1998, Ramyar and Hessami, 1972). The role of arthropod vector in the transmission of the disease has been suspected (Al–Ztabi et al., 2007).
Camelpox is most probably not zoonotic (Sophie et al., 2007). Azabi et al (2007) reported that no evidence for signs of human infection of people who took care of their infected animals. Though transmission to human is very rare but it can occur in immunocompromized individuals. Also infection was reported in non vaccinated humans in Somalia (Sophie et al., 2007).

1.5 Clinical signs:

Camelpox occurs naturally in old world camels including *Camelus dromedarius* and *Camelus bacteranus* (Wernery et al., 2002). The incubation period of the disease ranged from 9-13 days (Wernery and Kaaden, 2002). Clinically, two forms can be distinguished, the severe generalized form (most among young animals) and the milder localized form (mostly in old). In both forms initial multiplication of the virus occurs at site of entry. In systemic disease further viral multiplication in draining lymph node is followed by primary viraemia and virus replication in organs and tissues (Wernery et al., 2002). This results in a second viraemia subsequent infection of the skin. Eruptions over entire body are found in generalized form in which mortality rate can reach 28% (Jezek et al., 1983).

Khalafalla (1998) reported that acutely affected animals had a fever (morning temperature: 39-40° C), edema of face, lachrymation, lymph node enlargement, papules formation and pendulous lips. Papules and vesicles were first observed in the lips and nostrils and later involved the whole head, neck, buttock, abdomen legs and groin. Pox lesion observed in the trachea and lungs of young dromedaries (Wernery and Kaadan, 1995). Classical lesions in skin start as ertherematous macules which develop into papules and vesicles. Vesicles develop into pustules with depressed centers and raised erthermatous borders called pocks, after pustules
have rupture they become covered by crusts. Healing of pustules might take 4-5 weeks with or without scars (Munz, 1992).

Associated lymph nodes are often swollen (Munz, 1992). Mammary glands, genitalia and nasal area are also frequently affected (Kriz, 1982; Murphy et al., 1999). In some affected animals blindness develops due to the presence of pox lesions on the eyes. In young animals there is difficulty in suckling or eating due to ulceration of the buccal cavity by pox lesions, difficulty in respiration and occasionally pneumonia.

In the mild form, pustules appear in nostril, eyelid and nasal mucosa. The course of disease was found to range between 15 to 30 days. Occasionally the course may extend for 2 to 4 months with localized pox lesions (Khalafalla and Mohamed, 1998).

1.6 Diagnosis of camel pox

Preliminary diagnosis of CMLV is based on clinical, epizootiological and pathological findings (Buchnev et al., 1987). Several diagnostic methods are available and where possible, more than one should be used to make confirmatory diagnosis of disease. Confirmatory diagnosis may be accomplished by electron microscopy, detection of orthopox particles in pox lesions, cultivation of the virus in tissue culture cells or in chorioallantoic membrane (CAM) of embryonated chicken eggs as well as serological tests (Munz, 1992).

The systemization and laboratory differentiation is of great importance in demarcating the orthopox virus from parapox virus as both viruses can be found in the same camel (Wernery and Kaaden, 1995). The fastest method of laboratory confirmation of camelpox is by the demonstration of the characteristic brick-shape
orthopox virions in skin lesions, scabs or tissue samples using transmission electron microscopy (TEM). Camelpox virus is distinct from the ovoid–shaped parapox virus (Mayer and Czerny, 1990).

Camelpox can be confirmed by demonstration of the camelpox antigen in scabs and pox lesions in tissues by immunohistochemistry (Nothelfer et al., 1995). It is relatively a simple method that can be performed in laboratories where TEM is not available. In addition the paraffin-embedded sections can be stored for long period enabling future epidemiological, retrospective studies.

Chicken embryos are commonly employed for propagation of pox viruses on the CAM (Cunningham, 1973). Most camelpox virus isolates from different countries grow readily on primary inoculation of embryonated chicken eggs (Ramyar and Hessammi, 1972). Camelpox virus can be isolated on CAM of 11-13days oldembryonated chicken eggs. The eggs should be incubated at 37°C and after 5days the eggs containing living embryos are open and CAM examined for the presence of characteristic pock lesions. Camelpox virus produce several distinct pocks which are opaque and round (approximately 0.5-1.5mm in diameter) and without hemorrhagic or necrotic centers (Khalafalla and Mohamed, 1998).

In cell culture, CP virus replicates and produces complete cytopathic effects (CPE) in a wide range of cell cultures (Mayr et al., 1990). Clear CPE were produced in baby hamster kidney cells (BHK) and lamb lung, calf and lamb testis, chicken embryo kidney cell culture (Khalafalla and Mohamed, 1998). The CPE produced by VD45 isolate of CPV in Vero and porcine kidney cells has been observed under different forms; first infected cells may be seen as round refractive; second formation of syncytium by progressive fusion of greater numbers of affected cells which terminate by detachment of those cells to form plaque; the third form was
mixed type consist of syncytium of round cells and plaque. Intracytoplasmic eosinophilic inclusion bodies, characteristic of poxvirus infection, may be demonstrated in infected cells using haematoxylin and eosin staining (Pfeffer et al., 1996).

Various serological tests have been used for demonstration of antibodies against CP. These include gel diffusion test, neutralization test, and plaque reduction test. Those tests were found to be not suitable for large scale serological surveys since they either time consuming, insensitive or expensive (Khalafalla et al., 1998).

Khalafalla and Mohamed (1998) identified CMPV using virus neutralization, agar gel diffusion, immune fluorescence test and histopathological picture of skin lesions.

Munz et al (1992) described enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody for camelpox. The simplicity of ELISA makes it suitable for retrospective studies and also for epidemiological investigations (Azwi et al., 1995).

Nucleic acid hybridization techniques based on the polymerase chain reaction (PCR) are now widely used for detection and characterization of many viruses, including poxviruses (Khalafalla et al., 2005).

The presence of viral nucleic acid may be confirmed by polymerase chain reaction, and different strains of camelpox virus may be identified using DNA restriction enzyme analysis (Meyer et al., 1994). Binns and othres used PCR in coloning of thymidine kinase (TK) gene from camelpox (Binns et al., 1992).
Ropp et al (1995) developed a PCR strategy to differentiate between orthopox virus species including CMPV. They successfully used this strategy to identify virus DNA in clinical materials infected cell culture and CAM.

Khalafalla et al (2005) used PCR for rapid diagnosis and differentiation of parapox virus (PPV) and orthopox virus infections in camels. They found that the PCR proved to be more sensitive in viral DNA extracted directly from scabs specimens and also fast and specific diagnosis of PPV and OPV infection can be accomplished directly from scabs materials without the need for a DNA extraction step (Khalafalla et al., 2005).

Al–Ztabi and others (2007), reported that camelpox can be diagnosed by clinical symptoms in combination with PCR and electron microscope (Al –Ztabi et al., 2007).

The LAMP assay originally described by Notomi et al (2000) is based on the principle of autocycling strand displacement DNA synthesis. The assay utilizes a single DNA polymerase that is active at relatively high isothermal amplification temperatures, which diminishs the probability of non-specific priming (Boehme et al., 2007).

The reaction is performed by a DNA polymerase with high strand displacement activity and a set of two specially designed inner primers and two outer primers (Notomi et al., 2000). Further improvements in the time kinetics and sensitivity of the LAMP reaction by the use of two additional loop primers, termed accelerated LAMP, have been reported (Nagamine et al., 2002). LAMP is highly specific for the target sequence because of the recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences during the later stages of the LAMP reaction(Mori et al., 2001).
1.7 Epidemiology:

Camelpox is most frequently and widely reported viral disease of camel (Khalafalla et al., 1998). Its economic importance is mainly due to its relatively high mortality, loss of condition and fall in milk production and weight of infected camels (Jezek et al., 1993).

Although the causative virus for camelpox was not isolated until 1970 but it has been long recognized by its wide spread in many camel raising countries (Kriz, 1982). This viral infectious disease has been reported throughout Africa, North of equator, the middle east and Asia (Sophie et al., 2007). Some Asian countries (India, Pakistan and Afghanistan) regarded as the main endemic countries of camelpox (Azwai et al., 1996).

In the Middle East, camelpox disease is well known for its economic consequence, and is characterized by high morbidity and high mortality rates in young camels and pregnant females (Sophie et al., 2007).

Clinically two distinct types can be distinguished severe generalize form which appear more frequently among young animals and the milder localized form encounter more often in older.

Occurrence of outbreaks is often reported during wet seasons (Munz, 1992). Alhendi et al (1994) observed that camelpox outbreaks are becoming more severed mainly during rainy seasons, and when the disease developed during the dry season it usually fellow a mild course.

In United Arab Emirates Wernery et al (1997) reported that no camelpox disease occur during hot summer but occur only during winter seasons.
CMPV has been isolated from camel ticks *Hyaloma dormedarii*, it is generally believed that larger arthropod population build up during rainy season, forcing greater virus pressure and virus doses onto camel population (Wernery and Kaaden, 2002).

The morbidity rate of camelpox is variable and depends on whether the virus is circulating in the herd (Wernery et al., 1997).

Jezek et al (1993) observed that death occurred in 30% of the observed outbreaks with highest case of fatality in single outbreak being 28%.

Kriz (1982) and Davies et al (1975) reported an incidence of 50% of camelpox in Somalia and of 100% in Kenya.

The mortality rate is usually low (5-10%) and appear to be influenced by the presence of intercurrent disease (notably trypanosomiasis), stress, the age, structure and the general nutritional status of the herd at the time of outbreak as well as virulence of the virus strain involved (Azwai et al, 1996). Ramyar and Hessami (1972) reported that the mortality rate in infected herds range from 25-100% for young animals and 5-25% for older ones, and it was higher in males than in females. Kriz (1982) reported that the mortality rate in infected herds was 8.8% in males and 4.4% in females.

In the Sudan, camelpox was first reported in 1953 but it is known to exist in the country for years under Arabic name *algedderi* (Khalafalla et al., 1998).

Sudan is regarded as second most density camel populated country in the world after Somalia (Schwatz and Dioli, 1992). According to Shommein and Osman (1987) the disease was reported in the late severities in Kassala of eastern Sudan. Khalafalla and Mohamed (1996) reported that there were thirty five outbreaks of
Camelpox in Eastern Sudan between 1992-1994 with morbidity and mortality rate of 9 and 1.2%, respectively while the case of fatality rate was 14%. Most of affected animals were immature, less than five years of age with mean age of 2.7 years. All deaths occur in young animals less than 3 years with mean age of 1.7 years.

In four areas of Sudan Khalafalla et al (1998) found that the prevalence of seropositive animals was higher in adult more than four years old (87%) than in calves less than one year old (40%) and than in young animals of 1-4 years old (75%) and the prevalence rate was higher in female camels (76.5%) than in males (66-70%).

1.8 Prevention and control

Despite the economic importance of camelpox little has been published concerning the production of potent vaccine for it control.

Primitive methods are used by Bedouin camel herd to protect their stock by rubbing a suspension of pox crust in milk into scarified labia surface of calves. Similar method was also reported in Punjab (Hafez et al., 1992).

The eradication of camelpox infection in camel husbandry is of great importance by developing attenuated protective vaccine (Elharrak et al., 1991).

Orthopox virus (OPV) induce an immunity which protect against infection with other member of the genus and avirulent live vaccine (vaccinia virus strain) has been used to vaccinate camels against camelpox (Martin et al., 1995).

Sophie et al (2007) in their study suggested that treatment with antiviral agent could be another therapeutic approach to managing camelpox infection particularly in young camels by using the antiviral drug Cidovir.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Virus strains

2.1.1 Camelpox virus

Camelpox virus (CP/DB/92/3) used in this study is a field virus isolated from an outbreak of the disease in Butana area, eastern Sudan (Khalafalla et al., 1998).

2.1.2 Vaccinia, sheeppox and parapox viruses

Those poxviruses species were obtained from the virus stock of the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum and were used as controls in the LAMP test.

2.2 Preparation and sterilization of glassware

Flasks, beakers, Bijou and volumetric bottles, measuring cylinders, tissue culture bottles, tubes and other glassware were rinsed in tap water, brushed with soap and then rinsed several times in tap and deionized distilled water (DDW). The clean, dry glassware was sterilized in hot air oven at 160° C for one hour. Volumetric glass pipettes were soaked overnight in potassium dichromate then they were washed several times in distilled water (DW).

2.3 Preparation of cell culture

A flask containing confluent monolayer culture of Vero cell from the stock of the Virology Research Laboratory, Department of Microbiology, Faculty of Veterinary
Medicine was used. Growth media was removed then cells were washed with phosphate diluents (PD), then 0.5 ml of warm trypsin-versen solution was added and the flasks were incubated at 37°C until the cells flew freely when the flask is tilted. After that a few drops of bovine serum were added to stop the action of trypsin - versen. Then cells were diluted in Glassgow Minimum Essential Media (GMEM) growth medium and sub-cultured in appropriate plastic tissue culture flask and incubated at 37°C.

2.4 Virus propagation

25cm flasks containing semi-confluent monolayer cells were inoculated with 0.2 ml of each virus suspension. The inoculated flasks were kept at 37°C for 60 min (adsorption time). Inocula were then removed and monolayer washed twice with PD and refilled with maintenance media. Flasks were examined daily with inverted microscope and when cytopathic effect (CPE) involved 90% or more of the flask the whole culture was harvested after three repeated cycles of freezing and thawing.

2.5 DNA extraction

Harvested cultures were pelleted by ultra centrifuge (L7-65 ultra centrifuge TYPE-15.zonal and continuous fellow rotator, USA) for one hour at 20.000 rpm. The supernatant was discarded and the pellet drain was covered with 0.5 ml phosphate buffered saline (PBS) and kept at 4°C overnight. Then the pellet was transferred to 1.5 ml Eppendorf tube when it was dissolved.

DNA was extracted by using DNA extraction kit (PUREGEN E genomic DNA purification kit, Gentera system USA) by the following steps:
- 300µl of lysis solution was added to the cells by pippetting up and down and vortexing at high speed for 10 seconds. Then the tubes were heated at 65° C for 15-60 min to complete cell lysis.
- 1.5 µl of proteinase K solution (20mg/ml) was added to cell lysate and mixed by inverting the tube 25 times and incubated at 55° C overnight.
- 1.5 µl of RNAase solution was added to cell lysate, sample were mixed by inverting the tube 25 times and then incubated at 37° C for 15-60 min.
- Samples were cooled to room temperature by placing on ice for 1min then 100 µl of protein precipitation solution was added and vortexed at high speed at 13.000xg for 3 min.
- Supernatant containing DNA was poured into clean 1.5 ml microfuge tube containing 300 µl 100% isopropanol and then mixed by inverting tube 50 times and then centrifuged at 13.000xg for 1 min.
- Supernatant was poured off and tube was drained on clean absorbent paper then 300 µl of 70% alcohol were added and the tube inverted several time to wash the pellet. Then it was centrifuged at 13.000xg for 1min.
- Ethanol was poured off and the tube containing DNA pellet was inverted and drained on clean absorbent paper and allowed to air dry for 10 min.
- 50 µl of DNA hydration solution was added and kept overnight at room temperature.
- Then DNA was stored at -20° C until used.
- DNA concentration was estimated using a Nanodrop (Spectrophotometer N-D1000, South Africa).
2.6 Primers design:

Sequences of camelpox HA gene originating from different countries including Saudi Arabia, Somalia, Iran, Syria and the United Arab Emirates were obtained from the Genbank®. Regions of high homology in different camelpox virus strains were identified by sequence alignment using ClustalW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). Aligned strains showed a highly conserved sequence (99.9%) among these strains. Thereafter, HA gene sequences of camelpox, cowpox and vaccinia virus obtained from Genbank® were aligned together. Variations in the sequence between these other two members of the Genus orthopox and the camelpox virus were noted along the 960 nucleotides of the HA gene. The region of the gene between nucleotides 770 and 810, which is highly conserved in camelpox but different from the others was selected for primer design.

Primers were initially selected by using Primer Explorer V4 (http://primerexplorer.jp/e/) and then manually edited. The amplified region fell between nucleotide 585 and 811 with an amplicon size of 226

The primers were selected based on the criteria such as avoiding terminal dimer formation, 3′ hairpins and self-complementarity, and ensuring 40 to 60% G+C content.

The two outer primers were described as forward outer primer F3 and backward outer primer B3. The inner primers were described as forward inner primer FIP and backward inner primer BIP. Two additional loop primers, forward loop primer (LF) and backward loop primer (LB), were designed to accelerate the amplification reaction. FIP consisted of a complementary sequence of F1 and a sense sequence of F2. BIP consisted of a complementary sequence of B1 and a sense sequence of...
The LF and LB primers were composed of the sequences that were complementary to the sequence between the F1 and F2 and the B1 and B2 regions, respectively. Special care was taken to adjust the melting temperatures ($T_m$) of the primers in such a way that the $T_m$ were in the following order: F1c and B1c > F2 and B2 > F3 and B3. The length of the loop (the distance between F2 plus F1 and B1c plus B2c) was adjusted to between 40 and 60 bp to achieve optimal results. The location and the sequence of each primer in the target DNA were shown in Figure (1) and Table (1).

Table (1) LAMP primer set designed and used in this study

<table>
<thead>
<tr>
<th>label</th>
<th>length</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>21</td>
<td>ACA GTA AGT ACA TCA TCT GGA</td>
</tr>
<tr>
<td>B3</td>
<td>21</td>
<td>TCG TGA TGT TTT CTA CAG TTG</td>
</tr>
<tr>
<td>FIP</td>
<td>44</td>
<td>AGA CAG TGT CTG TGA CTG TAT GAT C – AA TCC ACA ACA GAC GAG AC</td>
</tr>
<tr>
<td>BIP</td>
<td>47</td>
<td>ACT ACT AAA TCA ACC ACC GAT GAT G – GG TGA TAC AGT AGA TGG TTC AT</td>
</tr>
<tr>
<td>LF</td>
<td>20</td>
<td>CTT TAT CAG TGA TTG GTT CCG G</td>
</tr>
<tr>
<td>LB</td>
<td>22</td>
<td>CGG ATC TTT ATG ATA CGT ACA ATG</td>
</tr>
</tbody>
</table>

Fig (1). Location of the LAMP primers within the Camelpox virus hemagglutnin (HA) gene. The amplified target sequence between nucleotides 586 and 811. Locations of primer binding sequences are indicated as follows: F3, bold; B3, bold and italic; F2c, double underlined; F1c, single underlined; B2, double underlined and italic; B1c, single underlined and italic; LF, dot underlined; LB, dot underlined and italic. Sequence shown is GenBank accession number AY902250.


2.7 LAMP reaction condition:

LAMP reaction was performed in a final volume of 25 µl containing 12.5 µl 2x reaction buffer (40 Mm Tirs HCl, 20 mM KCl, 16 mM Mg$_2$SO$_4$, 20 mM (NH$_4$)$_2$SO$_4$ 0.2% Tween 20, 1.6 M Betaine, 2.8 mM each deoxy nucleoside triphosphate, 1.3 µl primer mixture (40 pmol each of FIP and BIP, 20 pmol each of LF and LB and 5 pmol each of F3 and B3), 2 µl of target DNA and 8.2 µl of distilled water. Virus isolate and negative control without DNA were included in each assay. Samples and control were incubated at 65° C for 60 min.

2.8 Detection of LAMP products:

2.8.1 Agarose gel electrophoresis

The LAMP products were separated electrophoretically in 2% agarose gel (Sigma, UK) in Tris/Boric acid/EDTA running buffer (TBE buffer). Agarose gel was prepared by dissolving 1 g of agarose in 50 ml of TBE buffer. Ethidium bromide (1µl /40 ml agarose) was added and the gel was casted into the tray. Combs were placed and the gel inside the tray was allowed to solidify for 30 min. 5 µl of the LAMP products were mixed with 1 µl of 6x loading dye and transferred into the wells. Two microliters of 1 Kb DNA ladder (GeneRuler™ 1kb DNA Ladder Plus #SM1331, Fermentas, Germany) were loaded into the first well of the gel. The gel was allowed to electrophoerose for 45 min (120V and 30 mA), then DNA was visualized under UV light and the picture was documented using a gel documentation system (Bio-Rad, England).
2.8.2 SYBER green method

Naked-eye and UV light visualization for amplification of LAMP method was performed through observation of a color change following addition of 1 µl (1:1,000) of SYBR Green I dye to the tube.

2.9 PCR:

For assessment of sensitivity and specificity of LAMP assay in detection of camelpox hemagglutinin gene, PCR was performed with the LAMP outer primer pair (F3 and B3). The sequence and location of these primers were previously described.

PCR reaction was performed in a final volume of 25 µl that contained 16.5 µl of DW, 2.5 µl of reaction buffer, 0.75 of 50Mm of MgCl₂ solution, 0.5 µl of 10mM of each of dNTPs, 1 µl of 20 µM each primer, 0.25 µl of Tag DNA polymerase and 2 µl of DNA template.

PCR amplification was achieved by Initial denaturation at 94° C for 3 min followed by 35 cycles each included denaturation step at 94° C for 30 seconds, annealing step at 56° C for 30 seconds and extension step at 72° C for 30 seconds. A final extension step at 72° C for 7min was included. PCR products were then stored at 4° C.

Agarose gel electrophoresis and gel visualization under UV light was done for PCR product in a way similar to that described for LAMP.

2.10 LAMP sensitivity:

The sensitivity of the LAMP assay for camelpox virus was determined by testing 10fold serial dilutions of samples for which the virus DNA had previously been
quantified by Nanodrop (Spectrophotometer N- D1000, South Africa). Camelpol DNA at a concentration equivalent to 20ng / µl was serially diluted, and these diluted DNA samples were subjected to the LAMP reaction. Two microliters of diluted DNA was amplified in a 12.5-µl reaction mixture. Reactions were incubated at 65°C for 1 h, followed by heat inactivation at 80°C for 5 min.

For PCR using the F3/B3 primer set and the same serial dilutions of the same batch of DNA. The sensitivity of PCR was also conducted using the F3/B3 primer set. The same batch of the CP DNA serial dilution was amplified by (Initial denaturation at 94 °C for 3min followed by 35 cycles each include denaturation step at 94°C for 30 second, annealing step at 56 °C for 30 second and extension step at 72 ° C for 30 second. The final step was prolonged for 7min at 72°C and then stored at 4°C.
CHAPTER THREE

RESULTS

3.1 Preparation of camelpox virus stock:-

3.1.1 Growth of camelpox virus in Vero cell culture:

The strain of Camelpox virus (CP/DB/92/3) grew well in Vero cell culture. The CPE appeared after three days and was characterized by rounding of the cells and plaque formation.

3.2 DNA extraction:

The extracted DNA detected by gel electrophoresis and appeared as clear band near the wells of the gel. When the DNA was estimated by Nanodrop, the average concentration was 20ng/µl.

3.3 LAMP assay:

The extracted DNA of camelpox virus was subjected to LAMP reaction with 6 primers derived from camelpox hemagglutinin (HA) gene. As controls vaccinia virus, sheeppox virus and parapox virus DNA were also included in the test. DDW was used as negative control. Within one hour reaction time, only camelpox virus specifically produced positive LAMP ampilicons from the target gene of the camelpox hemagglutinin in gel electrophoresis analysis due to the formation of a mixture of stem-loops DNA strands with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand.
LAMP reaction with camelpox-specific primers demonstrated many bands of different sizes in agarose gel electrophoresis, while with other species (sheepox, vaccinia and parapox viruses) as well as negative control no amplicons were produced and no band was demonstrated in gel electrophoresis analysis (Fig 2).

3.4 PCR:

In order to check the specificity of LAMP in detection of camelpox virus, a PCR assay was carried out using the LAMP outer primer set consisted of F3 (forward) and B3 (reverse) primers. Only camelpox virus specifically produced positive bands of 220 bp from the target camelpox hemagglutinin gene in a gel electrophoresis analysis. No ampilicons were produced for the other species (sheepox and vaccinia viruses) and DDW (Fig 3).

3.3 Sensitivity of LAMP assay compared to PCR for camelpox virus:

The sensitivity of the LAMP assay for camelpox virus was determined by testing 10-fold serial dilutions of DNA samples in both LAMP and PCR and comparing the detection limits for both tests. For LAMP, positive reaction appeared as ladder-like pattern in a gel electrophoresis analysis for template different dilutions till the dilution of 0.075 ng/µl (Fig 4). For PCR using the F3/B3 primer set and the same serial dilutions of the same batch of DNA, no amplicons could be detected beyond the dilution of 1.25 ng/µl of template (Fig.5). Therefore, it can be concluded that the LAMP for detection of camelpox virus was 10-fold more sensitive than PCR.

3.4 Detection of LAMP reaction using SYBER green method:

In positive amplification, the original orange color of the dye changed to green, which was judged under natural light as well as under UV light. The original orange color of the dye is retained in negative samples (Fig 6).
Figure 2. Specificity of LAMP primers for detection of camelpox DNA. DNA template of different pox viruses was used in LAMP. Products were electrophoresed in agarose gel (2%) and visualized under UV light. Lanes: 1, camelpox; 2, sheep box; 3, vaccinia; 4, parapox; 5, negative control.
Figure 3. Polymerase chain reaction (PCR) to detect pox viruses.
PCR was performed with the camelpox specific LAMP outer primer set and different pox viruses DNA samples. Products were electrophoresed in agarose gel (2%) and visualized under UV light. Lanes: M, 100-bp molecular weight marker; 2, camelpox positive sample; 3, sheeppox; 4, vaccinia; 5, negative control (DW).
Figure 4. Detection limit of the *cmaelpox* LAMP
Different concentrations of camelpox DNA were used as template in LAMP. LAMP products were electrophoresed in agarose gel (2%) and visualized under UV light. Lanes: 1, 20ng/µl; 2, 10 ng/µl; 3, 5ng/µl; 4, 2.5ng/µl; 5, 1.25 ng/µl; 6, 0.625ng/µl; 7, 0.312 ng/µl; 8, 0.156ng/µl; 9, 0.075 ng/µl; 10, 0.038 ng/µl; 11, negative control without DNA (DDW).
Figure 5. Detection limit of conventional PCR assay.
Different concentrations of camelpox DNA were used as template in conventional PCR with LAMP outer (F3 and B3) primer set. PCR products were electrophoresed in agarose gel (2%) and visualized under UV light. Lanes:1, 20ng/µl;2, 10 ng/µl; 3, 5ng/µl;4, 2.5ng/µl;5, 1.25 ng/µl;6, 0.625 ng/µl;7, 0.312 ng/µl;8, 0.156 ng/µl;9, 0.075 ng/µl;10, 0.038 ng/µl ;11, negative control without DNA (DDW).
Figure 6. Detection of the LAMP product with SYBER Green dye.
Positive reaction showed green colour while negative reaction showed orange colour in daylight with black background.
CHAPTER FOUR

DISCUSSION

Camelpox (CP) is a highly contagious viral disease affecting mostly young animals and characterized by papular eruptions on the skin and mucus membrane. The morbidity rate in infected herds ranges from 25% to 100% for young animals and from 5% to 25% for older animals (Ramyar and Hessami, 1972; Kriz, 1982).

Camelpox virus (CMPV) and variola virus (VAR) are orthopoxviruses (OPVs) that share several biological features and cause high mortality and morbidity in their single host species (Gubser et al., 2002). Recently, increased attention has been paid to poxviruses isolated from animals, particularly to those which share properties with smallpox viruses (Babxy et al., 1975).

Diagnosis of CP can be accomplished by electron microscope and detection of orthopox viruses in tissue culture cells and in CAM of embryonated chicken eggs as well by serological tests (Munz, 1992).

Isolation of camelpox virus on CAM, which should be incubated at 37° C for 5 days, is time consuming, and as reported by Baxby (1972) when the virus is propagated in Vero cell culture, the CPE appearance could take 4-6 days in the first passage. The most conventional serological tests are very laborious and time consuming, which make them not suitable for primary diagnosis (Azwai et al., 1996).

Conventional PCR method also require laborious post PCR handling and include high risk of unwanted carry over contamination by the processed PCR product,
particularly when genotyping by restriction fragment length polymorphism analysis is used for differentiation (Loperen et al., 2001).

The recent invention of loop-mediated isothermal amplification (LAMP) of DNA provides a new alternative for molecular diagnosis (Notomi et al., 2000). The LAMP reaction is a very efficient and specific method for the amplification of DNA templates (Notomi et al., 2000; Mori et al., 2001; Nagamine et al., 2002; Fukuta et al., 2003).

The LAMP assay originally described by Notomi et al is based on the principle of autocycling strand displacement DNA synthesis. The assay utilizes a single DNA polymerase that is active at relatively high isothermal amplification temperatures, which diminishes the probability of non-specific priming (Boehme et al., 2007). The reaction utilizes a set of two specially designed inner primers and two outer primers (Notomi et al., 2000). LAMP is highly specific for the target sequence and specificity occurs through the recognition of six independent sequences in the initial stage and four independent sequences during the later stages of the reaction. Further improvements in the time kinetics and sensitivity of the LAMP reaction by the use of two additional loop primers (LF and LB) accelerated the LAMP reaction and cut the time needed for amplification by half (Nagamine., et al 2002). LAMP permits the use of simple and cost-effective reaction equipment and due to the isothermal nature of the assay, there is no time lost in temperature cycling, which leads to extremely high efficiency compared with regular PCR (Notomi et al., 2000 and Nagamine et al., 2001).

One of the useful features of LAMP is the different possibilities for detection of amplicons. Since the amplification of DNA is directly correlated with the production of magnesium pyrophosphate leading to turbidity, Mori et al (2001)
reported that LAMP products can be observed by the naked eye when a white precipitate of magnesium pyrophosphate is present in the reaction mixture. However, this detection limit is limited when the turbidity of reaction is low. To increase the sensitivity and the rate of recognition by the naked eye or by gel electrophoresis after ethidium bromide staining, SYBR Green can be added to the LAMP reaction mixture, which enables detection under UV light (Iwamotoe et al., 2003). Ethidium bromide had several limitations, such as generation of hazardous waste and sensitivity of 25 to 100 times less than that of SYBR Green I (Mori et al., 2001).

Recently the use of LAMP in the detection of the monkeypox virus (Itoe Iizuka et al., 2009) has been reported and in this study we report, for the first time, the development of LAMP assay for specific diagnosis of camelpox virus.

In the present developed LAMP method, camelpox virus DNA was amplified within 60 min. The technique was quite simple and did not require high expertise. Only four pipetting steps were required to prepare the master mix and the use of a primer mix (of 6 primers) has enabled a one step transfer for all the primers into the reaction master mix. Thermal cycler was used only for adjusting the required temperatures during the first evaluation steps, but regular laboratory water bath or a heat block that supplies a constant temperature of 65°C can be sufficiently used.

We first examined the specificity of the developed LAMP for detection of heamagglutnin gene of camelpox virus. No amplicons were detected for sheeppox, vaccinia or parapox viruses and efficient DNA amplification was observed only for camelpox virus. These results suggest that the primer set used for the LAMP assay is highly specific for the camelpox virus. This high specificity of the reaction can be justified by the fact that the hemagglutinnin (HA) gene is present in
orthopoxviruses, and no other poxvirus genus possesses such a gene. The hemagglutinin gene encodes a glycoprotein that can be detected on the infected cell plasma membrane and on the tegument covering the outer membrane of extracellular enveloped virions. The glycoprotein is synthesized late in infection by orthopoxviruses, which makes the presence of antibodies against the HA protein a good indicator of orthopoxvirus infections (Clarissa et al., 2000).

The specificity of the present LAMP for camelpox virus is also attributed to the choice of primers, which were designed after sequence alignment and analysis of the HA gene of different orthopox viruses species (camelpox, vaccinia and cowpox virus) from different geographical locations obtained from the Genbank®. Differences exist among HA gene sequences of different orthopoxviruses, which makes it useful for phylogenetic analysis (Ropp et al., 1995). When those species were aligned together they showed few mismatching and mismatching of two or three bases between aligned species, indicate significant difference in terms of detection of CMPV DNA by the LAMP assay and also this mismatching let the primer to be specific for camelpox only.

Though the primers were selected from the most conserved region in the sequence of camelpox isolates, further validation of the LAMP assay should include other camelpox as well as cowpox samples collected from different geographical areas in Sudan.

The sensitivity of LAMP compared to that of PCR was compared by running the two tests using the same template in serial dilutions. The outer primer pair for the LAMP (F3 and B3) was used for the PCR amplification. While the two tests had the same specificity (detecting only the camelpox virus with no false amplification of other pox viruses), LAMP exhibited a higher sensitivity than PCR. It had a
detection limit of 0.075 ng / µl which is 16 times higher than that of PCR (1.25 ng / µl). Higher sensitivity of LAMP assays compared to PCR are usual and have been reported elsewhere (Bakheit et al., 2008).

Recently a PCR specific for camelpox virus was described (Bhanuprakash et al., 2009) in which the detection limit for camelpox DNA was 0.4 ng / µl, which is still lower than the detection limit of our LAMP assay. The naked eye detection of the amplified products by using SYBER Green 1 dye also indicates that the rapid detection can be achieved within few minutes. The time for LAMP reaction was quicker, compared to two hrs for PCR.

In conclusion, this study constituted the first report on using LAMP for the detection of camelpox worldwide. The method was established successfully and high sensitivity and specificity were observed. Although limited numbers of samples was used in the present study, and further validation of the method by using more isolates of orthopox viruses is recommended, but it can be shown that LAMP could be a convenient tool for detection of camelpox virus due to its simplicity and cost effectiveness.
REFERENCES


Gubser, C., Smith, G.L., (2002). The sequence of camelpox virus shows it is most closely related to variola virus, the cause of smallpox. J gen Virol. 83(4), 855-72.


Detection of Orthopoxviruses and Simultaneous Identification of Smallpox Virus J Clin Microbiol. 42(5), 1940–1946


Preparation of solutions and cell culture media:

1. **Cell culture medium**:

Eagle’s minimum essential medium is either Earle’s or Hank’s (HBSS) balance salt solution and contain heat inactivated (56c for 30min) fetal bovine serum (FBS). Growth medium is used for the rapid proliferation of cells and usually contain FBS at final concentration of 7.5 to 10.0%.

Maintenance medium contain only 2% FBS and is used to maintain the cells in steady state of slow metabolic activities.

Outgrowth and maintenance medium were prepared according to Ali (1971) as shown below:

**Medium/liter**

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Outgrowth</th>
<th>Maintenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMEM</td>
<td>200ml</td>
<td>200ml</td>
</tr>
<tr>
<td>Lactalbumin hydolysate</td>
<td>25ml</td>
<td>25ml</td>
</tr>
<tr>
<td>Yeast extracts (1%)</td>
<td>25ml</td>
<td>25ml</td>
</tr>
<tr>
<td>Sodium bicarbonate (7.5%)</td>
<td>7.5ml</td>
<td>10ml</td>
</tr>
<tr>
<td>Penicillin – streptomycin solution</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Fungizon (5000micr/ml)</td>
<td>1ml</td>
<td>-</td>
</tr>
<tr>
<td>DDW</td>
<td>1L</td>
<td>1L</td>
</tr>
<tr>
<td>Tryptose phosphate broth</td>
<td>50ml</td>
<td>50ml</td>
</tr>
<tr>
<td>Bovine serum</td>
<td>100ml</td>
<td>20ml</td>
</tr>
</tbody>
</table>
2. Media additives:

2.1 Tryptose Phosphate Broth (TPB):
Three grams of TPB powder were dissolved in 100ml DDW, sterilized by autoclaving at 121c for 10min and stored at 4c.

2.2 Lactalbumin hydrolysate:
Five grams of lactalbumin powder were dissolved in 100ml DDW, sterilized by autoclaving at 121c for 10min and stored at 4c.

2.3 Yeast extracts solution:
One gram of yeast extract powder were dissolved in 100ml DDW, sterilized by autoclaving at 121c for 10min and stored at 4c.

2.4 7.5% Sodium bicarbonate solution (NaHCO₃):
7.5% Sodium bicarbonate gram (NaHCO₃) were dissolved in 100ml DDW, sterilized by autoclaving at 121c for 10min and stored at 4c.
Appendix (ii)

Medium and solutions required in cell culture:-

1. Thyoglycolate medium:-

Twenty nine and have grams of thioglycolate medium were dissolved in 100ml of DDW, dispensed in bijou bottles and autoclaved at 121°C for 10 min and stored at 4 °C

2. Preparation of stock trypsine (2.5%) :-

2.5grams of trypsine were dissolved in 100ml of PD, filtered through What Mann filter and stored at -20°C

3. Preparation of stock versin (5%) :-

Five gram of versin powder dissolved in 100ml of PD, autoclaved at 121°C for 15min

1. Trypsin Versin solution(T.V) :-

To prepare 100ml

<table>
<thead>
<tr>
<th>Trypsin (2.5)</th>
<th>6.0ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versin(5%)</td>
<td>4.0ml</td>
</tr>
<tr>
<td>Phosphate</td>
<td>90ml</td>
</tr>
</tbody>
</table>

Several drops of phenol red (0.2%) were added and the PH adjusted by IM NaOH until the colour is faint pink

2. Bovine serum :-

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

Buffers

1. Phosphate Buffer Saline (PBS):
To prepare 2 liters

Solution A

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>KCl</td>
<td>0.4gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>16.0gm</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.0g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.4gm</td>
</tr>
<tr>
<td>DWW</td>
<td>1000ML</td>
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Solution B

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>MgCl$_2$</td>
<td>0.2gm</td>
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<tr>
<td>DDW</td>
<td>200ML</td>
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Solution C

<p>| | |</p>
<table>
<thead>
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</tr>
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<tr>
<td>CaCl$_2$</td>
<td>0.26gm</td>
</tr>
<tr>
<td>DDW</td>
<td>200ML</td>
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</tbody>
</table>

Autoclaved and cool and add A to B then C. complete to 2000ml with DDW

2. Phosphate Diluent (PD):
Solution A in (PPS) was completed to 2 liters with DDW, then autoclaved and cooled before antibiotics were added.
Appendix (iv)

Preperation of LAMP solutions, primers, dNTP and master mix

1. LAMP reaction mix preparation

First prepare 2x LAMP reaction mix without dNTPs as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Total 900 ml</th>
<th>Total 90 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M TrisHCl (pH 8.8) stock sol.</td>
<td>40 ml</td>
<td>4 ml</td>
</tr>
<tr>
<td>KCl</td>
<td>1.49 g</td>
<td>0.15 g</td>
</tr>
<tr>
<td>*MgSO₄(MgSO₄.7H₂O)</td>
<td>*1.93 g (3.94 g)</td>
<td>*0.19 g (0.394 g)</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2.64 g</td>
<td>0.26 g</td>
</tr>
<tr>
<td>Tween 20</td>
<td>2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Betaine</td>
<td>187.4 g</td>
<td>18.7 g</td>
</tr>
</tbody>
</table>

If MgSO₄.7H₂O is used instead of MgSO₄, weights have to be corrected to the values in parenthesis.

[40 mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM (NH₄)₂SO₄, 16 mM MgSO₄, 0.2% Tween 20 and 1.6M Betaine],
**Tris-HCl (1M)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>121.1 g</td>
</tr>
<tr>
<td>ddH₂O to</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Dissolve in 800 ml of ddH₂O, adjust to pH 8.8 with concentrated HCl and add ddH₂O to 1 litre

---

**2. Primer Mix Preparation**

Primer mix contains six primers. Final concentration of FIP and BIP is 40 pmol each, LF and LB is 20 pmol each, and F3 and B3 is 5 pmol each.

<table>
<thead>
<tr>
<th>100 pmol/µ l stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIP 40</td>
</tr>
<tr>
<td>BIP 40</td>
</tr>
<tr>
<td>LF 20</td>
</tr>
<tr>
<td>LB 20</td>
</tr>
<tr>
<td>F3 5</td>
</tr>
<tr>
<td>B3 5</td>
</tr>
</tbody>
</table>
Take 1.3 µl per reaction

3. Preparation of 25 mM dNTP stock

25 mM dNTP stock

<table>
<thead>
<tr>
<th>100mM Datp</th>
<th>1 vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM dCTP</td>
<td>1 vol.</td>
</tr>
<tr>
<td>100mM dGTP</td>
<td>1 vol.</td>
</tr>
<tr>
<td>100mM dTTP</td>
<td>1 vol.</td>
</tr>
</tbody>
</table>

Use this formula to prepare reaction mix working solution:

2x LAMP reaction buffer (Working solution)

<table>
<thead>
<tr>
<th>2x LAMP reaction buffer without dNTPs</th>
<th>900 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>25mM dNTP stock</td>
<td>100</td>
</tr>
</tbody>
</table>
4. Preparation of Master Mix (should be conducted on ice)

For one sample

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>8.2 µl</td>
</tr>
<tr>
<td>2 x Reaction buffer</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Primer mix</td>
<td>1.3 µ</td>
</tr>
<tr>
<td><em>Bst</em> DNA polymerase</td>
<td>1 µl (8 units)</td>
</tr>
<tr>
<td>DNA template</td>
<td>2 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>25 µl</td>
</tr>
</tbody>
</table>
Appendix (iv)

Preparation of materials for gel electrophoresis:

**Agarose 2%**

0.75 gm of agarose (Sigma, UK) was dissolved in 25 ml of TBE buffer heated in microwave for 45 seconds, left to cool and poured onto electrophoresis chamber.

**1- Storage Buffer** (TE buffer)

10 mM Tris-HCl (pH 7.6), 1 mM EDTA.

**2- 6X Loading Dye Solution**

10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA.

**3 ladder**

DNA Ladder 1µl

6X Loading Dye Solution 1µl

**4 loading dye**

<table>
<thead>
<tr>
<th>Bromophenol blue</th>
<th>11%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>40ml</td>
</tr>
<tr>
<td>DDW</td>
<td>50ML</td>
</tr>
</tbody>
</table>

**5- Ethidium bromide**

Stock solution (PROMEGA, Madison) and protected from light.