

ADDIS ABABA UNIVERSITY SCHOOL OF VETERINARY MEDICINE

**INVESTIGATION OF RESPIRATORY VIRUSES IN CAMELS SLAUGHTERED AT
ADDIS ABABA AKAKI ABATTOIR**

By:

WOSSENE NEGASH

June, 2011, Ethiopia

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**A thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial
Fulfillment of the Requirements of Masters Degree in Veterinary Microbiology**

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ACKNOWLEDGEMENT	I
TABLE OF CONTENTS	II
ABBREVIATIONS.....	IV
LIST OF ANNEXES	VI
LIST OF TABLES	VII
LIST OF FIGURES	VIII
ABSTRACT	IX
1. INTRODUCTION	1
2. LITERATURE REVIEW	6
2.1. Respiratory viruses of camels	8
2.1.1. PPR disease.....	8
2.1.2. Respiratory syncytial virus (RSV).....	10
2.1.3. Camelpox virus.....	12
2.1.4. Infectious bovine rhinotracheitis (IBR) disease.....	13
2.1.5. Parainfluenza viruses (PIV).....	13
2.1.6. Adenovirus.....	14
2.1.7. Influenza viruses.....	16
2.2. Diagnosis of respiratory viruses	17
2.2.1. Conventional and novel molecular amplification techniques.....	18
2.2.2. Serological tests.....	26
2.2.3. Electron microscopy	28
2.2.4. Virus culture methods.....	29
3. MATERIALS AND METHODS.....	32
3.1. Study areas	32

3.2. Study population.....	33
3.3. Study design	33
3.5. Study methods.....	34
3.5.1. Virus isolation in cell culture system.....	34
3.5.2. Molecular virus characterization methods.....	35
3.6. Data management and analysis	44
4. RESULTS.....	45
4.1. Postmortem examination and virus infection prevalence.....	45
4.2. Virus cultivation and cytopathic effects	46
4.3. Molecular characterization methods	52
4.3.1. PCR detection of DNA and RNA respiratory camel viruses by DOP-PCR.....	52
4.3.2. Conventional PCR	53
5. DISCUSSION.....	61
6. CONCLUSION AND RECOMMENDATIONS.....	68
7. REFERENCES	69
8. ANNEXES	92
9. SIGNED DECLARATION SHEET.....	106

ABBREVIATIONS

BRSV	Bovine Respiratory Syncytial Virus
BVD	Bovine Viral Diarrhea
CAHW	Community Animal Health Workers
CDC	Center for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
CPE	Cytopathic Effect
CSA	Central Statistical Agency
DEPC	Diethyl Pyrocarbonate
DOP-PCR	Degenerate Oligonucleotide Primer – Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbant Assay
FAO	Food and Agricultural Organization
FMD	Foot and Mouth Disease
GLIPHA	Global Livestock Production and Health Atlas
GMEM	Glasgow Minimum Essential Medium
HI	Haemagglutination Inhibition
HIV	Human Immunodeficiency Virus
HRSV	Human Respiratory Syncytial virus
IBR	Infectious Bovine Rhinotracheitis
ICTVdB	International Committee on Taxonomy of Viruses (data base)
ILAR	Institute of Laboratory Animal Resource
Kbp	Kilo base pair
MDBK	Madin Darby Bovine Kidney
M-MMLV	Mouse Mooney Murine Leukemia Virus
NAHRC	National Animal Health Research Center
NCBI	National Center for Biotechnology Information
NVI	National Veterinary Institute

OIE	Office of International des Epizootics
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIV1	Parainfluenza 1 virus
PIV2	Parainfluenza 2 virus
PIV3	Parainfluenza 3 virus
PPR	Peste des Petits Ruminants virus
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial virus
RT	Reverse Transcription
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SNNP	Southern Nations Nationalities and People
TAE	Tris Acetate EDTA (ethylene diamine tetra acetic acid)
TWAS	Third World Academy of Sciences
USAHA	United States Animal Health Association
VIDISCA	V irus D iscovery by c DNA – A FLP (Amplified Fragment Polymorphism)
WHO	World Health Organization

LIST OF ANNEXES

PAGES

Annex-1: Tissue processing procedure for virus isolation.....	92
Annex-2: Composition of GMEM and its preparation protocol.....	93
Annex-3: Viral RNA extraction protocol.....	93
Annex-4: DNA extraction Protocol.....	96
Annex-5: cDNA synthesis (Reverse Transcription) protocol.....	96
Annex-6: Conventional PCR master mix preparation and procedure	97
Annex-7: DOP- PCR master mix preparation and amplification protocol.....	98
Annex-8: Electrophoresis gel preparation and gel reading.....	99
Annex-9: Digital photographs of some pneumonic camel lungs sampled.....	100
Annex-10: Daily virus cultivation result registration sheet.....	103
Annex-11: Pneumonic camel lung tissue sample collection format for camel virus investigation from Addis Ababa - Akaki Abattoir.....	105

LIST OF TABLES

PAGES

Table 1: Sequences of oligonucleotide primers used for the detection of camel respiratory viruses	43
Table 2: The prevalence of camel viral pneumonia by cell culture results.....	46
Table 3: Summary of conventional PCR results	60

LIST OF FIGURES

PAGES

Figure 1: Polymerase chain reaction (PCR) thermal cycles.....	21
Figure 2: Infected vero cells showing morphological changes, cytopathic effects (CPEs), (top and bottom left corners) with respective controls (top and bottom right corners), respectively.....	47
Figure 3: Infected vero cells exhibiting morphologic changes, cytopathic effects (CPEs), (top and bottom left corners) with respective controls (top and bottom right corners), respectively.....	49
Figure 4: Infected vero cells exhibiting morphologic changes, cytopathic effects (CPEs), (top and bottom left corners) with respective controls (top and bottom right corners), respectively.....	51
Figure 5a and b: Digital photography of Ethidium bromide stained agarose gel electrophoresed DOP- PCR amplified DNA and RNA products analyzed by UV transillumination.....	52
Figure 6: Digital photography of ethidium bromide stained agarose gel electrophoresed PCR amplified products of six <i>Respiratory syncytial virus</i> (RSV) samples analyzed by UV.....	54
Figure 7: Digital photography of Ethidium bromide stained agarose gel electrophoresed PCR amplified RSV products of ten samples analyzed by UV.....	55
Figure 8: Digital photography of electrophoresed agarose gel analyzed by UV illumination of six (6) viral RNA samples using PPRV specific primer amplified PCR products.....	56
Figure 9: Digital photography of ethidium bromide stained electrophoresed gel analyzed by UV illumination of six (6) PCR amplified DNA products using <i>Adenovirus</i> specific primer.....	57
Figure10: Digital photography of ethidium bromide stained agarose gel electrophoresed PCR amplified products of six (6) viral cDNA samples using PIV1, PIV2 and PIV3 specific primers analyzed by UV illumination.....	59
Figure11: Flowchart of RNA extraction using RNeasy mini kit.....	95

ABSTRACT

This study was conducted from September 2010- June 2011 at Debre Zeit National Veterinary Institute (NVI) with an attempt to isolate and characterize respiratory viruses from pneumonic lungs of camels slaughtered at Addis Ababa Akaki abattoir. Accordingly, pneumonic camel lung tissues were collected and transported in ice box to NVI virology laboratory and temporarily stored at -85°C and then processed and inoculated on confluent vero cell monolayers, incubated at 37°C and followed for the development of CPE for 4-15 days. All CPEs observed developed between 4-14 days. Thirty five (35) pneumonic camel lungs were cultured among which 27 of them were CPE positive characterized majorly by initial rounding of vero cells, elongation, syncytia formation, swelling and rounding, floating and sloughing of cells. Some of the supernatant suspensions of those samples exhibiting CPEs were taken and extracted both for DNA and RNA viral genomes. cDNAs were synthesized from RNA extracted samples. The DNAs and cDNAs were used to test both DNA and RNA viruses, respectively using universal (DOP- PCR) and conventional PCR techniques. *Respiratory syncytial virus*, *Adenovirus*, *Pest des petitis ruminants virus*, *Parainfluenza 1 virus*, *Parainfluenza 2 virus* and *Parainfluenza 3 virus* were tested. All six samples tested were negative for PPRV and *Parainfluenza viruses* 1-3. But nine of ten samples tested were positive for *Respiratory syncytial virus* and two of six samples tested were positive for *Adenovirus*. Finally, further investigative, epidemiological and molecular characterization studies were forwarded.

Key words: *Abattoir, Camel, Polymerase chain reaction, Respiratory viruses, Virus isolation*

1. INTRODUCTION

Ethiopia is located at 80°N and 38°E (The world Fact book, 2006). The total land area of the country is 1.1 million Km² (Kilometer squares) and the total human population is estimated at 79,221, 000 (CSA, 2008). More than 80% of the population is dependent on agriculture of which livestock production played a significant role (Bureau of African Affairs, 2006), where the contribution of livestock and livestock products to the agricultural economy accounts 40%, excluding the values of draught power, transport and manure (Winrock international, 1992). Although Ethiopia holds the largest livestock population in Africa, estimated at about 50.88 million heads of cattle, 25.98 million sheep, and 21.96 million goats, 0.807 million camels , 42.05 million chicken, 2 million horses 4.72 million asses, 0.37 million mules and 4.6 million beehives (CSA, 2010), the total national production remains amongst the lowest in the world, even by African standards.

Ethiopia is a home for more than 12-15 million pastoralists who reside in 61% of the nation's landmass. The pastoral areas are estimated to comprise 42% of the national total livestock population. Moreover, the pastoral areas are rich in biodiversities, mineral and water resources as well as energy resources and untapped tourist attractions. The major problems in pastoral areas include lack of appropriate livestock marketing, education, public health, veterinary services and water both for human and livestock and rural finance (FAO, 2008).

Pastoralists in Ethiopia are mainly found in four lowland regions, Afar, Oromyia, Somali, and the Southern Nations Nationalities and Peoples (SNNP) regional states. The main livelihoods of pastoralists include pastoralism, farming and ex-Pastoralists (ex-pastoralists later engaged in petty income earning activities) (Behnke *et al.*, 2007).

Camels are the most capable animal species in utilizing marginal areas and in survival and production under harsh environmental conditions (Abbas and Tilley, 1990; Schwartz, 1992). The

camel is well adapted to the climatic extremes and is well appreciated for its significance in the pastoral economy (Raziq and Younas, 2006).

Camels can live in areas that are inhospitable to other domestic animals and are therefore an important factor in the capacity of humans to survive and make use of these drier regions (Dirie and Abdurahman, 2003).

Many pastoral groups and communities in diverse ecozones throughout the world are depending on camels for their livelihood. This dependence consists of utilization of camel meat, milk, leather, wool and exportation of live camels (Kohler-Rollefson, 1992; Snow *et al.*, 1992). Their dung is used for fires and they are also used for riding and transport; they are means of investment and long-term savings; they are source of prestige for their owners and there is a large market for trade in live camels. Sales of surplus milk, livestock or livestock products are sources of cash income for pastoral families (Dirie and Abdurahman, 2003) and they also serve as transport of people as well as goods (Kappeler, 1998).

According to FAO statistics (Global Livestock Production and Health Atlas - GLIPHA, 2006), the world population of camels is about 20 million animals, mainly in arid zones, of which 15 million camels live in Africa and 5 million in Asia (GLIPHA, 2006). In most countries, the camel population is increasing after a period of decreasing number due to the introduction of modern transport facilities (Farah, 2004). Ethiopia is estimated to have the third largest camel herd in the world after Somalia and Sudan, followed by Mauritania and Kenya in that order (FAO, 2008).

In Ethiopia, there are about 0.87 million camels (CSA, 2010). Pastoralists often dwell in border areas; highly volatile and insecure environments that are often beyond the reach of formal health services. Accordingly, disease control activities are often absent in these areas or are not adapted to the pastoralists' mobile lifestyle (Abdi *et al.*, 2010).

The major problems of livestock production include camels are housing, health and feeding (Parsani *et al.*, 2008). According to Wahba and Refail (2003), high incidence of diseases is one of the major constraints associated with camel production.

The camel seems to be spared from the devastating epidemic infections which threaten other livestock species, the animal is however affected by many other diseases, some of which are unknown to date. Camel diseases that are shared with other species of livestock are comparatively well-known, while other camel-specific diseases, although well-known to pastoralists for generations, still remain a mystery to the scientific community (Dirie and Abdurahman, 2003).

Infectious and parasitic diseases appear to be the major constraints that are hampering the potential performance of the animals (camels). Scarce research information on diseases reveals that camels may be either carriers of, susceptible to or suffering from a vast array of infectious and parasitic diseases. Trypanosomiasis, camel pox, contagious skin necrosis, pneumonia, mange mite infections and internal parasites are among the major health problems previously reported in camels in Borena areas (Richard, 1979; Demeke, 1998). Similarly, Odeh *et al.* (1999) and Bekele (2002) indicated that although camels seem to suffer from fewer diseases than other domestic animals and epidemics are rare. In many African countries, information regarding camel production and disease are available (Abbas *et al.*, 1993; Khalafalla *et al.*, 1994). Camels may also be susceptible to Bovine viral diarrhea (BVD), contagious ecthyma, rinderpest, foot and mouth disease (FMD), Infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PIV3), *Respiratory syncytial virus* (RSV) and rift valley fever (RVF) (Brown, 2004; Odeh *et al.*, 1999). In fact, the dromedary of East Africa, particularly Ethiopia, Kenya and Somalia have been suffering from different diseases for the past so many years and conventional intervention measures seemed to be difficult due to the very reason that the animals are reared in marginal areas where veterinary services are not available or very limited (Dirie and Abdurrahman, 2003).

The diseases of Ethiopian camels have not been very extensively investigated in comparison with those of other domesticated species, probably owing partly to the non-sedentary nature of the

herds: constantly moving in search of grazing and water. It is only in a few places, where the animals are found in favorable environments, often alongside other species. In addition, the dominance of other ruminant species over camels perhaps might have masked the potential contributions of these animals to the national and household economy. As a result, the camels have been neglected, or at least their importance underestimated, by the society as such. Consequently, livestock production planners and researchers have overlooked its usefulness for the local community and roles in national economy and have not so far considered the animals in any research and development agenda (Bekele, 2010).

Respiratory infections are considered one of the main factors that decrease animal production. Respiratory tract infections are of common occurrence in various species of domestic and farm animals (Mohamed and Abdelsalam, 2008). Viruses, bacteria, fungi and parasites have been incriminated as the main causative agents of pneumonia in mammals (Cotran *et al.*, 1999).

Of the many diseases, respiratory diseases are the major threats to the camel population of Ethiopia (Roger *et al.*, 2001). However, in Ethiopia, few studies were conducted on the extent of respiratory problems of camels compared to other species of livestock animals (Bekele, 2008). Generally, camel respiratory problem has received little considerations though it is becoming one of the emerging diseases causing considerable loss of production and death (Bekele, 1999; Rufael, 1996).

Pulmonary diseases are among the emerging problems of camels that are causing considerable loss in production and death (Zubair *et al.*, 2004; Kane *et al.*, 2005). However, the definitive etiologies of most respiratory diseases of camels have not yet been determined as a variety of viruses, fungi, bacteria and parasites are to be the possible causes of respiratory outbreaks among camels (Schwartz and Dioli, 1992). These agents may represent risk to camels, other livestock and even human population (Bardonnet *et al.*, 2002; Teshome *et al.*, 2003). Most of the studies on camels were about parasitic infections (Al-Rawashdeh *et al.*, 1999). Al-Tarazi (2001) and other authors had isolated and characterized many bacterial agents from pneumonic camel lungs.

However, there is little or no information on possible viral etiologies of camel pneumonia or viral flora in camels.

The major lesions encountered on different portions of the camel lung includes emphysema, fibrosis, adhesion, atelectasis, interstitial pneumonia, bronchopneumonia, fibrenious pneumonia, pulmonary abscess, foreign bodies, hydatid cyst and lymphoid tumor (Bekele, 2008; Bhardwaj *et al.*, 2008).

Recently, in pastoralist areas of Ethiopia, there was a report of mysterious disease that caused sudden death to camels in pastoralist area (Focus on Ethiopia, 2005). During 1995 camel respiratory disease outbreak, the whole camel population of Ethiopia has been affected and the causative agent is not yet clearly identified (Roger *et al.*, 2001). Recently, in 2005/06 in Afar region and kereyu area of Oromia region, there was a huge camel mortality due to respiratory disease outbreak which was characterized by sudden death (Wernery *et al.*, 2006). In 2007, similar disease was reported from Somali region, Guji and Borena zones of Oromia region. Researchers from National Veterinary Institute (NVI), National Animal Health Research Center (NAHRC) and other regional laboratories of the country have been doing disease investigation following the occurrence of the outbreak, but the exact etiologic agent of the disease has never been identified yet (Asegedech, 2009).

Accordingly, in Ethiopia, limited work has been undertaken in view of improving the productivity of camels, identify and characterize health problems that threat their existence in the respective regions. Thus, there is an urgent need to address the problems of pastoralists posed by variety of camel diseases with a particular focus on investigation of viral etiologies of camel respiratory diseases.

Therefore, the objective of this study was

To isolate and characterize camel respiratory viruses

2. LITERATURE REVIEW

In zoological taxonomy, *Camelids* are classified in the suborder *Tylopoda* (pad footed animals) that represents with the suborders *Suiformes* (pig-like) and *Ruminantia* (ruminants) and the order *Artiodactyla* (even-toed ungulates). Thus, *Camelids* (family *Camelidae*) as ruminating animals are classified in proximity to ruminants but developed in parallel and are not part of the suborder *Ruminantia*. Some differences as foot anatomy, stomach system and the absence of horns underline this fact (Schwartz and Dioli, 1992; Fowler, 1998).

The family *Camelidae* is divided into three genera; the old world camels (genus *Camelus*) and the new world camels (genus *Lama* with the species *L. glama*, *L. guanicoe*, *L. pacos* and genus *Vicugna* with the species *V. vicugna*) (Wilson and Reeder, 2005).

Two domesticated species of old world camels exist, the dromedary or one humped camel (*Camelus dromedarius*) that has its distribution in the hot deserts of Africa and Asia and the Bactrian or two-humped camel (*Camelus bactrianus*) that can be found in the cold deserts and dry steppes of Asia (Wilson, 1984).

Camels (*Camelus dromedarius*) are vital domestic animal species that are best adapted to harsh environments and fluctuating nutritional conditions of arid and extreme arid zones. The one humped camel or dromedary is one of the world's hardest domesticated animals (Yagil, 1985). These animals are endowed with extraordinary features that enable them to survive and perform in such hard conditions (Teka, 1991).

The camel is an important animal component of the fragile desert eco-system. With its unique bio-physiological characteristics, the camel has become an icon of adaptation to challenging ways of living in arid and semi-arid regions (Bikaner, 2008). The proverbial "Ship of Desert" earned its

epithet on account of its indispensability as a mode of transportation and draught power in desert but the utilities are many and are subject to continuous social and economic changes.

Camels play an important socio-economic role in arid and semi arid areas where most of the resource poor farmers in Africa live (Guilye *et al.*, 2007). Camels have been known to have particular or peculiar physiological features by which they regulate body temperature to changes in ambient temperatures, which enable them to survive and adapt to such hard conditions (Wilson, 1984). Camels can live in areas that are inhospitable to other domestic animals and are therefore an important factor in the capacity of humans to survive in and make use of these drier regions (Schwartz, 1992). The role of camels in traditional areas has been highlighted (Mehari *et al.*, 2007). The camels were and still are valued as riding, baggage, draught animals, hair hides, as well as the best food providers in the arid areas (Tigani *et al.*, 2005).

Although camels are well adapted to their environment and seem to be spared from devastating epidemic infections which threaten other livestock species in the same region, there are however a number of economically important diseases that affect camels (Kane *et al.*, 2003; Dia, 2006).

This paper had intended to revise some important camel respiratory viruses including *Peste des petitis ruminants virus*, *Influenza viruses*, *Parainfluenza viruses*, *Adenovirus*, *Respiratory syncytial virus* (RSV), *Infectious bovine rhinotracheitis* (IBR) and respiratory *Camelpox virus*.

Paramyxoviruses

The family *Paramyxoviridae* is subdivided into the subfamilies *Paramyxovirinae* and *Pneumovirinae* (Murphy *et al.*, 1999). The subfamily *Paramyxovirinae* in the family *Paramyxoviridae* comprises the five genera *Respirovirus*, *Morbillivirus*, *Rubulavirus*, *Avulavirus*, and *Henipavirus* as well as a group of yet unclassified viruses (Lamb *et al.*, 2005) and the latter subfamily contains the genera *Pneumovirus* and *Metapneumovirus* (Murphy *et al.*, 1999).

Members of the family *Paramyxoviridae* are pleomorphic enveloped viruses possessing a non segmented negative-strand (NNS) RNA genome between 15 and 19 kb in length (Lamb and Kolakofsky, 2001). The genus *Pneumovirus* contains *bovine respiratory syncytial virus*, *ovine respiratory syncytial virus*, *murine/pneumonia virus of mice* and *human respiratory syncytial virus* where as the genus *Metapneumovirus* contains *turkey rhinotracheitis virus*, *avian metapneumovirus* and *human metapneumovirus* (ICTVdb, 2006).

Paramyxovirus virions are pleomorphic in shape (spherical as well as filamentous forms occur), 150-300 nm in diameter. Virions are enveloped, covered with large peplomers and contain a herringbone-shaped helically symmetrical nucleocapsid. The genome consists of a single linear molecule of negative-sense, single-stranded RNA, 15-16 kb in size. The envelope contains two glycoproteins, a hemagglutinin (in most species with neuraminidase activity also) and a fusion protein (Murphy *et al.*, 1999). On the basis of morphology, growth in cell culture, the nucleic acid composition and antigen and physiochemical properties, PPR virus is classified as fourth member of the genus *Morbillivirus* of the *Paramyxoviridae* family and grouped together with *canine distemper*, *human measles* and *rinderpest viruses* (Gibbs *et al.*, 1979).

2.1. Respiratory viruses of camels

2.1.1. PPR disease

PPR is an economically important viral disease of sheep and goats first described by Gargadennec and Lalane (1942). PPR is caused by a virus belonging to the genus *Morbillivirus* of the family *Paramyxoviridae* (Van Regenmortel *et al.*, 2000) and is the causative agent of one of the highly infectious, Office Internationale des Epizooties (OIE) notifiable and economically important transboundary viral diseases of sheep and goats (Venkataramanan *et al.*, 2005).

The genome of PPR virus is non-segmented single stranded RNA of negative polarity. PPRV has 15,948 nucleotides making it the longest member of the genus *Morbillivirus* (Diallo, 1990). PPRV has six structural proteins: fusion protein (F), heamagglutinin protein (H), polymerase or

large protein (L), matrix protein (M), nucleocapsid protein (N) and phosphoprotein (P). The outer layer forms the matrix protein (M), which plays a significant role in the budding process. The haemagglutinin (H) helps the virus to bind to a host cell receptor and the fusion protein (F) helps fuse the viral envelope to the host cell membrane; as a result, a viral nucleocapsid enters the host cell cytoplasm and starts multiplication (Diallo *et al.*, 2006).

PPR is distributed in parts of Africa (West, Central and North) and Middle East countries, some Asian countries and indeed in most third world countries where people rely on agriculture and animal products (Diallo *et al.*, 2007).

Given that this is a small ruminant's disease, sheep and goats are the common hosts. While cattle, buffalo, camel and pig can be infected, they do not show clinical signs, or, rarely, they show only mild clinical signs (Chauhan *et al.*, 2009).

The incubation period can range from two to 10 days; in most cases, clinical signs appear in 2-6 days (OIE,2008).The clinical signs of PPR is characterized by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhea and death (Gibbs *et al.*, 1979). In general, the clinical feature of PPR in camels is not different from those reported in sheep and goats (Abubakar *et al.*, 2009; OIE, 2009).

PPRV is transmitted by direct contact with secretions and excretions of infected animals. Inhalation is thought to be an important route of spread. It is highly contagious and all discharges can carry virus (Chauhan *et al.*, 2009). It probably occurs in milk. Although close contact is the most important way of transmitting the disease, it is suspected that infectious materials (fomites) can transmit (FAO, 1999).

Peste des petits ruminants virus is epitheliotropic and lymphotropic. The virus enters via the respiratory tract. Following infection, there is a 3–4 days incubation period during which the virus replicates in the draining lymph nodes of the oropharynx before spreading via the blood and

lymph to other tissues and organs including the lungs causing a primary viral pneumonia (Hamito, 2009).

In the respiratory system, small erosions and petechiae may be visible on the nasal mucosa, turbinates, larynx and trachea. The lungs commonly exhibit pneumonia of a bronchointerstitial type, characterized by congestion, consolidation and enlarged regional lymph nodes. There may be serofibrinous pleuritis with inflammatory effusion within the pleural cavity. The spleen may be slightly enlarged and congested. Most lymph nodes throughout the body are enlarged, congested and edematous (USAHA, 2008). Bronchopneumonia in anteroventral areas, consolidation/atelectasis of lungs and pleuritis may be present in respiratory cases of PPR. The lungs have pneumonic lesions and look dark red to purple. Blood and exudates with many small bubbles can be found in the trachea and the tracheal mucosa has multifocal hemorrhages (Thomas *et al.*, 2006).

2.1.2. Respiratory syncytial virus (RSV)

RSV is a member of the *Pneumovirinae* subfamily and is the type-species member of the *Pneumovirus* genus (Morris *et al.*, 1956). Historically, HRSV was first isolated in laboratory chimpanzees in 1955 (Morris *et al.*, 1956) and ten years later, BRSV was isolated from calves in Switzerland (Paccaud and Jacquier, 1970).

Electronmicroscopy (EM) and negative staining have shown that RSV is pleomorphic and consists of a nucleocapsid surrounded by a lipid envelope with a diameter of 150–300 nm (Norrby *et al.*, 1970). The RSV genome is composed of single-stranded negative sense RNA encoding 11 proteins namely non-structural (NS2, NS1), nucleocapsid (N), phosphoprotein (P), matrix (M1, M2), small hydrophobic (SH), surface attachment glycoprotein (G), surface fusion glycoprotein (F), second matrix (M2) and RNA-dependent RNA polymerase (L). The lipid envelope that surrounds the nucleocapsid is derived from the host cell plasma membrane during the budding process and contains three virally encoded surface transmembrane glycoproteins: G, F and SH. The F protein mediates cell-to-cell fusion of RSV and the formation of syncytia

(Beeler *et al.*, 1989). The G protein is responsible for attachment to respiratory cells (Levine *et al.*, 1987).

In humans, there are two major groups of RSV, strains A and B and both strains may circulate and cause concurrent infection (Peret *et al.*, 1998). Humans and cattle are considered to be the natural hosts of HRSV and BRSV, respectively, but antigenically-related RSV has been isolated in sheep and goats (Evermann *et al.*, 1985) and antibodies to BRSV have been detected in other species (Van der Poel *et al.*, 1995).

RSV is predominantly a human virus though it has also been recovered from chimpanzees, cattle, goats and sheep. It is now known to occur worldwide in all bovine species as well as in sheep, goats and other animals (Murphy *et al.*, 1999), Chimpanzees (Morris *et al.*, 1956) and mice (van Regenmortel *et al.*, 2000).

Although cattle are the natural host of BRSV, it is possible that other species such as *Ovine*, *Caprine*, *Bison*, *Chamoix* or *Camelids* may play an epidemiological role in certain circumstances (Sausker and Dyer, 2002; Citterio *et al.*, 2003). The virus is related closely to Human respiratory syncytial virus (Murphy *et al.*, 1999).

RSV is a fragile virus that is transmitted from animal to animal mainly in aerosolized secretions (Baker, 1986). Infection can occur when droplets from a cough or sneeze come into contact with the eyes, mouth, or nose, or when someone touches an infected surface or object and then their eyes, mouth, or nose (CDC, 2009).

The airway epithelium is the main target of RSV infection. After infection, RSV replicates in the respiratory mucosa, leading to epithelial damage and perivascular mononuclear infiltration (Atreya and Kulkarni, 1999). Infected epithelial cells respond by producing a number of potent immunomodulatory and inflammatory mediators, including cytokines and chemokines (Zhang *et al.*, 2001). The infection results in the loss of cilia, sloughing of epithelial cells into the airway, collection of desquamated epithelial cells, polymorphnuclear leukocytes, fibrin, lymphocytes;

mucus within the airway and edema around the airway (Matsuse *et al.*, 2000; Harris and Werling, 2003).

RSV infection is characterized by sudden onset of fever, abdominal breathing, lethargy, rhinitis, nasal discharge and cough. High morbidity and low mortality are general (Pirie *et al.*, 1981; Murphy *et al.*, 1999). The infection of *Respiratory syncytial virus* leads to complete loss of ciliated epithelium, 8-10 days post-infection. Post-mortem lesions are bronchioleitis and interstitial pneumonia, edema, emphysema in all lung lobes which may progress to bronchopneumonia and death. The characteristic finding of the disease is the presence of syncytial cells in the lungs which are larger than those in PIV3 infection (Murphy *et al.*, 1999).

2.1.3. *Camelpox virus*

Camel pox is a wide-spread infectious viral disease of Old World *Camelids*. New World *Camelids* are also susceptible. It occurs throughout the camel-breeding areas of Africa, north of the equator, the Middle East and Asia causing economic impact through loss of production and sometimes death. The *Camel pox virus* belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Orthopoxvirus* (OIE, 2008).

The disease is characterized by fever, local or generalized pox lesions on the skin and in the mucous membranes of the mouth, respiratory and digestive tracts. The clinical manifestations range from inapparent infection to mild, moderate and less commonly severe systemic infection and death. The disease occurs more frequently and more severely in young animals. Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The role of insects in transmission has been suspected because the disease is often observed after rainfall. *Camelpox virus* is very host specific and does not infect other animals. Only one suspected case of human camel pox involving mild skin lesions has been described, underlining that camel pox is of no public health importance (OIE, 2008).

2.1.4. Infectious bovine rhinotracheitis (IBR) disease

IBR, caused by *Bovine Herpes virus-1* (BHV-1) of subfamily *Alpha herpesvirinae* within the *Herpesviridae* family, is one of the most economically important diseases of farm animals despite low mortality rate. BHV-1 genome consists of a single linear molecule of double stranded DNA. The virus is distributed worldwide and the disease is characterized by clinical signs of upper respiratory tract, nasal discharges, conjunctivitis, fever, depression, loss of appetite and reduced milk yield (Murphy *et al.*, 1999).

An outbreak of IBR in dairy cattle in Saudi Arabia characterized by fever, lacrimation, salivation and nasal discharge was reported (Abu *et al.*, 2008). In Sudan, IBR virus was detected in a calf suffering from acute respiratory disease: It was showing pyrexia, profuse bilateral watery nasal discharge and respiratory distress (Eisa, 1983). Prevalence of BHV-1 in camels is poorly studied; detection of antibodies to IBR in camels was reported in Tunisia (Burgemeister *et al.*, 1975) and in Egypt (Eisa, 1998). Nawal *et al.* (2003) was the first to report the detection and isolation of BHV-1 from camels in Egypt. The widespread of BHV-1 infections in New World *Camelidae* (NWC) was documented. BHV-1 was isolated from llamas with bronchopneumonia (Mattson, 1994). Intisar *et al.* (2009) detected BHV-1 antigen from camel lungs collected from abattoir using ELISA and FAT. Intisar *et al.* (2009) also isolated BHV-1 by cell culture confirmed by PCR from pneumonic camel lungs in Sudan.

2.1.5. Parainfluenza viruses (PIV)

Parainfluenza viruses are typical members of the family *Paramyxoviridae* and belong to the genus *Paramyxovirus*. The *Paramyxovirus* group includes mumps virus, four types of *Parainfluenza viruses*, simian virus 5 (SV5) and Newcastle disease virus (NDV) of birds (Kingsbury *et al.*, 1978). The *Parainfluenza viruses* (PIV) are members of the family *Paramyxoviridae* and are divided into types 1-4, with type 4 divided into subtypes A and B. These RNA viruses are pleomorphic enveloped particles that are 150 to 300 nm in diameter. The family *Paramyxoviridae* are RNA viruses containing a linear, non-segmented, negative-sense

RNA strand of about 15,000 nucleotides (Corne *et al.*, 1999; Henrickson, 2003) and consists of six or seven genes that encode eight or nine proteins, some of which are not detected in the virion but participate in the virus replication cycle in the cell. The virion consists of a filamentous, herringbone-like nucleocapsid core surrounded by a lipid envelope with virus specific glycoprotein spikes.

The PIV are important respiratory pathogens and have been shown to cause a wide range of respiratory illnesses including upper respiratory tract symptoms, croup, bronchitis, bronchiolitis and pneumonia both in humans and animals (Nawal *et al.*, 2003; Intisar *et al.*, 2010a).

Singh and Ata (1967) examined 52 sera from Sudanese camels at Cairo abattoir for the detection of PIV3 antibodies using haemagglutination inhibition (HI) and serum neutralization (SN); two camel sera were found to be positive. Maurice *et al.* (1968) reported widespread infection with PIV3 in the Saharan area of Chad. Frigeri and Arush (1979) examined sera from 138 dromedaries in Somali land for PIV3 antibodies using HI. Hedger *et al.* (1980) found neutralizing antibodies to PIV3 in camels (80%) in Oman. Isolation of PIV3 in tissue culture has been reported previously (Van Der Maaten, 1969); Nawal *et al.* (2003) was the first to report the isolation of PIV3 from camels. It is shown in this document parainfluenza-3 was found from Ethiopian camels as a primary causative agent with the association of *M. haemolytica* serotype A2 serologically and indicated as evidence for the camel respiratory disease outbreak occurred (Kebede and Gelaye, 2010).

2.1.6. *Adenovirus*

The *Adenoviridae* family includes a group of icosahedral nonenveloped viruses containing a linear double-stranded DNA genome. The widely diverse populations of Adenoviruses infect a broad spectrum of species; 51 human, 6 porcine, and 10 bovine serotypes have been described along with *Adenoviruses* infecting other hosts, such as birds or amphibians. The *Adenoviridae* were classically divided into the *Mastadenovirus* and *Aviadenovirus* genera, comprising mammalian and avian isolates, respectively. Recently, according to the available data from DNA

sequencing, phylogenetic analyses and the genome arrangements observed, two new genera, *Atadenovirus* and *Siadenovirus* have been accepted as including some bovine serotypes and the increased number of serotypes identified from amphibians and reptiles in particular (Allard *et al.*, 2001; Benko *et al.*, 2002).

Adenovirus (AdV) associated acute respiratory disease (AdARD) epidemics have been widely reported among human and animals worldwide. The virus enters through nasopharyngeal, oral, and conjunctival routes. Most cases involved only mild, acute, febrile and respiratory illness. However, occasionally, infections with pneumonia and more severe sequelae may happen (Trei *et al.*, 2010).

In cattle about 10 serotypes are identified, all causing asymptomatic disease or mild upper respiratory illness. However, in 1993, an epidemic in mule deer in California characterized by pulmonary edema and erosions, ulcerations and hemorrhage and abscess of intestinal tract was found to be caused by *Adenovirus* (Murphy *et al.*, 1999). Eisa (1973) was the first to report the detection and isolation of *Adenovirus* 1 from a bovine calf in Sudan. In Sudan, *Adenovirus* antigen was detected in camel lung tissue specimens using ELISA. Low percentage of positivity was observed (1.3%), but it is sufficient to indicate that *Adenovirus* is responsible for the pneumonia since all specimens were collected from lungs that showed pneumonia. This is the first report of *Adenovirus* antigen detection in camels in Sudan and probably elsewhere. This finding was supported by the previous reports on the association of *Adenovirus* with pneumonia in *camelidae* (Galbreath *et al.*, 1994) as well as the isolation of *Adenovirus* from diarrheic llamas and alpacas in USA (Mattson, 1994). With regards to serological evidence of *Adenovirus* infection in *camelidae*, Olaleye *et al.* (1989) reported the detection of *Adenovirus* antibodies in 1.3% of camel sera in Nigeria and Hadia *et al.* (2001) detected *Adenovirus* antibodies from camel sera in Egypt.

2.1.7. Influenza viruses

The family *Orthomyxoviridae* comprises the genera *Influenza virus A*, *Influenza virus B*, *Influenza virus C*, and *Thogoto virus*. *Influenza A viruses* are pathogens of horses, swine, mink, seals, whales, fowl and humans. *Influenza B viruses* are pathogens of humans only and although *influenza C viruses* infect humans and swine, they rarely cause serious disease.

Orthomyxovirus virions are pleomorphic, often spherical but predominantly filamentous in fresh isolates, 80- 120nm in their smallest dimension. Virions consist of an envelope with large peplomers surrounding eight (genera *Influenzavirus A* and *Influenzavirus B*), seven (genus *Influenzavirus C*), or six (genus *Thogotovirus*) helically symmetrical nucleocapsid segments of different sizes (Murphy *et al.*, 1999).

Influenza viruses of the family *Orthomyx-oviridae* are enveloped negative-strand RNA viruses with segmented genomes containing seven to eight gene segments (Palese and Shah, 2007). One genus includes *Influenza A* and *B viruses* and the other comprises *Influenza C viruses*. The three virus types differ in host range and pathogenicity. Type B and C *Influenza viruses* are isolated almost exclusively from humans, although *Influenza B viruses* have been isolated from seals and *Influenza C viruses* have been isolated from pigs and dogs (Youzbashi *et al.*, 1996; Osterhaus *et al.*, 2000). *Influenza A viruses*, however, infect a wide variety of warm-blooded animals including birds, swine, horses, humans and other mammals.

Influenza A and *B viruses* have a similar structure, whereas *Influenza C virus* is more divergent. A and B type viruses contain eight discrete gene segments, each of them coding for at least one protein. They are covered with projections of three proteins: HA, NA, and matrix 2 (M2). *Influenza C viruses* have seven segments and only one surface glycoprotein (Palese and Shah, 2007). Each influenza RNA segment is encapsidated by nucleoproteins to form ribonucleotide-nucleoprotein complexes (Palese and Shah, 2007). Serologic studies on camel influenza viruses are available. The presence of antibodies to influenza virus types A and B and virus was reported by Burgemeister *et al.* (1975) in Tunisia and Semushkin (1968) in Asia. *Influenza A* and *B virus*

antibodies were also reported in camels by Dioli and Stimmelmarmy (1992) and Olaleye *et al.* (1989).

2.2. Diagnosis of respiratory viruses

The diagnosis of viral diseases requires the identification of the viral pathogen in the clinical sample and a subsequent correlation between the identified virus and the clinical disease. Conventional methods of viral detection include isolation and in vitro culture and immunological methods such as direct fluorescence antibody or enzyme immunoassay. With some viral disease agents, identification is readily accomplished. Other viruses (e.g., *Norovirus*), however, are refractory to in vitro culture. Immunological methods, dependent on the quality and availability of antibodies, may also be ineffective for identifying viruses with constantly evolving serotypes. Although PCR has revolutionized viral diagnostics by increasing detection sensitivity and specificity, the maximum number of viruses detectable in a single assay is relatively small. In diseases where different viruses are present or where no etiologic agent has been identified, the value of these conventional methods may be limited (Liu *et al.*, 2006).

Respiratory viruses are one of the most important causes of morbidity and mortality throughout the world (Mizgerd, 2006; WHO, 2010). The presence of infectious virus or viral structural components can be investigated directly from various clinical specimens either by virus isolation, nucleic acid detection assays, or antigen detection assays (Vainionpa and Leinikki, 2008). Specific virus diagnostics can be used to determine the etiology of acute viral infection or the reactivation of a latent infection. Two approaches can be used: demonstration of a specific antibody response or the presence of the virus itself. Serological methods are used for measuring the antibody response while the presence of virus can be demonstrated by cultivation or demonstration of specific antigens or gene sequences. For the latter, molecular diagnostic methods have become more and more widely applied (Vainionpa and Leinikki, 2008).

In diagnostic virology, there are two general techniques, namely, culture methods and non culture methods. Cell culture techniques, explant cell, laboratory animal assay and embryonated chicken

egg assay are categorized under culture techniques while electron microscopy, antigen detection (serology) and molecular tests are among non culture methods (Zuckerman *et al.*, 2009).

It is clear that no single approach is optimal for detecting all viruses in all clinical situations. Therefore, it is vital to combine culture and nonculture methods to optimize viral disease diagnosis, yielding medically useful, cost effective and labor saving viral testing results. The following are the frequently used nonculture diagnostic techniques in diagnostic virology (Cutrín *et al.*, 2009; Zuckerman *et al.*, 2009).

2.2.1. Conventional and novel molecular amplification techniques

Acute respiratory tract infections (ARTI) are a major cause of morbidity and mortality worldwide (Arnold *et al.*, 2006). Viruses are responsible for the majority of ARTI, with *Rhinoviruses* (HRV), *Respiratory Syncytial virus* (RSV), *Influenza virus* (INF) and *Parainfluenzaviruses* (PIV) considered the major pathogens. Traditionally, the diagnosis of these infections relied on the isolation and identification of the viral agent by cell culture or detection of viral antigens by direct immunofluorescent assays (DFA) (Arnold *et al.*, 2006). However, using these methods a large proportion of respiratory infections remained undiagnosed.

Conventional virus detection systems used are virus isolation in cell cultures with the identification of typical cytopathic effects and direct detection of viral antigens using different techniques (Germann and Telenti, 1995). Classically, viral detection in patient samples employed tissue culture, which can take a long time to obtain results (average 3–5 days). Modern molecular technologies are more sensitive, more dependable and more rapid than classical tests. For this reason they have joined tissue culture as a gold standard for respiratory virus diagnosis and are more useful for rapid diagnosis (WHO, 2010). The development of PCR analysis in 1985 (Saiki *et al.*, 1985) made possible the diagnosis of viral infection through sensitive detection of specific viral nucleic acids and any virus can potentially be detected in this way.

Nucleic acid technology presents the opportunity to detect the pathogen directly, targeting the genetic material and augmenting or replacing culture, serological or histological techniques (Wagener, 1997; McKeever and Rege, 1999).

Polymerase chain reaction (PCR)

Recent advances in molecular biology, particularly the introduction of the polymerase chain reaction (PCR) assay, have greatly improved the detection of viral respiratory pathogens (Louie *et al.*, 2005). This is the most rapidly developing area in diagnostic virology, providing both qualitative and quantitative results (Zuckerman *et al.*, 2009).

PCR is a technique that started as a research tool and was eventually integrated into medical diagnostic applications. The Polymerase Chain Reaction (PCR), conceived by Kary Mullis in 1983 (Mullis and Faloona, 1987) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA.

The Principle of the polymerase chain reaction (PCR)

This technique uses a thermostable DNA polymerase to extend oligonucleotide primers complementary to the viral DNA genome target (Saiki *et al.*, 1988). Consecutive cycles of denaturation, annealing and extension result in an exponential accumulation of target DNA. RNA genomes require transcription to complementary DNA (reverse transcription) prior to the PCR reaction (Zuckerman *et al.*, 2009).

PCR has become one of the most widely used technologies in veterinary research and molecular diagnostics of infectious disease. The wide range of applications include the detection of DNA or RNA derived from infectious bacterial, viral, mycoplasmal, parasitic, protozoal, or fungal organisms; evaluation of specific genetic defects; and quantization of cytokines and cellular growth factors (Dahlhausen, 2010). Nucleic acid detection involves the amplification of nucleic acid and the assay is particularly important for viruses that are difficult or impossible to cultivate,

virus that grow slowly in culture, and viruses for which antigen detection cannot be applied because of antigenic diversity or because the level of viral antigen is too low in clinical specimen to permit successful detection (Carman, 2001). PCR testing can be used on a wide range of samples including tissue from biopsies or necropsies, blood, feces, tissue, soil, cerebrospinal fluid, and swabs of mucosal surfaces (Dahlhausen, 2010).

PCR uses the highly specific molecular recognition ability of Watson-Crick base pairing to provide the selectivity needed for a nucleic acid probe to bind to a targeted DNA sequence and allow for its exponential amplification. Specific fragments of DNA are replicated by cyclical heating and cooling through repeated temperature stages in a thermal cycler unit (Dahlhausen, 2010).

PCR comprises repetitive cycles of a three step amplification procedure. In the first step, nucleic acid isolated from a clinical specimen is denatured (mostly at 94°C). In the second step, 2 short oligonucleotide primers complementary to a specific microorganism's genome are allowed to anneal (commonly at 55°C). The oligonucleotide primers are designed based on known sequence and are typically positioned a few hundred nucleotides apart on the genome. In the third step, the oligonucleotide is extended by polymerase (commonly at 72°C). These 3 steps are repeated 20 to 40 times yielding a product of defined size in reactions containing the microorganism of interest (Kim and Chae, 2004). Each cycle can double the amount of existing target DNA. Repeatedly amplifying the target sequence over 30 to 40 cycles theoretically allows for millions (2^{30}) of DNA copies to be produced. PCR assays are thus extremely sensitive. They are capable of detecting femtograms (10⁻¹⁵g) of target DNA sequence in a large mixture, or up to a microgram (10⁻⁶ g) of non target DNA. The DNA obtained at the end of the reaction can be used for a variety of applications, including sequencing, cloning, or as a probe for in situ hybridization (Dahlhausen, 2010).

1. Melting (denaturing) step - Hybridization of the complementary DNA strands is disrupted by heating of the mixture to 95°C.

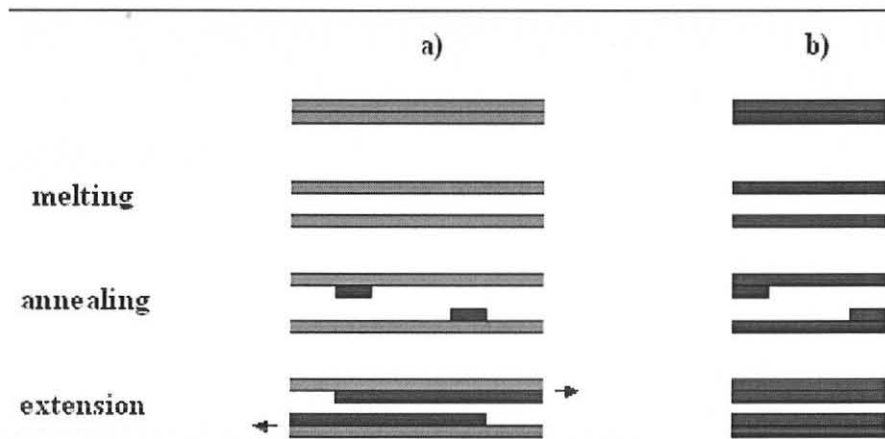


Figure 1: PCR thermal cycle: a) first cycle, b) typical n^{th} cycle

2. Annealing step - Temperature is lowered to $\sim 55^{\circ}\text{C}$ to allow binding primers to the single stranded DNA template.
3. Extension step - The primers are readily extended by a polymerase by complementation to the template strands at $\sim 72^{\circ}\text{C}$.

Ideally, in each cycle the number of copies of the DNA strand is doubled resulting in an amplification factor which is exponential in the number of cycles (Syvanen, 2001; Lipson, 2002).

Multiplex PCR

It is often desirable to perform several PCR reactions together, or in a multiplexed manner. Multiplex PCR is a reaction in which several target sequences are co-amplified, each with its own different primer pair, so that significant amplification of all targets is achieved simultaneously (Lipson, 2002).

Molecular-based testing for multiple pathogens in a large number of animals is expensive. The development of new variations of PCR have allowed for simultaneously performing multiple tests on a single diagnostic sample, thereby affording some savings on a per-test basis. In

multiplex PCR, multiple primer pairs are used to amplify more than one gene or pathogen in a single PCR reaction. Multiplex assays must be carefully designed for specific primer selection. Primers selected must be to regions that are uniquely different and characterized by low affinity for primer-to-primer interactions (Dahlhausen, 2010). Post-amplification analyses of DNA fragments produced by multiplex PCR are routinely carried out by agarose gel electrophoresis.

RT-PCR (Reverse Transcription –Polymerase Chain Reaction)

Standard PCR techniques cannot be used to detect the presence of RNA-based viruses in clinical samples. The direct amplification of RNA in PCR systems is not possible because of the polymerase enzyme's inability to synthesize DNA from an RNA template. The inability to synthesize DNA from an RNA template is overcome by the use of a reverse-transcriptase (RT) enzyme before the PCR reaction. In RTPCR, RNA is converted into a complementary DNA (cDNA) strand by an RNA-dependent DNA polymerase (reverse transcriptase). The cDNA produced is then used as a template in a conventional PCR reaction. It is important that all genomic DNA within the test sample be destroyed before the RT-PCR to prevent masking the presence of cDNA derived from the RT step (Mhlanga and Malmberg, 2001).

Analysis of PCR products/ PCR Product Analysis

There are many ways to analyze PCR products depending upon the information required:

- The presence or absence of the target DNA sequence

- The length of the amplified fragment

- The yield of PCR product to quantitate the relative or absolute amounts of the starting DNA or RNA

It will often be possible to predict the size(s) of expected PCR products, which are of a length defined by the positions of the PCR primers. Often PCR products are relatively small, in the range of 0.2–3kbps and even in many genomic cloning experiments they are less than 10 kbps in length. The simplest and most direct methods to analyze PCR products involve gel electrophoresis.

Gel electrophoresis

The most common and rapid way of analyzing PCR products is by standard agarose gel electrophoresis. Depending on the expected size of the amplified fragment, a fraction of the PCR reaction should be loaded onto a 0.8–3% agarose gel containing ethidium bromide. Usually one-tenth or one-fifth of the reaction volume is loaded and the remainder is stored at 4°C or -20°C for subsequent use. An aliquot of loading dye containing glycerol and a marker such as bromophenol blue should be added to the sample to assist both loading on the gel and visualization of the sample migration through the gel (McPherson and Møller, 2006).

Appropriate molecular size markers such as a 100bps or 1kbps DNA ladder or bacteriophage lambda or ϕ X174 restriction enzyme digests, available from a range of manufacturers, or previously characterized PCR products should be loaded in adjacent lanes of the gel. The amplified fragment(s) should be readily visible under ultraviolet transillumination and the gel can be photographed using a camera or digital imaging system to record the results (McPherson and Møller, 2006).

Unknown Virus investigation methods

Conventional PCR methods are used frequently to identify or exclude the presence of a viral pathogen suspected to be present in a biological sample. PCR is both very sensitive and specific, especially when coupled with sequencing of PCR products. However, its reliance on specific primers complementary to the pathogen genome sequence makes conventional PCR analyses unsuitable for screening of biological samples for the presence of unknown viruses (Nanda *et al.*, 2008).

The detection of unknown viruses is still one of the most challenging problems in diagnostic virology. If a known virus is suspected to be present in a biological sample, it is usually not difficult to use the known viral sequence to design specific PCR primers and to implement a specific detection assay. However, in an era of emerging pathogens, improved methods for virus

discovery and detection that are less dependent upon the availability of sequence information are needed (Nanda *et al.*, 2008).

Degenerate oligonucleotide primed- polymerase chain reaction (DOP-PCR)

DOP-PCR was developed in order to representatively amplify DNA for cytogenetic studies (Telenius *et al.*, 1992). DOP-PCR is a reliable tool to amplify DNA accurately and uniformly. DOP-PCR was developed to allow random amplification of DNA from any source (Speicher *et al.*, 1993). DOP-PCR uses a partially degenerate sequence in a PCR protocol with two different annealing temperatures. It has been successfully applied for amplifying entire genomes such as human, mouse, and fruit fly, as well as isolated human chromosomes and cosmid (Scalzi *et al.*, 1998). The technique has also been used to increase the amount of sample for genotyping (Cheung and Nelson, 1996) and genomic fingerprinting (Sayada *et al.*, 1994). The DOP-PCR primer consists of three regions. The 5'-end carries a recognition sequence for *Xho*I (C·TCGAG), a restriction endonuclease that cuts rarely within the human genome. The sequence is then followed by a middle portion containing six nucleotides of degenerate sequence (NNNNNN, where N = A, C, G, or T in approximately equal proportions) and a 3'-end sequence containing six specific bases (ATGTGG) which primes the reaction approximately every 4kbs (Telenius *et al.*, 1992).

The DOP-PCR protocol is a combination of two different cycling programs. During the first five cycles, the sample is amplified under low stringency conditions, then 30- 40 cycles of high stringency PCR is performed. DOP-PCR primers are usually 22bps long and contain a 6bps well defined 3'end which is followed by 6bps of degenerate nucleotides. The 5'end contains a rare restriction endonuclease recognition site. During the low stringency cycles, only the 3' end of the primer initiates elongation from the sites that contain the 6bps well-defined sequences. The degenerate part of the primer ensures that all possible combination of the 12bps will be recognized and extended (Kiss *et al.*, 2002).

The principle of the technique is that at a sufficiently low annealing temperature only the six specific nucleotides included in the 3'-end of the degenerate oligonucleotide will anneal to the genomic strand allowing the primer to initiate PCR. The PCR fragments are then generated which contain the full length of the oligoprimers at one end and its complementary sequence at the other end. Subsequently, the temperature is increased to the level required for the full length of the degenerate primer to anneal (Telenius *et al.*, 1992).

Virus Discovery using cDNA AFLP (VIDISCA) method

Identification of unknown pathogens using molecular biology tools is difficult because the target sequence is not known, so genome-specific PCR primers cannot be designed. To overcome this problem, the VIDISCA method was developed based on the cDNA-AFLP technique. The advantage of VIDISCA is that prior knowledge of the sequence is not required as the presence of restriction enzyme sites is sufficient to guarantee PCR amplification (Bachem *et al.*, 1996).

As CPE-positive, but serologically untypable cell cultures occur regularly during routine diagnostics, it would be desirable to have a simple and inexpensive method for the characterization of viruses from supernatants (van der Hoek *et al.*, 2004). Virus Discovery by cDNA AFLP (VIDISCA) uses cell culture supernatants treated by DNase digestion in a modified cDNA Amplified Fragment Length Polymorphism (AFLP) analysis (van der Hoek *et al.*, 2004) and this method has been described originally in the context of the discovery of a novel human *Coronavirus* in 2004 (van der Hoek *et al.*, 2004). In that study, it was used to amplify an untypable virus from the supernatant of a cell culture showing a cytopathic effect (CPE).

The input sample can be either blood plasma or serum or culture supernatant. Whereas cDNA-AFLP starts with isolated mRNA, VIDISCA begins with a treatment to selectively enrich for viral nucleic acid including a centrifugation step to remove residual cells and mitochondria. A DNase treatment is also used to remove interfering chromosomal and mitochondrial DNA from degraded cells (viral nucleic acid is protected within the viral particle). Finally, by choosing frequently cutting restriction enzymes, the method can be fine-tuned such that most viruses will

be amplified (Bachem *et al.*, 1996). AFLP employs restriction enzyme digestion sites in an unknown DNA sequence to ligate oligonucleotide adaptors which are then used as primer binding sites for PCR amplification (van der Hoek *et al.*, 2004).

2.2.2. Serological tests

Antigen detection

Conventional virus detection systems used are virus isolation in cell cultures with the identification of typical cytopathic effects and direct detection of viral antigens using different techniques (Germann and Telenti, 1995).

Antigen detection assays are also widely used in respiratory tract infections like *Influenza* and *Respiratory syncytial virus* infections. Antigen detection systems are generally less sensitive but more rapid than culture and are useful and cheap tools for the detection of viruses which are excreted in high concentrations and for which cell culture isolation is not easily available, e.g. *Hepatitis B virus* (HBV) and rotavirus (Germann and Telenti, 1995). The lack of requirement for viability is another important advantage over viral culture, especially when specimen transport time is prolonged or otherwise suboptimal. Methods used for viral antigen detection include fluorescent antibody (FA) staining, immunoperoxidase staining, and enzyme immunoassay (EIA) (Weisbroth *et al.*, 1998; Cann, 2005; Cutrín *et al.*, 2009; Zuckerman *et al.*, 2009). For direct fluorescent antibody staining, a fluorescent label is conjugated to the antibody that recognizes the viral antigen. In the indirect format, the antiviral antibody is unlabeled and is detected by a second fluorescent labeled antibody that recognizes the antiviral antibody. Different respiratory viruses such as *Influenza virus* A and B, PIV1-3 and RSV, enteric viruses such as *Adenovirus* and *Rotavirus* and blood viruses such as *Hepatitis virus* and *Human immunovirus* have been diagnosed using the technique. Immunoperoxidase staining is similar in principle to FA staining except that horseradish peroxidase is used in place of fluorescent label. After incubation, the substrate for peroxidase is added that changes color when acted on by peroxidase and the staining can be viewed by light microscope. In ELISA, the same principles are used as in case of enzyme

linked immunosorbent assay (ELISA) except it is antigen that is detected in this case (Williams *et al.*, 1994; Knipe and Holey, 2001).

Complement Fixation Test (CFT)

The complement fixation test (CFT) is a classical laboratory diagnostic test which is still used for determination of virus antibodies in patient sera or cerebrospinal fluid samples during an acute infection. CFT is convenient and rapid to perform; the demand on equipment and reagents is small and a large variety of test antigens are readily available (Iman *et al.*, 1997). However, there is now a trend to replace the CFT with more direct, sensitive and rapid techniques such as RIA and EIA, but it is a very exacting procedure because five variables are involved (Cann, 2005; Ali *et al.*, 2008).

In the actual test, the complement in the patient's serum is first destroyed by heating; the serum is then mixed with appropriate viral antigen and after incubation; when the antigen-antibody complexes are formed, exogenous complement (usually from fresh guinea pig serum) is added. This complement then binds to the complexes and having been fixed, it is then no longer able to cause lysis of added indicator red cells. Usually, sheep red cells coated with antishsheep red cell antibodies are used as indicator to measure the presence of any residual complement. The tests are usually carried out on microtiter plates and the results are observed by eye (Vainionpa and Leinikki, 2008).

Hemagglutination inhibition (HI) test

Many viruses bind to hemagglutinin molecules found at the surface of red blood cells of various animal species and this can cause aggregation of red cells in suitable conditions. Prevention of this aggregation, called hemagglutination inhibition, by specific antiviral antibodies in the patient's serum has been widely used for diagnostic purposes. The test known as hemagglutination inhibition test, has important diagnostic and public health applications in certain infections, most notably in Influenza where antibodies measured by this test show

additional specificity compared to other tests and therefore provide more detailed information about the immunity and past infections of individuals. However, for the diagnosis of individual patients, the assay is no longer widely used and is replaced by more modern immunoassays (Vainionpa and Leinikki, 2008).

Enzyme linked immunosorbant assay (ELISA)

Solid-phase systems for antigen detection are still used widely. ELISA is based on the capture of antibody in a clinical specimen to a solid-phase (such as the base and walls of a well in a microtitre plate, or multiple magnetic microparticles/beads) via a capture antigen and subsequent detection uses an enzyme linked specific antibody that produces a color change in the presence of a suitable substrate (Azwai *et al.*, 1996; Abraham *et al.*, 2005; Albayrak and Gur, 2009). ELISA is also readily used for the detection of specific antigen. The use of synthetic peptides or recombinant antigens instead of whole viral lysates and improvements in signal detection has led to more sensitive, specific and rapid methods for measuring virus specific antibody levels (Intisar *et al.*, 2010b). The immunoassay format is versatile, and new assays can be designed quickly to cope with clinical demands, for example the investigation of new viruses such as SARS associated *Coronaviruses*. Most of the assays employ horseradish peroxidase, alkaline phosphatase, or B-D-galactosidase. The most interesting recent developments have been in new methods to detect these enzymes rather than the use of new enzymes. The use of monoclonal antibodies has led to many improvements in ELISA systems such as higher sensitivity, higher specificity and higher practicality. Solid phase immunoassays for the detection of antibody are essentially of three types (Xu *et al.*, 2007; Alonso-Padilla *et al.*, 2010).

2.2.3. Electron microscopy

Viral infections can occasionally be diagnosed using electron microscopy for the direct visualization of viral particles in specimens. It is the only technique available for directly visualizing viruses and therefore has many applications beyond purely diagnostic ones and relies on the detection and identification of viruses on the basis of their characteristic morphology

(Zuckerman *et al.*, 2009). With the introduction of negative staining in the late 1950s (Brenner and Horne, 1959) and the wider availability of electron microscopes, electron microscopy became essential in characterizing many new isolates detected in cell cultures and clinical samples (Plummer *et al.*, 1985; Biel and Madeley, 2001). Pattern recognition (e.g. information of size, particle morphology) leads to rapid identification of infectious agents. The “open procedure” of electron microscopic testing allows an unbiased, rapid detection of viruses and other agents if sufficiently high particle concentrations exist. Due to this capability, electron microscopic testing must be a frontline method applied to cell cultures of cultivable agents. A specimen can be ready for examination and an experienced virologist or technologist can identify by electron microscopy, a viral pathogen morphologically within a few minutes of arrival in the electron microscopy laboratory. Other advantages of electron microscopy include the lack of requirement for viral viability, the fact that many different kinds of viral particles can potentially be seen and allow the differential diagnosis of many agents contained in the specimen (Biel and Madeley, 2001).

The main disadvantage of EM is its inability to examine multiple specimens coincidentally (Cutrín *et al.*, 2009). Other disadvantages include the cost and complexity of maintaining an electron microscope, the need for a skilled operator and relative lack of sensitivity related to the fact that a relatively high concentration of viral particles (10^5 to 10^6 per milliliter) is required for visualization (Miller, 1995).

2.2.4. Virus culture methods

Culture of viruses is a ‘gold standard’ that enables isolation, identification and propagation of viruses. Cell culture has been the widely used technique in culture of viruses (Kim and Chae, 2004). However, embryonated chicken eggs or explant cultures may be used as alternatives to tissue culture where culture of some viruses in cell culture is particularly difficult. The same way, some viruses cannot be propagated in either mammalian tissue culture systems or embryonated chicken eggs. For these viruses, the use of laboratory animal is mandatory to propagate the virus *in vivo* (Cann, 2005; Cutrín *et al.*, 2009; Zuckerman *et al.*, 2009).

Cell culture

Viral culture is the original method used for diagnosing respiratory virus infections and culture for viruses using primary and immortalized cells was expanded for wider diagnostic use in the 1950s. The main advantage of traditional cell culture methods is the ability to isolate and identify a wide range of viruses (Kesson, 2007).

Cell culture is the process by which cells are grown under controlled conditions referring to the culturing of cells derived from multicellular eukaryotes, especially animal cells and are separated into 3 types (Burleson *et al.*, 1992; Dunham and Guthmiller, 2008). First, primary cells which are prepared directly from animal or human tissues and primary monkey or baboon kidney are one of the examples of primary cells. Second, semi-continuous diploid cells are the one which are derived from human fetal tissue and can be subcultured 20 to 50 times. Third, continuous cell lines derived from tumors of human or animal tissue can undergo continuous multiplication (NCBI, 2006).

To date, there are many defined cell lines that are available for utilization in diagnostic virology in which most of them are of human origin. However, there are cells originated from animals such as large animals, mouse, murine, dog, primate (ape), rat, hamster and arthropods (Cann, 2005; Ali *et al.*, 2008; Dunham and Guthmiller, 2008). Virus multiplication can be detected in several ways such as morphologic alteration of cell (cytopathic effect, CPE), the formation of giant cells and syncytia, ballooning of cells and the appearance of cytoplasmic or nuclear inclusion bodies (Burleson *et al.*, 1992; Cann, 2005; Zuckerman *et al.*, 2009). Other means including change in pH of the medium, change in hemadsorption property of cell membrane, serologic technique to detect viral antigen, the ability of noncytopathic virus to modulate the CPE of other virus and assay of cell culture material in another host systems are also useful to detect virus proliferation in cell culture. Some viruses require multiple passages before cytopathic effects are evident. Other viruses such as *Influenza* and *Parainfluenza* viruses never cause a visible cytopathic effect, but can be recognized by hemagglutination, reactivity of infected cells with virus-specific antibody, or molecular techniques (Carman, 2001; Ogilvie, 2001).

Embryonated chicken eggs and explant culture

Culture of some viruses directly from clinical specimens in cell culture is particularly difficult. For these viruses, embryonated chicken eggs or explant cultures may be used as alternatives to tissue culture. At one time, embryonated chicken eggs were commonly used for virus propagation. However, with the development of immortalized cell lines, embryonated eggs are now used infrequently for diagnostic evaluations. Explant or organ cultures are similar to primary cell cultures except that the tissue is not treated with trypsin to disperse the cells (Williams *et al.*, 1994; Weisbroth *et al.*, 1998). Explant cultures allow the virus to proliferate *in vitro* but obviate the virus need to adapt to a mammalian cell line, a process that may take many passages for some viruses. Explant cultures have been shown to be particularly useful for initial isolation of some viruses, including rodent *Parvoviruses* (Jacoby *et al.*, 1991).

Laboratory animals

Some viruses cannot be propagated in either mammalian tissue culture systems or embryonated chicken eggs. An example is *Mouse thymic virus*, which must be propagated *in vivo* in laboratory animals such as neonatal mice (Osborn, 1982). A number of additional diagnostic techniques have been developed and are now commonly used to identify potential pathogens in laboratory mice and rats (Valera *et al.*, 2008). Identification of potential pathogens in laboratory animals is accomplished using a variety of methodologies. The technique used to identify a specific virus depends on a number of factors, including the type of virus, the selectiveness of the virus, the immunological status of the host, the tissue or site of localization of the virus in the animal and the particular tests that have been developed to detect and identify the virus (ILAR, 1996). In general, because virus isolation is very labor intensive and often results in false negative results, it is seldom used as a primary diagnostic test (Weisbroth *et al.*, 1998; Kostomitsopoulos *et al.*, 2008; Valera *et al.*, 2008).

3. MATERIALS AND METHODS

3.1. Study areas

The study camels slaughtered at Addis Ababa Akaki abattoir were originated from Borena and Bale zones, Fentale district of East Shoa zone and Meiso district of West Hararghe zone of Oromia regional state, Ethiopia.

Borena, one of the 12 administrative zones of Oromiya Regional State, is located in the far south of Ethiopia bordering Kenya in the south. It is located about 570kms) south east of Addis Ababa (Negussie, 1999). Administratively, Borana Zone consists of 12 Woredas. The total area of the zone is about 69, 373.3 kms². It is divided into six highland and six lowland districts (Zonal atlas of Borana zone). Borena is boarded with Kenya in the South, SNNP in the West and North-East, Somali regional state in the East and Bale zone in the north east directions (Duba, 1998).

The livestock population of borena zone is estimated to be; cattle (1,977,130), sheep (454,863), goats (819,538), horse (11,648), donkey (88,425), mule (26,790), camel (161,581), poultry (301,976) (Borena zone pastoral development office, 2010). The adaptations of borena zone ranges from semi arid (hot, lowland) to humid (cool, highland). Annual temperatures in the Borena area vary from 19 to 24°C with little seasonal variation. Average annual rainfall varies from about 400 to 1100mm (with the average of 700mm) and rainfall increases with 64mm for every 100m-elevation increase (Coppock, 1994). Rainfall delivery is bimodal: Two rainy seasons are recorded in the area: the spring and autumn rains, referred to as “gannaa” and “hagayyaa,” respectively. The natural vegetation is forest and lowland bushy savannah or steppe-type vegetation (Duba, 1998). The rangelands are dominated by tropical savannah vegetation with varying proportions of open grasslands and perennial woody vegetation (Homann *et al.*, 2007). The majority of community members in the zone are pastoralists or agro-pastoralists. The main agricultural crops are maize, teff, sorghum and haricot beans (Demeke, 1998).

Few study camels slaughtered at Addis Ababa akaki abattoir were also originated from Meiso woreda of Western Hararghe, Fentale district of East Shoa and bale zones of Oromia regional state, Ethiopia.

3.2. Study population

The study animals were the local camels (*Camelus dromedaries/Old world camels/one humped camels*) slaughtered at Addis Ababa- Akaki abattoir. Most of the camels presented for slaughter were dominantly adult femaes with few males whose ages were estimated above 15years. Lorry car was the transportation system used to bring the camels from their local markets (Borena and Bale zones, Fentale and Meiso areas) to the abattoir.

3.3. Study design

The study was conducted for a defined period of a year using cross –sectional type of design.

3.4. Sampling method

The nonrandom sampling method used was purposive type of sampling. During the study period, autopsic tissue samples from pneumonic lungs of camels slaughtered at Akaki abbatoir (Addis Ababa) originated from Borana, West Hararghe and Bale zones, Fentale district of East Shoa zone of Oromia regional state were collected for isolation and characterization of camel respiratory viruses.

3.5. Study methods

3.5.1. Virus isolation in cell culture system

Dubca cell lines (derived from camel skin fibroblast cells) were proposed for alternative camel virus isolation. However, due to sloughing of Dubca cell lines from cell culture flask, these cell lines were not used for virus isolation during the study period. This study had been conducted using vero cell lines for virus cultivation throughout study time. Vero cells are derived from two words, verda Reno (which means green and kidney, respectively). Owing to their universal susceptibility to many viruses, vero cell lines (derived from the kidney of adult African green monkey) were used for cultivation of the camel lung tissues to check the growth of viral agent/s/ on these cell lines and finally to isolate respiratory camel viruse/s/ therein.

Camel lung tissue sample collection and transportation

At Addis Ababa-Akaki abattoir, on average 8 apparently health camels were slaughtered each day. Before slaughter, camels were examined antemortally for any observable disease symptoms. Following slaughtering, those pathologic lungs physically observed to have lesions suspected of having viral infections excluding lesions due to parasitic, pyogenic bacteria, foreign body and tuberculoid cases were collected. As such, all carcasses were examined and about 6 grms of camel lung tissue samples were aseptically/carefully sliced and taken using clean scalpel blade and gloved hands. Each tissue taken was then put into labeled sterile/clean/ capped universal glass and plastic bottles and the labeled bottles placed in an ice box containing ice packs and transported to National Veterinary Institute (NVI) virology laboratory for virus processing and cultivation. Fresh tissues were preserved at -85°C till processing. Samples were collected before the lungs contacting with other tissues and the ground. At the abattoir level, contaminations between consecutive samples were also minimized by taking the specimen from inside portion of the tissues and washing hands frequently.

Preparation of the camel lung tissues for virus cultivation

Thirty five pneumonic camel lung tissue samples collected from the abattoir were processed and cultured on vero cell monolayer during the study period where about 1 gram of each pneumonic camel lung tissue was taken and washed three times using phosphate Buffered Saline (PBS) on petridish, washed tissue was transferred to mortar and cut into small pieces using scissor and minced by scalpel blade. The minced tissues were then ground and homogenized using pestle. Nine ml of phosphate Buffered Saline (PBS) was added to the ground and homogenized tissues and well mixed. The homogenized tissues were transferred to test tube and centrifuged at 3400 rpm for 10min. 0.5ml of the supernatant was inoculated on to confluent vero cells and incubated at 37°C for 1hour. Following incubation, the inoculated cell lines were washed using PBS and about 10ml of 2% Glasgow Minimum Essential medium (GMEM) whose preparation protocol is given in (Annex-2) was added and incubated at 37°C to see the development of cytopathic effect (CPE) (Burleson *et al.*, 1992). (Procedures and methods for tissue processing is provided (Annex-1).

3.5.2. Molecular virus characterization methods

Viral DNA/RNA extraction methods from cell culture supernatants

Molecular methods are the only accurate methods and the techniques applied in this study included novel and conventional PCR techniques. Nevertheless, before molecular characterization such as novel universal PCR and conventional PCR, viral genomes (DNA or RNA) were first extracted using standard viral RNA or DNA extraction protocols described below. Positive samples exhibiting observable CPE on vero cell cultures were assumed to contain either DNA or RNA or both viral agents. Therefore, both DNA and RNA extraction protocols were conducted for each sample exhibiting CPE.

RNA extraction using RNeasy principle

RNA was extracted using (Qiagen RNeasy mini spin column kit). The RNeasy procedure represents a novel technology for RNA isolation. This technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 50 µl of water. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are isolated. The procedure provides enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs which together comprise 15–20% of total RNA) are selectively excluded (Qiagen, 2006a). Materials required and the detailed procedure is provided (Annex- 3).

RNA extraction

Due to intracellular nature of viruses, their genome purification requires host cell/vero cell membrane and nucleic acid destruction. Accordingly, 460 µl lysis buffer RLT was added to a 1.5 ml eppendorf tube containing 460 µl of infected cultured vero cell suspension. Four hundred sixty microliters (µl) 70% ethanol was applied to precipitate nucleic acids released from disrupted host/vero/ cells due to lysis buffer RLT followed by homogenization. The homogenized suspension was transferred to RNeasy spin column and centrifuged where viral nucleic acids released bound to the silica membrane; the fluid part passed through the membrane down to the collection tube and the flow through discarded. The nucleic acids bound to the membrane was washed using 700 µl wash buffer RW1 followed by addition of 500 µl RPE buffer and centrifuged to dry the membrane and the flow through discarded. Finally, the nucleic acids bound to the silica

membrane were eluted into eppendorf tube using 100µl RNase free water and the eluted RNA was used for further procedures.

Viral DNA extraction

Principle

DNA was extracted using (QIAamp DNA Mini and QIAamp DNA Blood Mini Kits). QIAamp DNA Mini and QIAamp DNA Blood Mini Kits are designed for rapid purification of an average of 6µg of total DNA (e.g., genomic, viral, mitochondrial) from 200µl of whole human blood and up to 50µg of DNA from 200µl of buffy coat, 5×10^6 lymphocytes, or cultured cells that have a normal set of chromosomes (Qiagen, 2006b).

QIAamp Mini spin columns fit into most standard microcentrifuge tubes. In the spin protocol, due to the volume of the filtrate, 2ml collection tubes (provided) are required to support the QIAamp Mini spin column during loading and wash steps. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp Mini spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. DNA bound to the QIAamp membrane is washed in 2 centrifugation steps. The use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding. DNA is eluted using Buffer AE in collection tube and used for molecular tests (Qiagen, 2006b).

DNA extraction

To purify only viral DNAs from host cells containing host cell DNAs and others like mitochondrial DNAs, host cell /vero cells and the DNAs should get destroyed. One hundred

eighty microliters (180µl) of infected cultured cell suspension and 180µl ATL lysis buffer were added to a 1.5ml reaction tube and vortexed. Proteinase K was added and incubated at 56°C for 1.30 hours in water bath. 200µl of each AL buffer and ethyl alcohol were added and the mixture was transferred to QIAamp DNA mini Spin column. The DNeasy Spin column was placed into a 2ml collection tube and centrifuged at 8000 rpm for 1min where the flow through in the collection tube was discarded. The DNA sample bound to the silica gel membrane was washed and the membrane dried using 500µl of each wash buffers AW1 and AW2 by centrifugation. Finally, the DNA sample bound to membrane was eluted into a 1.5ml eppendorf tube using 100µl elution buffer AE, the eluted DNA was stored at -20°C till further processing (Annex-4).

cDNA synthesis (Reverse Transcription)

The direct amplification of RNA in PCR systems is not possible because of the polymerase enzyme's inability to synthesize DNA from an RNA template. The conversion of RNA to its complementary (cDNA) is overcome using an RNA-dependent DNA polymerase reverse-transcriptase (RT) Revert Aid™ M-MLV RT (derived from *Mouse Moloney Murine Leukemia Virus*) enzyme. This enzyme has been engineered to reduce RNase H activity and provide increased thermal stability. RNAs extracted from infected vero cell cultures were converted to DNA using cDNA synthesis kit. cDNA synthesis was performed in the first step using total RNA primed with random primers using M-MLV RT enzyme. In the second step, the cDNA produced was used as a template for universal or DOP-PCR virus detection, conventional PCR and multiplex PCR to characterize the suspected viral etiologic agents.

cDNA Synthesis reaction

cDNA was synthesized combining two chemical mixtures prepared separately: primarily, a mixture containing 3µl RNase free water, 1µl random hexamer primer, 1µl dNTPs and 5µl extracted RNA sample were mixed and incubated at 65°C for 5 minutes. Second, the cDNA synthesis mix containing 2µl of 10X Dream Taq RT buffer, 4µl of 25mM MgCl₂, 2µl of 0.1M

DTT, 1µl of RNase OUTTM(40U/µl), and 1µl of Revert AidTM*Mouse Moloney Murine leukemia Virus (M-MMLV-RT)* enzyme were homogenized. The first and the second reaction mixtures were mixed together in a single tube, centrifuged and incubated at 25°C for 10min followed by 50°C for 50 minutes. The reaction was stopped by incubating at 85°C for 5min and 1µl of RNase H enzyme was added to destroy unconverted and contaminating RNAs. cDNA produced was used for virus characterization (Annex-5).

Conventional PCR

Polymerase Chain Reaction (PCR) is a technique that started as a research tool and was eventually integrated into medical diagnostic applications. PCR is a technique for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In principle, this technique uses a thermostable DNA polymerase to extend oligonucleotide primers complementary to the viral DNA genome target. Consecutive cycles of denaturation, annealing and extension result in an exponential accumulation of copies of the target DNA/gene/ (Kim and Chae, 2004).

PCR reaction

PCR reaction was carried out in a total volume of 50µl in a 0.2ml reaction tube containing 32.6µl RNase free water, 5µl of 10X Dream Taq buffer, 3µl of 25mM MgCl₂, 1µl of 10mM dNTPs, 1µl of each forward and reverse primers, 0.4µl of dream Taq DNA polymerase enzyme and 10µl of the cDNA/DNA.

Once all the reagents were mixed, the tube was placed into the PCR machine. The amplification was carried out according to the following programme. The reaction was initiated by maintaining the mixture at 95°C for 2 minutes (initial denaturation) followed by 35cycles of 94°C for 40 seconds, 68°C for 30 seconds and 72°C for 40 seconds. The reaction was stopped by keeping the mixture at 72°C for 7min and maintained at +4°C as the PCR amplified product. Ten microliters (10µl) of the PCR products and 2µl loading were mixed and loaded into the wells of Ethidium

bromide stained agarose gel and the gel electrophoresed, analyzed by UV illumination where the result was digitally photographed and documented (Annex-6).

Conventional PCR was performed for *Parainfluenza virus* 1, *Parainfluenza virus* 2, *Parainfluenza virus* 3, *Respiratory syncytial virus* (RSV) and *Adenovirus* using specific primers (Table 1). *Peste des petits ruminants virus* (PPRV) was also investigated using PPRV specific primers whose forward and reverse primer sequences were:

NP4 (5'- CCTCCTCCTGGTCCTCCAGAATCT-3' and
NP3(3'TCTCGGAAATCGCCTCACAGACTG-5'), respectively
(Invitrogen, Carlsbad, California, USA).

Degenerate oligonucleotide primed– polymerase chain reaction (DOP-PCR)

DOP-PCR was developed to allow random amplification of DNA from any source and does not depend on the availability of sequence information.

The DOP-PCR primer

The sequence of this primer is (5'-CTCGAGNNNNNNATGTGG-3') and consists of three regions. The 5'-end carries a recognition sequence for *Xho*I (C·TCGAG), a restriction endonuclease that cuts rarely within the human genome. The sequence is then followed by a middle portion containing six nucleotides of degenerate sequence (NNNNNN, where N = A, C, G, or T in approximately equal proportions) and a 3'-end sequence containing six specific bases (ATGTGG) which primes the reaction approximately every 4kbp (Telenius *et al.*, 1992).

The principle of DOP-PCR is that at a sufficiently low annealing temperature only the six specific nucleotides included in the 3'-end of the degenerate oligonucleotide anneal to the genomic strand allowing the primer to initiate PCR. PCR generated fragments contain the full length of the oligoprimer at one end and its complementary sequence at the other end. It is a random amplification of the whole regions of the unknown DNA is incubated with slightly

differing 4096 primers at low temperature. As a chance, some of these primers may find complementary regions and anneal/bind to the unknown DNA genome and produce copies of the regions ahead.

DOP-PCR reaction

A master mix containing 35.1µl RNase free water, 5µl of 10X Dream Taq buffer, 1.5µl of 25mM MgCl₂, 1µl of 10 X mM dNTPs, 1µl of universal primer and 0.5µl Taq DNA polymerase was prepared for each reaction. Then 45µl of the master mix and 5µl of the cDNA sample were added to an eppendorf tube. Once all the reagents were mixed, the reaction tube was placed in the PCR machine. The amplification was carried out according to the following programme: A cycle of initial denaturation at 95°C for 5min, 5 cycles of 94°C for 1min, 55°C for 1min and 72°C for 3min followed by 35 cycles of 94°C for 1 minute, 55°C for 1min and 72°C for 3min with final extension at 72°C for 7 minutes. Ten microliters (10µl) of PCR products and 2µl loading dye were mixed and loaded into the agarose gel wells being electrophoresed on a gel (1.5%) which was then analyzed by UV transilluminator and digitally photographed. Detailed Procedure and materials required is provided (Annex -7).

Multiplex PCR

Principle

Molecular-based testing for multiple pathogens in a large number of animals is expensive. The development of new variations of PCR have allowed for simultaneously performing multiple tests on a single diagnostic sample, thereby affording some savings on a per-test basis. Multiplex PCR is a reaction in which several target sequences are co-amplified, each with its own different primer pair, so that significant amplification of all targets is achieved simultaneously. In multiplex PCR, multiple primer pairs are used to amplify more than one gene or pathogen in a single PCR reaction. Primers selected must be to regions that are uniquely different and characterized by low affinity for primer-to-primer interactions.

Testes for five respiratory viruses

The modification of molecular diagnostic technique that was developed by Osiowy, (1998) to permit the rapid and sensitive detection of the three major groups of respiratory viruses involved in lower respiratory tract infection was applied for this purpose (Corne *et al.*, 1999); this assay permits detection of RSV, PIV1, PIV2, PIV3 and respiratory tract associated AdV, in a single step multiplex RT-PCR. The various virus types are differentiated by their unique amplicon sizes following separation of PCR products on agarose gels. Expected sizes for the five virus type amplicons are as follows: for RSV, 348bp; for PIV3, 234bp; for *Adenovirus*, 215bp; for PIV2, 164bp; and for PIV1, 84bp (Osiowy, 1998).

According to Osiowy (1998) five sets of oligonucleotide primers were designed for RT and amplification of (i) the nucleocapside gene of RS, (ii) the nucleocapside gene of PIV1, (iii) the nucleocapside gene of PIV2, (iv) the nucleocapside gene of PIV3 and the hexon genes of *Adenovirus* types 1 to 7 (sequences available from gene bank). All primer sets were designed to have similar melting temperatures (range, 65 to 70°C) and a higher G/C content to allow a higher annealing temperature to be used during amplification (Osiowy, 1998). The sequences of these primers are given (Table 1).

Multiplex PCR reaction

For multiplex RT-PCR, a reaction tube contained the following in a final volume of 50µl: 45µl Platinum® PCR Supermix containing 22U/ml complexed recombinant Taq DNA polymerase with anti platinum Taq antibody, 22Mm Tris. HCl (PH 8.4), 55 mM KCl, 1.65 mM MgCl₂ and 220µM concentrations (each) of dATP, dCTP, dGTP and dTTP (Invitrogens); 0.2mM concentrations (each) of RSV specific primers (RSVN3 and RSVN5) and PIV1specific primers (PIV1PR3 and PIV1PR5); 0.1 mM concentrations (each) of PIV2, PIV3, and *Adenovirus* specific primers

(PIV2PR3 and PIV2PR5, PIV3PR3 and PIV3PR5 and ADHEX3 and ADHEX5, respectively); and 3µl cDNA (DNA in case of *Adenovirus*) from each suspected virus. RT-PCR was performed in thermal cycler under the following conditions: 94°C for 3 minutes and 35 cycles of 94°C for 40 seconds, 68°C for 30 seconds and 72°C for 45 seconds (with a 5 seconds primer extension added per cycle). A final primer extension step of 5 minutes at 72°C completed the cycle (Osiowy, 1998; Corne *et al.*, 1999).

Table 1: Sequences of oligonucleotide primers used for detection of respiratory viruses in camels

Primer	Gene ^a	Position ^b	Sequence ^c
Primers			
RSVN3	N	426–451	GGGAGAGGTGGCTCCAGAATACAGGC
RSVN5	N	748–773	AGCATCACTTGCCCTGAACCATAGGC
PIV1PR3	NP	64–89	TCTGGCGGAGGAGCAATTATACCTGG
PIV1PR5	NP	122–147	ATCTGCATCATCTGTCACTCGGGC
PIV2PR3	NP	360–385	AACTATGTCCAGAGGAGAGGTGCTGG
PIV2PR5	NP	498–523	CCATGCCTGCATAAGCACACTGTAGC
PIV3PR3	NP	416–441	ACCAGGAACTATGCTGCAGAACGGC
PIV3PR5	NP	624–649	GATCCACTGTGTCAACGCTCAATACC
ADHEX3	Hexon	154–179 ^d	CCTACGCACGATGTGACCACAGACCG
ADHEX5	Hexon	343–368 ^d	GTGTTGTAGGCAGTGCCGGAGTAGGG

Source: (Osiowy, 1998).

^aN, nucleocapsid gene of RSV; NP, nucleocapsid gene of PIV

^bRelative to the translation start site.

^cSequences are shown 5' to 3'.

^dPositions shown are according to the adenovirus type 5 hexon gene sequences

PCR product analysis by gel electrophoresis

The most common and rapid way of analyzing PCR products is by standard agarose gel electrophoresis. Electrophoresis is a migration of charged ions in an electric field separating DNA fragments based on their molecular size by applying electric force across the dish.

In order to prepare an agarose gel, 1.8 gms of agarose powder and 120ml of 1X TAE buffer was mixed and boiled in a microven at 500°C for 4 minutes and cooled to 50°C. In the liquid state, 6µl of ethidium bromide was added and poured onto agar plate to form a semi solid gel of 1.5 % electrophoresis gel. Wells were formed by placing a comb into the poured gel. Ten microliters (10µl) of the amplified PCR product and 2µl of loading dye was mixed on parafilm (plastic paper), loaded into the gel wells formed by the comb. The agar plate containing the loaded gel was placed in the electrophoresis dish filled with 1XTAE buffer. All wells were loaded with the samples except the first well which was loaded with loading dye and a mixture of molecular markers differing by 100bps. The agar plate containing the loaded gel was placed in the electrophoresis dish filled with 1XTAE buffer. The electrophoresis chamber was connected to an electric current and run for one hour. Then the gel was taken and placed on UV transilluminator to detect separated DNA bands, which was then photographed by digital imaging system and transferred to the computer for visualization and documentation. Details for agarose gel preparation and gel reading is provided (Annex-8).

3.6. Data management and analysis

Available data on cell culture results, duration of CPE development and type of CPE observed for each sample were tabulated and finally organized. Some of those cell culture positive camel samples were tested for six respiratory camel viruses using universal and conventional PCR. Molecular characterization results were recorded and tabulated (Table 3).

4. RESULTS

4.1. Postmortem examination and virus infection prevalence

During the study period, 389 apparently healthy camels presented to the abattoir for slaughter were examined antemortally of which 35 of them were found pneumonic upon postmortum examinations. However, it should be underlined that these lesions were exclusive of those lesions caused by parasites, foreign bodies, pyogenic bacteria and others including tuberculoid cases.

Virally suspected pneumonic camel lung tissues were collected mainly based on the nature of lesions observed and the status of lymphnodes (prescapular and mediastinal lymphnodes). The nature of most frequently encountered and collected pathologic lung lesions observed were acutely and moderately inflamed lungs and a few of them included chronically infected, interstitially pneumonic, hemorrhagic, hepatized, atelectatic and adhesive lung lesions. Postmortem examinations were also assisted by palpation and incision. Accordingly, the total prevalence of virus suspected camel pneumonia was determined to be 8.99% (35 pneumonic camel lung tissues out of 389 camel lungs) examined (Table 2). The prevalence of viral infection was determined based on cell culture results and hence the prevalence of viral pneumonia in camels as well as the proportions of CPE exhibiting pneumonic lung tissues is summarized below (Table 2).

Table 2: The prevalence of camel viral pneumonia by cell culture results

Number examined (camels)	Suspected viral infection rate (%)	Viral infection rate by cell culture (%)	Virus culture results with respect to total pneumonic tissues collected (35)	
			CPE positives (%)	CPE negatives (%)
389	35/389(8.99)	27/389(6.94)	27 /35(77.14)	8/35(22.86)

Keys: %: (percentage) and CPE: Cytopathic effect

4.2. Virus cultivation and cytopathic effects

Of the 35 pneumonic camel lung tissue samples collected, processed and cultured, 27 samples exhibited morphologic alterations (CPEs) on vero cell monolayer. The presence of viral agents in the pneumonic samples collected were evidenced by initial swelling and rounding of infected vero cells, sloughing of infected cells from cell culture flasks. The most predominant and frequently observed type of CPEs was the aggregation of infected cells together forming networked mesh (syncytia). Digital photography of some key representatives is provided in the following consecutive figures (2 and 3). Morphologic alterations of all tissue samples exhibiting CPE were compared against controls (controls were those cell cultures with no sample inoculation).

The CPEs observed yet were not characterized by single type of CPE rather the majority of CPEs observed were mixed type of CPEs (elongating, rounding, singles and pairing, syncytia, sloughing and floating). However, there were variations among the CPEs observed in the degree of expression of the above type of CPEs. As a result, some of the representative CPE types observed were accordingly described below (Figures 2, 3 and 4) and in (Annex-10).

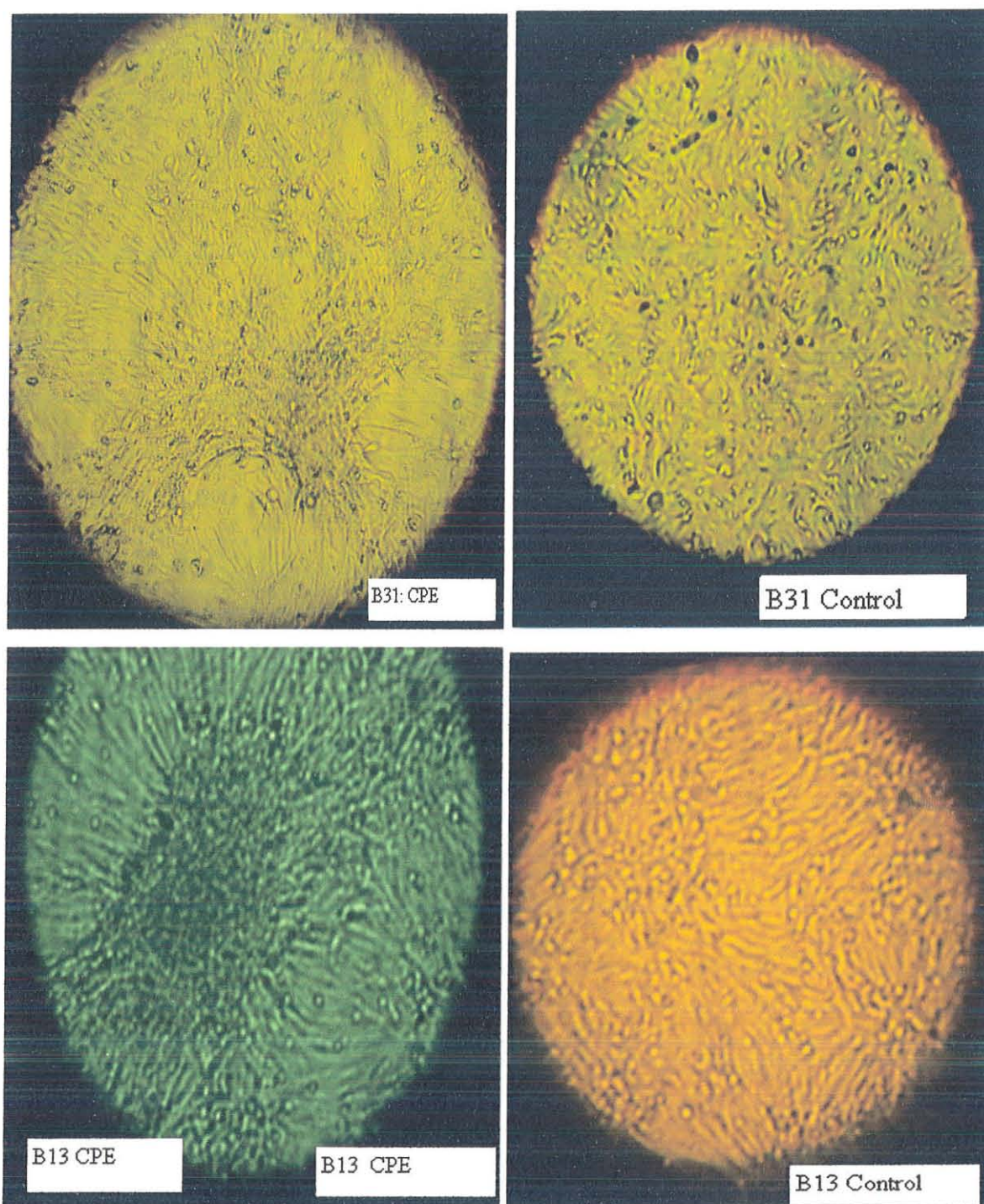


Fig.2: Infected vero cells showing morphological changes, cytopathic effects (CPEs), (top and bottom left corners) and control vero cells (top and bottom right corners), respectively.

The cytopathic effect shown on figure 2 (top left corner-B31) is characterized by extreme elongation (compared to the next one, B31) and moderate syncytia formation with small cells rounded while figure 2 (left bottom corner-B13) is characterized by extremely heavy syncytium formation. In addition, B13 also exhibited moderate elongation and few of them swollen, rounded and scattered singly and pairly. Figures indicated on the left parts are negative controls in these consecutive pictures including the above ones.

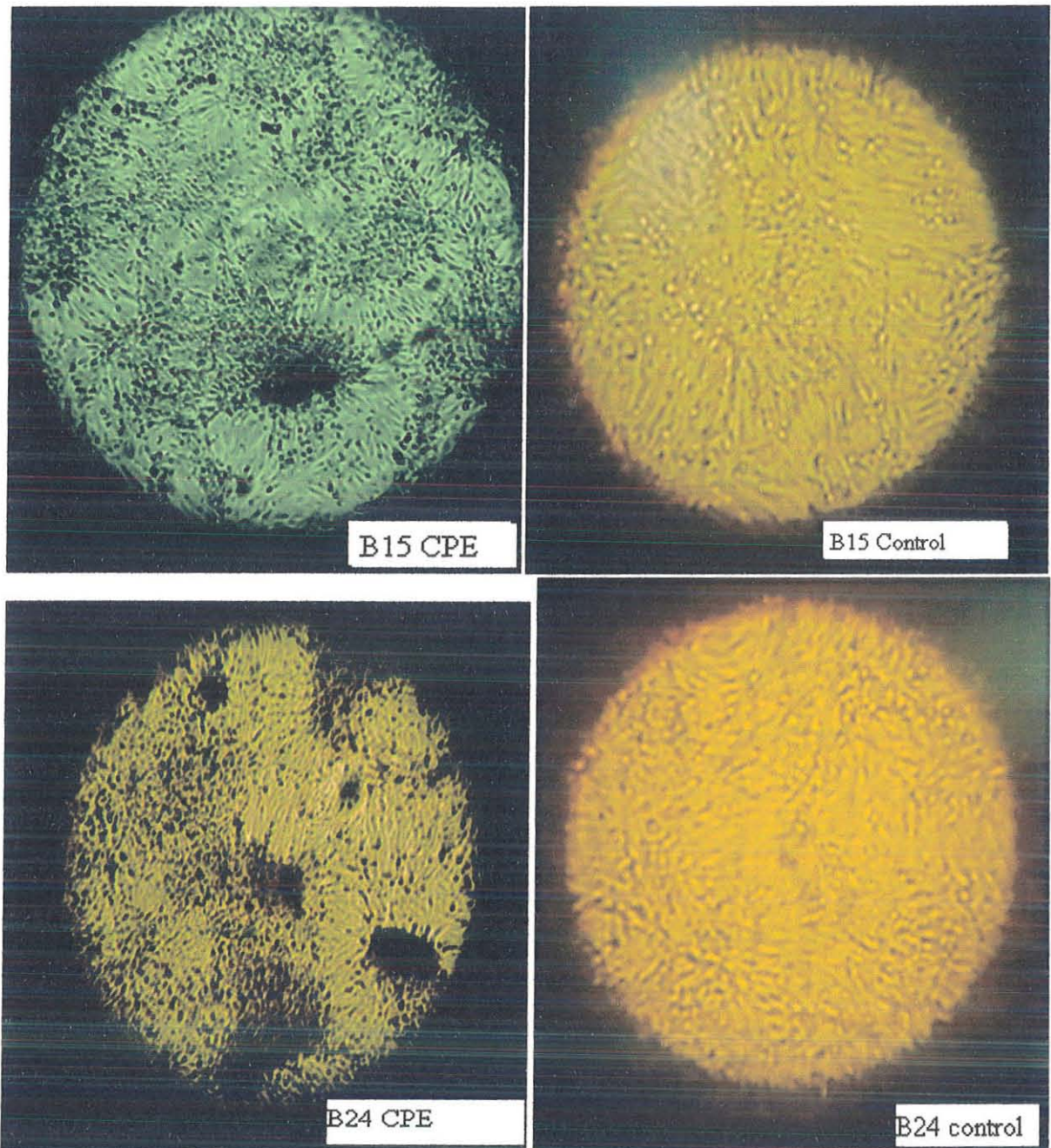


Fig.3: Infected vero cells exhibiting morphologic changes, cytopathic effects (CPEs), (top and bottom left corners) with respective controls (top and bottom right corners), respectively.

The cytopathic effects observed on figure 3 (top left corner- B15 and left bottom corner- B24) are both closely related to each other since they are characterized by sloughing of infected vero cells (clear areas among darkened aggregated cell groups (syncytia)), but the typical CPE observed in both of them is syncytial type of CPE. Elongation is also exhibited and few cells are also singly and pairly scattered. But, elongation is not pronounced as the previous ones (Fig.2 (B31) and Fig.2 (B13)).

Below are the representative photographs of second viral subcultures undertaken and most of them revealed identical morphologic changes characterized typically by pronounced swelling and rounding of infected vero cells. The left top and bottomed two photos (Figure 4) are characterized by floating of few infected vero cells, swollen singled and paired cells and less heavily aggregated as indicated below on figure 4. However, it can be seen that in both photos (Fig.4; B25 and B24), elongation is totally absent.

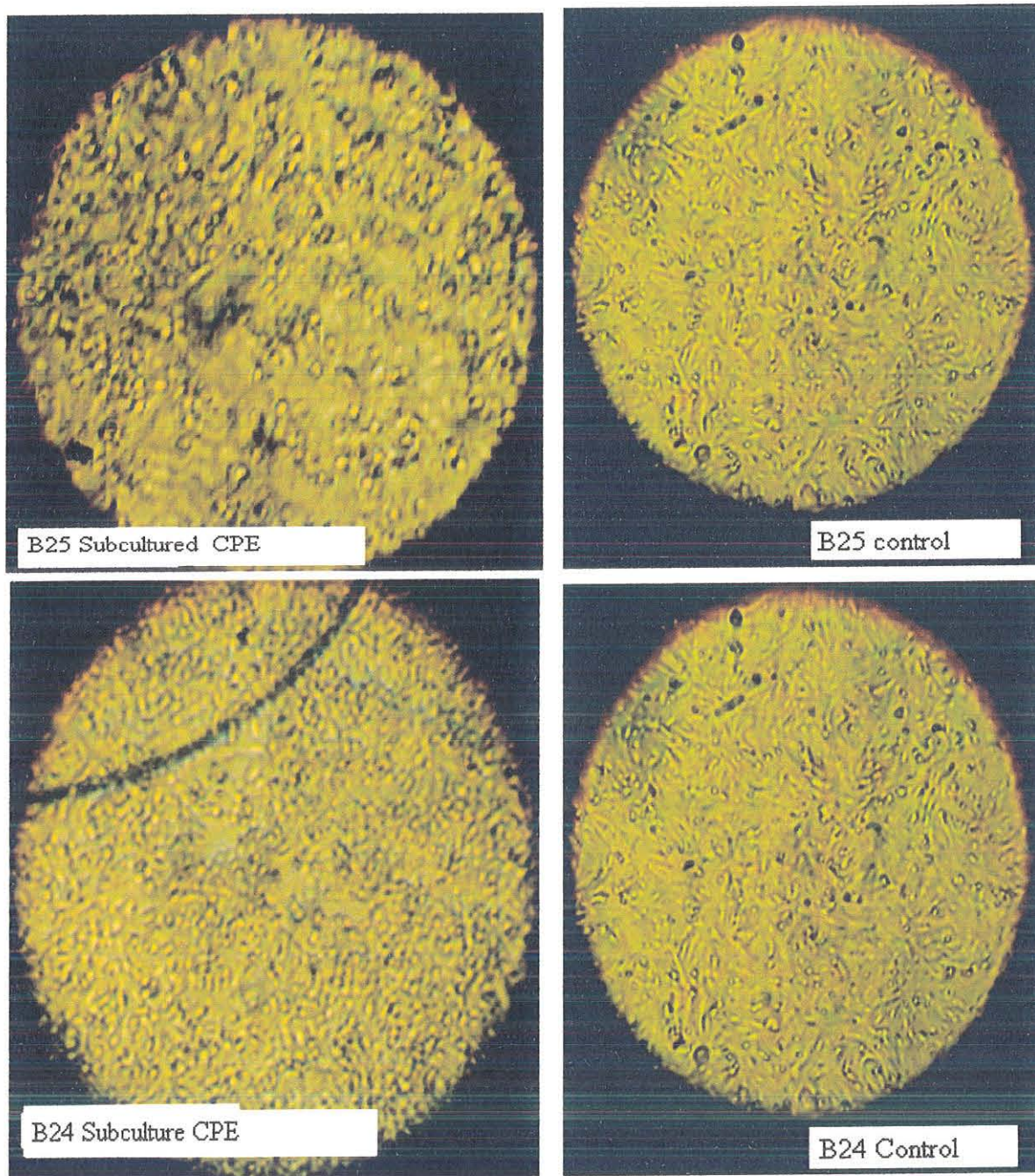
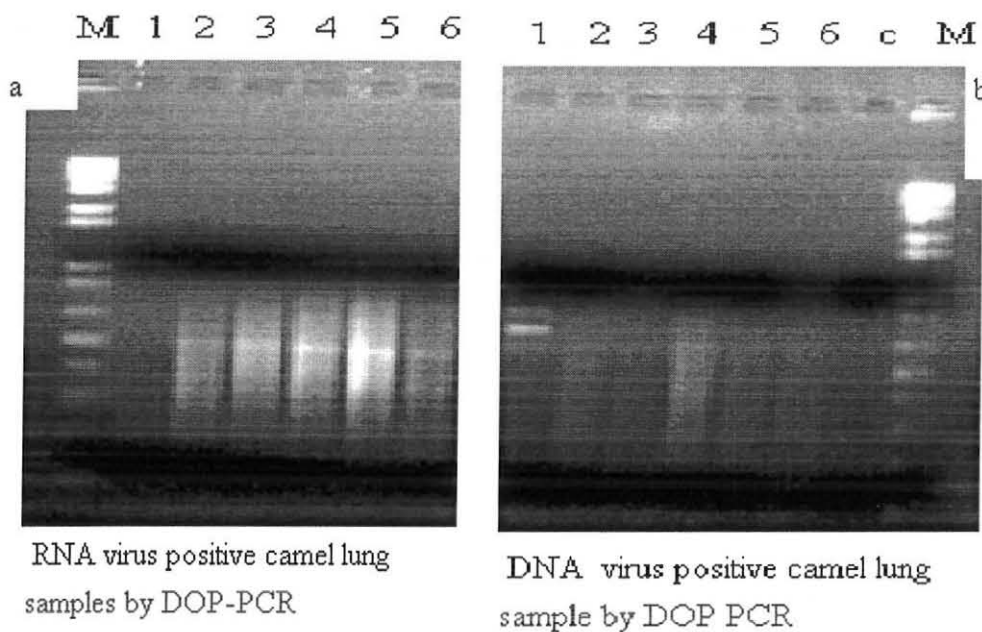


Fig.4: Infected vero cells exhibiting morphologic changes, cytopathic effects (CPEs), (top and bottom left corners) with respective controls (top and bottom rights), respectively.

4.3. Molecular characterization methods

4.3.1. PCR detection of DNA and RNA respiratory camel viruses by DOP-PCR

DOP-PCR master mix for six samples was prepared and 5 μ l of each viral DNA (DNA extracted) was mixed and added into the PCR reaction tube. The same procedure (master mix) was performed using the same samples RNA extracted to detect viral RNA using 5 μ l cDNA samples. Ten microliters (10 μ l) of each DOP- PCR amplified products were mixed with 2 μ l of loading dye and the mixtures were loaded into agarose gel containing ethidium bromide. The samples were electrophoresed and the gel was analysed by UV transilluminator and revealed the results depicted below (Fig.5).



Keys: M= Molecular weight Markers and C= Control (Negative). The molecular sizes of the markers ranged from 100 bps (bottom most) to 1000 bps (top most).

Fig.5a and b. Digital photography of Ethidium bromide stained agarose gel electrophoresed DOP- PCR amplified DNA and RNA products analyzed by UV illumination.

As indicated on figure 5a, sample number one or lane 1 was negative for RNA virus but positive for DNA virus (Fig.5b lane 1) as amplified by universal primer DOP; the rest five samples (Fig.5a lanes 2-6) indicated that those five camels were infected with unspecified RNA viruses. On the other hand, samples 1 and 4 (Fig.5b) indicated that the samples contained DNA viruses of unspecified species implying that the animals from which these samples taken were infected by unknown DNA viruses. Lane 4 (on Figures, 5a and b) revealed that the sample was positive on both sides (DNA and RNA extracted samples) implying that the sampled animal was infected by both DNA and RNA viruses. Besides, lanes 2, 3, 5 and 6 (Fig.5b) were negative for DNA viruses which resembled negative control (lane 7).

4.3.2. Conventional PCR

PCR detection of *Respiratory syncytial virus* (RSV)

Five microliters (5 μ l) viral cDNA of each sample was mixed with master mix of the six samples prepared and amplified using RSV specific primers. Ten microliters (10 μ l) of the PCR amplified products were mixed with the loading dye and added into ethidium bromide stained agarose gel wells followed by electrophoresis. The gel was analyzed by UV transilluminator and photographed (Fig.6).

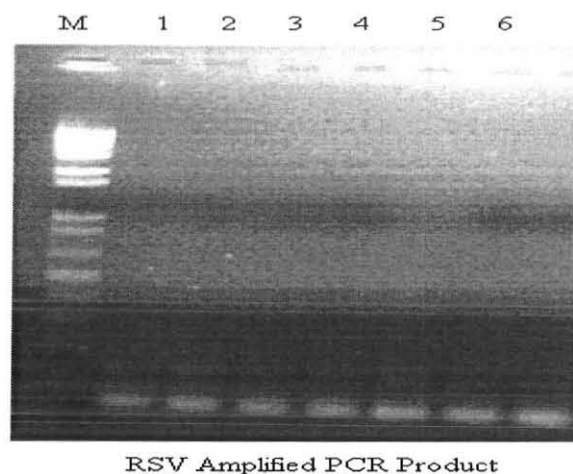
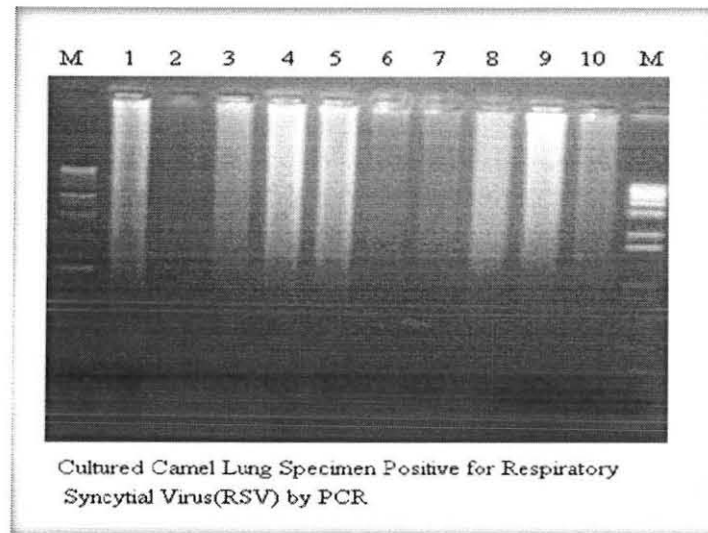


Fig.6: Digital photography of ethidium bromide stained agarose gel electrophoresed PCR amplified products of six *Respiratory syncytial virus* (RSV) samples analyzed by UV.

The above (Fig.6) showed that all samples are positive for *Respiratory syncytial virus* indicated by forming smears and dimmy bands at the bottom just above the visible bands formed by primer dimmers particularly at lanes 2, 5 and 6 or other very closely related virus amplified by RSV specific primer. To make bands visible, optimization was done with indicated results below (Fig.7). Technically, the procedure followed was same except that the template used was the amplified PCR products taken from previous slightly PCR positive samples for RSV. Master mix of these samples was PCR amplified and Ethidium bromide stained electrophoresed agarose gel was analyzed by UV whose output is depicted below (Fig.7).



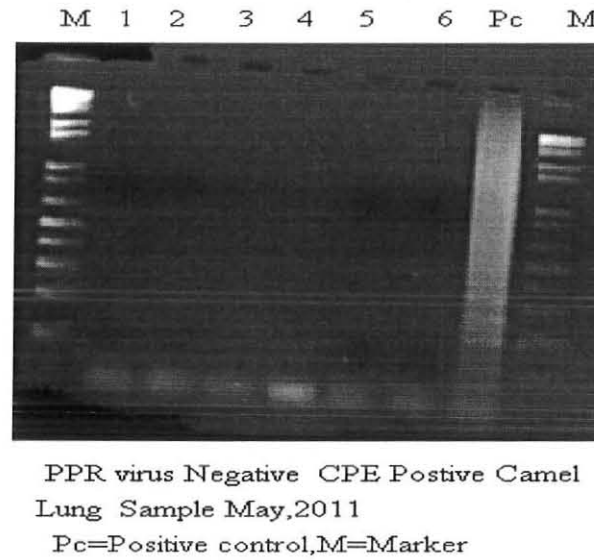
Key: M=Molecular weight marker

Fig.7. Digital photography of Eth.Br stained agarose gel electrophoresed PCR amplified RSV products of ten samples analyzed by UV.

It can be observed from the above digitally photographed agarose gel photo showing that all samples except sample two or lane 2 (lanes 1, 3-10) are positive for *Respiratory syncytial virus* in camels which were sourced from pneumonic camel lungs. But, all samples need further optimization to find discrete bands using more specific primers designed based on the genomic sequences of camel specific *Respiratory syncytial virus* (RSV).

PCR detection of PPRV

Master mix of six samples was prepared. Forty five microliters (45 μ l) of the master mix was transferred to each PCR tube; 5 μ l of each viral cDNA was added to each PCR tube and amplified using PPRV specific primer. Ten microliters (10 μ l) of PCR amplified products were mixed with loading dye and added into Ethidium bromide stained agarose gel wells followed by electrophoresing. UV analyzed gel provided the following results.



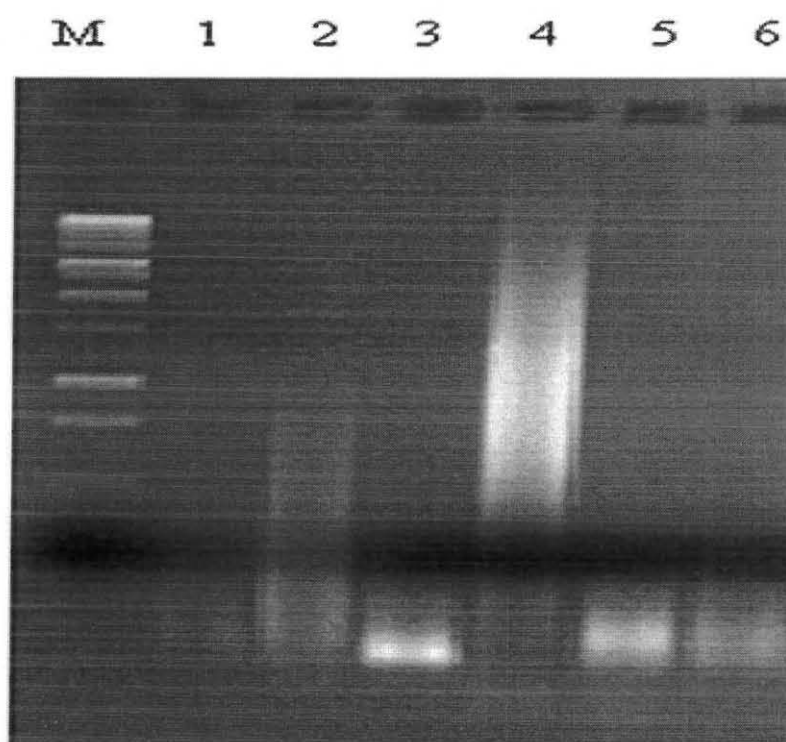
Keys: Pc= Positive control and M= Molecular weight marker

Fig.8: Digital photography of electrophoresed agarose gel analyzed by UV illumination of six (6) viral RNA samples using PPRV specific primer amplified PCR products.

All the six different cDNA samples (lanes 1-6) were not amplified by PCR using PPRV specific primer of N (nucleocapsid) gene of PPRV indicating that these samples and hence the animals from which the samples collected were negative of PPRV infection.

PCR detection of *Adenovirus*

The total amount of PCR mix for amplification including the 5 μ l template was 50 μ l. A master mix of 45 μ l was prepared. But, the template used was not cDNA product, instead 5 μ l of the PCR amplified DNA products were used as template and PCR amplification was done using *Adenovirus* specific primer. Amplified PCR products were electrophoresed. The EthBr stained gel was analyzed by UV transilluminator and revealed the following results (Fig.9).



Cytopathic effect positive camel lung sample and PCR :
positive for *Adenovirus*

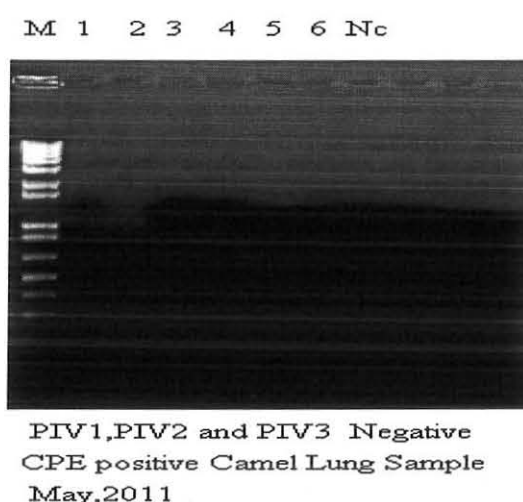
Key: M= molecular weight marker

Fig.9: Digital photography of ethidium bromide stained electrophoresed gel analyzed by UV illumination of six (6) PCR amplified DNA products using *Adenovirus* specific primer.

The above figure evidenced the possible role of *Adenoviruses* as the causative agent of camel respiratory disease since the samples were collected from pneumonic camel lungs forming smears (lanes 2 and 4), implying the positivity of the samples for *Adenovirus* but requiring optimization using primers designed based on camel specific adenovirus gene/genome/sequences.

PCR detection of *parainfluenza viruses*

A 45µl master mix was prepared for each six samples containing specific primers of PIV1, PIV2 and PIV3. To each six master, 5µl of the six different RNA extracted cDNA samples were added and mixed in a PCR tube. The PCR tube was then placed in the PCR machine and programmed accordingly to run. Ten microliters (10µl) of each PCR amplified products were mixed with the loading dye and added into ethidium bromide stained agarose gel wells followed by electrophoresing. The gel was analyzed by UV illumination revealing the following results.



Keys: M= molecular marker, Nc = Negative control

Fig.10. Digital photography of ethidium bromide stained agarose gel electrophoresed PCR amplified products of six (6) viral cDNA samples using PIV1, 2 and 3 specific primers analyzed by UV illumination.

Agarose gel analysis indicated that no amplified PCR products were available. This is an implication of no infection of those sampled animals with these viruses suspected though these same samples provided precise bands (Fig.5) except one sample by DOP- PCR indicating the presence of other respiratory RNA viruses capable of inducing camel pneumonia.

Table 3: Summary of conventional PCR results

No. of samples tested	Virus species tested	PCR Results	
		Positives	Negatives
6	Adenovirus	2	4
6	Peste des petits ruminants virus(PPRV)	-	6
10	Respiratory syncytial virus (RSV)	9	1
6	Parainfluenza virus 3 (PIV3)	-	6
6	Parainfluenza virus 2 (PIV2)	-	6
6	Parainfluenza virus1(PIV1)	-	6

5. DISCUSSION

Respiratory tract diseases are among the emerging health hazards to camels worldwide that are incurring considerable loss of life and production (Abubakar *et al.*, 2010; Kebede and Gelaye, 2010). Respiratory infection is considered to be one of the main factors that decrease animal production. Camels are known to be less susceptible to various diseases affecting ruminants than cattle and sheep; however, evidence for a significant role of respiratory infections in camels had been reported. Dioli and Stimmelmery (1992) reported that viruses that have been associated with respiratory infections in camels are *Parainfluenza virus 3* (PIV3), *Influenza viruses A and B*, *Adenovirus*, *Respiratory syncytial virus* (RSV) and *Bovine herpes virus-1* (BHV1). Various lower respiratory tract diseases have been reported in camels, but the definitive etiology of most respiratory diseases is not determined. With the camel respiratory disease outbreaks, the whole camel population of Ethiopia had been affected and the causative agent is not yet clearly identified which may or may not be associated with these viruses isolated and characterized in other camel rearing countries.

A serological study conducted by Olaleye *et al.* (1989) revealed the following viral agents with their respective prevalences from camel sera in Nigeria; *Parainfluenza virus-1* (22.3%), *Parafluenza virus 3* (18.5%), was followed in decreasing order by Influenza B (12.7%), *Parafluenza virus 2* (2.5%), *Adenovirus* (1.3%), Influenza A (0.6%) and RSV (0.6%).

The present study had also determined the overall prevalence of viral pulmonary infection in Ethiopian camelids. The prevalence of virally suspected pneumonia was determined to be 8.99 % (35 camels suspected virally pneumonic of 389 camels examined physically). On the basis of cell culture results from pneumonic tissue samples collected from camels slaughtered at the abattoir, the overall prevalence by cell culture was calculated to be 6.94% (27 CPE positives of 389).

The duration of CPE development for all samples ranged from 4-14 days. Few CPEs were characterized by larger swelling and rounding up, detaching and floating of singled, paired and

clumped infected vero cells suspected of *Adenoviruses* (Fig 4: Top and bottom left corners). The majority of CPEs were characterized by initial rounding, elongation, syncytium formation, general characteristics of *Respiratory syncytial virus*, *Parainfluenza viruses* and *Peste des petits ruminants virus* (PPRV). Dark heavy syncytia formation was also observed which are characteristic for *Respiratory syncytial virus*, *Parainfluenza 2 virus* and *Pest des petitis ruminants virus* (Fig.3: Top and bottom left corners, Fig.2: Left bottom corner-B13). On the other hand, fig.2 (B31), top left corner showed extreme elongation (long process like cells) and some clumping and rounding of infected vero cells more characteristic for *Parainfluenza 3 virus*.

Adenovirus is known to cause various diseases in different animal species as well as humans. In cattle, about 10 serotypes are identified, all causing asymptomatic disease or mild upper respiratory illness (Murphy *et al.*, 1999).

The present study was aimed to investigate the occurrence of *Adenoviruses* and other respiratory viruses in Ethiopian dromedary camels where respiratory camel virus information is unavailable. Accordingly, a cross sectional study was designed and pneumonic camel lung tissues were collected and cultured on confluent vero cells. *Adenovirus* was detected in 6 CPE positive samples.

Accordingly, six DNA extracted CPE positive samples were tested for by DOP-PCR and agarose gel analysis of amplified PCR products suggested the presence of DNA viruses in 2 samples (Fig.5b, lanes 1 and 4). These DOP-PCR detected six samples were tested for *Adenovirus* using *Adenovirus* specific primers providing less visible PCR output. The amplified PCR products of six previous *Adenovirus* tested samples were used as template and tested again for *Adenovirus* using *Adenovirus* specific primers and 2 out of six gave visible positive PCR results forming smears (Fig.9, lanes 2 and 4) implying the role of *Adenoviruses* in camel respiratory disease since the samples were collected from pneumonic camel lungs. The finding of the present study is the first report of *Adenovirus* in Ethiopian camelids and evidences of this virus in camels reported in other countries by different authors is indicated bellow.

Serologically, few works on *Adenoviruses* in camels were reported. With regard to serological evidence of *Adenovirus* type 3 infection in *camelidae*, Olaleye *et al.* (1989) reported the detection of *Adenovirus* antibodies in 1.3% of camel sera in Nigeria and Hadia *et al.* (2001) detected adenovirus type 3 antibodies in 35.8% of 120 camel sera collected from slaughter houses in Egypt. In Sudan, *Adenovirus* antigen was detected in pneumonic camel lung tissue specimens collected from lungs that showed pneumonia using ELISA. This was the first report of *Adenovirus* antigen detection in camels in Sudan and probably elsewhere which was supported by the previous reports on the association of *Adenovirus* with pneumonia in *camelidae* by Galbreath *et al.*, (1994). Similarly, a serological study conducted by Intisar *et al.* (2010c) reported a prevalence of 90% using indirect enzyme linked immunosorbant assay (ELISA) test confirmed by FAT technique on sera collected from abattoir houses in Sudan. The PCR based *Adenovirus* finding of this present study agrees with another consolidative serologic finding of Dioli and Stimmelmery (1992) for *Adenovirus* antibodies in camels also agreed with the present report of *Adenovirus* from the lungs of Ethiopian pneumonic Dromedary camels. A serologic investigation conducted by Eisa (1973) reported the presence of adenovirus antigen in camel lung tissue specimens using ELISA.

Pest des petitis ruminants (PPR) is an acute and highly contagious viral disease of small ruminants, which is characterized by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro intestinal tract leading to severe diarrhea (Gibbs *et al.*, 1979). Of the four distinct lineages of PPR-virus, lineage III is found in Eastern Africa (Ethiopia).

In Ethiopia, goats react more severely to PPR virus exposure compared to sheep and they exhibit striking clinical signs while sheep undergo a mild form of the disease (Taylor, 1984) and cattle have a sub-clinical infection (Anderson and McKay, 1994). PPR antibodies were first detected and reported by Abraham *et al.* (2005) in Ethiopian cattle and camels. Though many serological and molecular investigations made at federal and regional laboratories following repeated episodes of the unknown camel disease and PPRV outbreaks, the exact etiologic agent is not identified yet (Asegedech, 2009). In Ethiopia, though PPRV was confirmed serologically by

Roger *et al.* (2001) to affect camelids, which caused enormous mortalities and morbidities on camels, the virus is not yet isolated and molecularly characterized in Ethiopia.

This study was thus designed to investigate respiratory camel viruses among which PPRV is an economically important disease of camels and small ruminants. Six CPE positive camel lung specimens were RNA extracted producing cDNAs. Agarose gel electrophoresed PPRV specific primer amplified PCR products were analyzed by UV transillumination providing negative results. Suggestive reasons include; it may be as a result of smaller samples investigated. It may also be due to the failure of the primer used to amplify due to frequent virus evolution (strain variation). Another suggestive reason is that the PPRV isolated in Sudan by Khalafalla *et al.* (2010), used MDBK cells for virus cultivation, but in this study vero cells were used for virus culture. But, the present finding agrees with an investigation made by Dejene (2010) from outbreak samples.

The camel was not reported as a possible host to PPR until Ismail *et al.* (1992) in Egypt, detected the infection through serology in Sudanese camels. In general, the clinical feature of PPR in camels is not different from those reported in sheep and goats (Abubakar *et al.*, 2009; OIE, 2009). Then serological surveys have indicated the susceptibility of camels to PPRV (Haroun *et al.*, 2002; Abraham *et al.*, 2005; Abubakar *et al.*, 2008; Albayrak and Gür, 2010) although clinical signs have not been observed.

Some years before, in June–July 1992, a contagious acute disease characterized by pneumonia (named camel contagious pneumonia), lacrimation and difficulty in respiration caused a mortality of about 5% affected camels in Kassala, Sudan (Khalafalla, unpublished). The cause of that disease was not determined but later a similar disease was reported in Ethiopia in 1995 and laboratory investigations confirmed the involvement of a PPRV (Roger *et al.*, 2000). These authors revealed that the new acute febrile epizootic disease in camel in Ethiopia during 1995–1996 was characterized by highly contagious respiratory syndrome with elevated morbidity and low mortality rates. This was the first documented outbreak of PPR on camels by Roger *et al.* (2000, 2001). Similarly, in Sudan, a study conducted on camel PPRV by Khalafalla *et al.* (2010)

indicated positive result using PPRV specific primer from camel lung tissue samples cultured on MDBK cells.

Respiratory syncytial virus is one of the known causes of respiratory infection in human and various animal species (Murphy *et al.*, 1999). In Ethiopia, no information regarding *Respiratory syncytial virus* is available to explore the role of RSV in causation of respiratory infections in animals specially camels. This study was also concerned with the investigation of camel *Respiratory syncytial virus* and accordingly, 21 CPE positive samples characterized by initial rounding of infected vero cells, elongation and aggregation (syncytia formation) were tested for RSV. Among PCR positive samples, 10 of them were selected for further optimization. Amplified PCR products of these samples were used as templates for optimization. Nine of them revealed better PCR positive results though bands were smeared (Figure 7).

Respiratory syncytial virus (RSV) is one of the main viruses associated with respiratory infections in various animal species (Ganaba *et al.*, 1995; E-Hakim, 2003). Antibodies to RSV were demonstrated in different animal species (Yesilbag and Güngör, 2008). Antibodies to RSV were detected in 0.6% of apparently healthy camels in Nigeria (Olaleye *et al.*, 1989). In Egypt, camel sera tested for RSV were found positive and indicated the prevalence rate of 9.8 % (Shaker, 2003). Furthermore, Instar *et al.* (2010b) isolated RSV from the lung specimens of Sudan camels by cell culture, RT-PCR and serological methods.

Based on RSV characteristic CPEs observed during virus cultivation and PCR positive indications, this is the first attempt to isolate RSV from pneumonic Ethiopian camels though it needs further definitive molecular tests like sequencing left for confirmation. This finding agrees with the findings of Intisar *et al.* (2010b) in Sudan. The established finding of this study also agreed with other serologic findings of Roger *et al.* (2001) and Dioli and Stimmelmarty (1992).

Parainfluenza virus 3 is one of the viruses known to cause respiratory infection. In Ethiopia, information on camel respiratory viruses is very scarce compared to parasitic and bacterial pathogens in general and PIV3 in particular. According to Kebede and Gelaye (2010),

Parainfluenza-3 was found as primary responsible agent of the camel respiratory disease outbreak in Ethiopia. *Parainfluenza virus 3* was found as primary responsible agent of the camel respiratory disease outbreak as indicated serologically by Kebede and Gelaye (2010) from Ethiopian camels. Serologic based PIV3 detection in camel sera is available in other countries. The detection and isolation of PIV3 using hemagglutination inhibition (HI) from imported Djiboutian camels in Egypt were reported for the first time by Nawal *et al.* (2003). Similarly, Shaker (2003) reported the isolation of PIV3 from camel lungs in Egypt. Intisar *et al.*, 2009) isolated PIV3 from camel lung specimens in MDBK cell cultures observing the typical CPE of PIV3: rounded retractile cells, cell elongation and sloughing with some syncytia formation, which was similar to that described by Henrickson (2003). The virus was identified by FAT and ELISA; this was the first report of the isolation of PIV3 from camels in Sudan.

In the present study, six CPE positive samples characterized by initial rounding of vero cells, elongation and syncytia formation of infected vero cells were RNA extracted, cDNA synthesized and PCR amplified using PIV3 specific primers. However, no amplified PCR product was detected following electrophoresed gel UV illumination. The absence of a PCR product could be the following: Firstly, this study used vero cell lines for virus culture instead of the commonly used MDBK cells by other researchers which are more susceptible for *parainfluenza viruses*. The other reason may be due to the primer used which was designed based on nucleocapsid gene of human *Parainfluenza virus 3* because camel *Parainfluenza virus* may have slightly different genomic sequences and hence the primer designed for human virus may not amplify *Parainfluenza virus 3* infecting camels though relatedness could have existed. This finding is thus contradictory to the findings for PIV3 in camels conducted by other authors described above, but agrees with the finding of Dejene (2010).

There is no available information on camel *parainfluenza viruses* 1 and 2 in Ethiopia to date. Unfortunately, this study had attempted to investigate these viruses from pneumonic camel lung specimens collected from the abattoir indicated. Six DOP-PCR positive samples were multiplex PCR tested for RNA using specific primers of PPRV, *Adenovirus*, RSV, PIV1, PIV2 and PIV3 and indicated poor amplification except slight positive results for RSV. Multiplex PCR was

followed by detection of individual sample using specific primers of PIV1 and PIV2 viruses with no positive results obtained for each. This absence of PCR product can be suggested as: Primer specificity is suggested as a major factor for failure of amplification since the primers were designed based on conserved nucleocapsid genes of human *parainfluenza viruses* 1 and 2. Furthermore, these primers were not updated once and hence the viruses (*parainfluenza viruses* 1 and 2) might have been evolved leading to the failure of PCR amplification. Evidence for these viruses was reported serologically in Nigeria, consequently, agroecology may influence the occurrence of these viruses in Ethiopia or pneumonic lesions were caused by other viruses providing negative PCR output.

This finding came to contradict the serological findings reported by Olaleye *et al.* (1989) in Nigeria and Schwartz (1992) who reported the common occurrence of *Parainfluenza* 1 virus and *Parainfluenza* 2 virus in camel rearing areas despite the above reasons described, but agrees with the finding of Dejene (2010).

6. CONCLUSION AND RECOMMENDATIONS

Though the causative etiologies of camel respiratory diseases were still remained mysterious, this study had found out that viruses were among the hidden threats to camel health problems based on postmortem observations, cell culture and molecular technique results. Specifically, this study was concerned with the investigation of camel respiratory viruses from abattoir slaughtered pneumonic lungs of camels and partially reported and established the presence of both pathogenic DNA and RNA viruses on the basis of cell culture results further confirmed by conventional and universal PCR technologies. Ten and six CPE positive samples were detected by PCR and nine among ten samples tested were positive for *Respiratory syncytial virus* and two of six samples were positive for *Adenovirus* at PCR level. On the other hand, six CPE positive samples were also PCR tested for other suspected camel respiratory camel viruses including *Peste des petits ruminants virus*, *Parainfluenza 1*, *Parainfluenza 2* and *Parainfluenza 3* viruses though none of them were detected in the six samples. However, all CPE positive samples were still not fully characterized even those PCR positive ones.

Based on the findings of this study, the following recommendations were forwarded

- Continuous and detailed molecular epidemiologic investigations should be conducted to determine the viral species prevailing in Ethiopian dromedary camels.
- Effective prevention and control measures should be designed on the basis of molecular epidemiology.
- Genomic investigations should be conducted to establish the existing relationships among viral respiratory etiologies of human, bovine, camel and other animals.
- Investigation of other none viral infectious microbial pathogens capable of causing camel pneumonia should be continuously undertaken.

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8. ANNEXES

Annex- 1: Tissue processing procedure for virus isolation

Procedure

1. Clean the mouths of media, reagent, and other containers with alcohol in the BSC
2. Weigh approximately 1gm of tissue sample and place on the petridish and wash with PBS to remove any dirty substance including blood
3. Hold the tissue sample washed in step-2 with forceps and place in mortar
4. Mince into pieces with blade and Grind and homogenize the tissue using pestle
5. Add 9ml of PBS to the homogenized tissue
6. Transfer the solution (mixture) into test tube(s) and centrifuge at 3500RPM for 10min
7. Take the supernatant into new test tube and discard the sediment
8. Filter using filter membrane to remove any bacterial contaminants and others so that only viral particles pass through the membrane
9. Label cell culture flask containing monolayer
10. Using pipette, take 0.5 ml of viral suspension and inoculate into the flask and incubate at 37°C for 45-60 min to allow adsorption of the virus to the cell monolayer.
11. Then take after incubation, wash the flask with PBS to remove unabsorbed viral particles and other tissue debris since unabsorbed virus or host cell debris inhibit the growth of the monolayer cells.
12. Finally, add 10ml of maintenance medium (G-MEM) into the flask and incubate at 37°C and check daily for CPE development.

Annex-2: GMEM media preparation procedure

Procedure

1. Weigh the powdered GMEM, at the rate of 125 gm for 10L fluid media preparation.
2. While stirring the water (should have temperature between 15⁰C and 20⁰C), the powdered media added. Do not heat.
3. Rinse original package with small amount of water to remove all traces of powder.
4. Add 2.75gm of NaHCO₃ or 36.7 ml of NaHCO₃ solution (7.5%W/V) for each liter of final volume of medium being prepared and stir to dissolve.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Sterility test for bacteria and fungus should be conducted on thioglycollate media and SBCDM.
9. Aseptically dispense the medium in to sterile container to store at 2⁰C to 8⁰C in dark.

Annex- 3: Viral RNA extraction protocol

Procedure

1. Add 460µl homogenized sample in to 1.5 ml eppendorf tube.
2. Add the same amount of buffer RLT (β-ME added) to the sample, vortex and wait for 40 sec.
3. Centrifuge lysate for 3min at maximum speed in a microcentrifuge and transfer the supernatant into a new eppendorf tube.
4. Add 460µl of 70% ethanol to the cleared lysate and mix well by overtaking.

5. Transfer up to 700µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2ml collection tube (supplied). Close the lid gently and centrifuge for 15 sec at 8000 x g (10,000 rpm). Discard the flow through. Reuse the collection tube in step 6. If the sample volume exceeds 700µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow through after each centrifugation.
6. Add 700µl buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 sec at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow through. Reuse the collection tube in the next step.
7. Wash with 500µl buffer RPE onto RNeasy column, Close the lid gently and centrifuge for 15 sec at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow through. Reuse the collection tube in the next step.
8. Repeat the wash with 500µl buffer RPE onto RNeasy column, and this time centrifuge for 2min at maximum speed (13400rpm) to dry the RNeasy membrane. Discard flow through and the collection tube.
9. Place the RNeasy spin column in a new 2ml collection tube and Centrifuge at full speed for 1 min. discard the collection tube with filtrate.
10. Transfer RNeasy column into a new 1.5ml eppendorf tube (remove the lid) and pipette 30-50µl of DEPC treated water directly onto the RNeasy membrane. Centrifuge for 1 min at maximum speed to elute.
11. If the expected RNA yield is >30µg, repeat step 10 using another 30–50µl RNase free water, or using elute from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10.
12. Label the RNA with date, virus name and name of the extractor and store it under -20°C for further utilization.

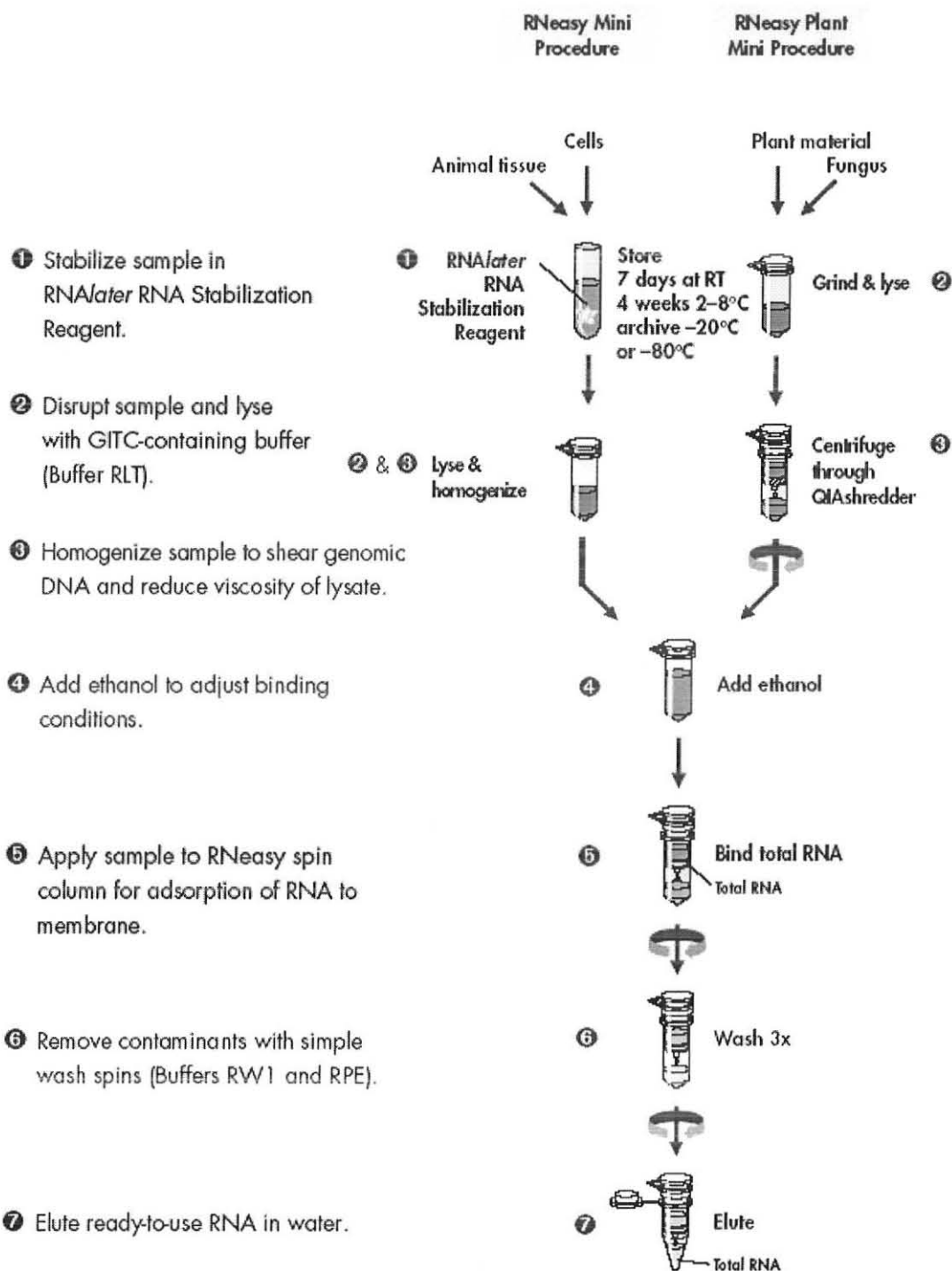


Figure 11: Flowchart of RNA Extraction using RNeasy Mini Kit

Annex- 4: DNA extraction Protocol

Procedure

1. Add 180µl of your sample to the eppendorf tube.
2. Add 180µl of ATL buffer and vortex
3. Add 20µl of protienase K enzyme
4. Incubate at 56°C for 1 and half hour in water bath and vortex
5. Add 200µl AL buffer and vortex
6. Add 200µl 100% Ethyl alcohol
7. Transfer the mixture to DNeasy mini spin column
8. Centrifuge at 8000 rpm for 1min, discard the flow through and change the collection tube.
9. Add 500µl AW1 wash buffer, centrifuge at 8000 rpm for 1min, discard the flow through and change the collection tube
10. Add 500µl AW2 wash buffer, centrifuge at 13,400 rpm for 3min, discard the flow through and change the collection tube by eppendorf tube.
11. Add 100µl elution buffer AE to the sample and centrifuge at 8000 rpm for 1min
12. Store at -20°C or use immediately for PCR

Annex-5: cDNA synthesis (Reverse Transcription)

Procedure

1. Mix and briefly centrifuge each component before use.
2. Combine the following in a 0.2- or 0.5-ml tube:

<u>Component</u>	<u>Amount</u>
DEPC-treated water	3µl
Random hexamer	1µl
10 mM dNTP mix	1µl
RNA extracted sample	5µl

3. Incubate at 65°C for 5 min, then place on ice for at least 1 min.
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

<u>Components</u>	<u>1Rnx</u>
10X RT buffer	2µl
25 mM MgCl ₂	4µl
0.1 M DTT	2µl
RNaseOUT. (40 U/µl)	1µl
M-MMLv/ (200 U/µl)	1µl

5. Add 10µl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.
Random hexamer primed: 10 min at 25°C, followed by 50 min at 50°C
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1µl of RNase H to each tube and incubate for 20 min at 37°C.
8. cDNA synthesis reaction can be stored at -20°C or used for PCR immediately

Annex-6: Conventional PCR master mix preparation and procedure

1. Add the following PCR master mix components in to the PCR tube in indicated order

RNase free water	32.6µl
Dreram Taq buffer	5µl
10mM dNTPs	1µl
25mM MgCl ₂	3µl
Primers	1µl
Dream Taq DNA polymerase	0.4

2. Homogenize the above mixtures by centrifugation and program the thermocycler as follows

Initial denaturation at 94°C for 2minutes followed by 35 cycles of

- Denaturation at 94°C for 40 seconds
- Annealing at 55°C for 30 seconds
- Extension at 72°C for 1min followed by final extension of 72°C for 5 min and take the amplified PCR product and store at +4°C or use immediately.

Annex-7: DOP- PCR master mix preparation and amplification protocol

Procedure

1. Mix and prepare the following DOP PCR master mix

RNase free water	35.1 µl
10 X Dream Taq buffer	5 µl
25mM MgCl ₂	1.5 µl
10mM dNTPs	1 µl
Universal Primer	1 µl
Dream Taq DNA polymerase	0.5µl, mix these and add 5µl of the cDNA sample

2. Program the thermocycler cycles as follows:

Initial denaturation at 95°C for 5 minutes followed by 5 cycles of

- denaturation at 94°C for 1min
- annealing at 55°C for 1min
- extension at 72°C for 3min followed by 35 cycles of
- denaturation at 94°C for 1min
- annealing at 55°C for 1min
- Extension at 72°C for 3min with final elongation at 72°C for 7minutes.

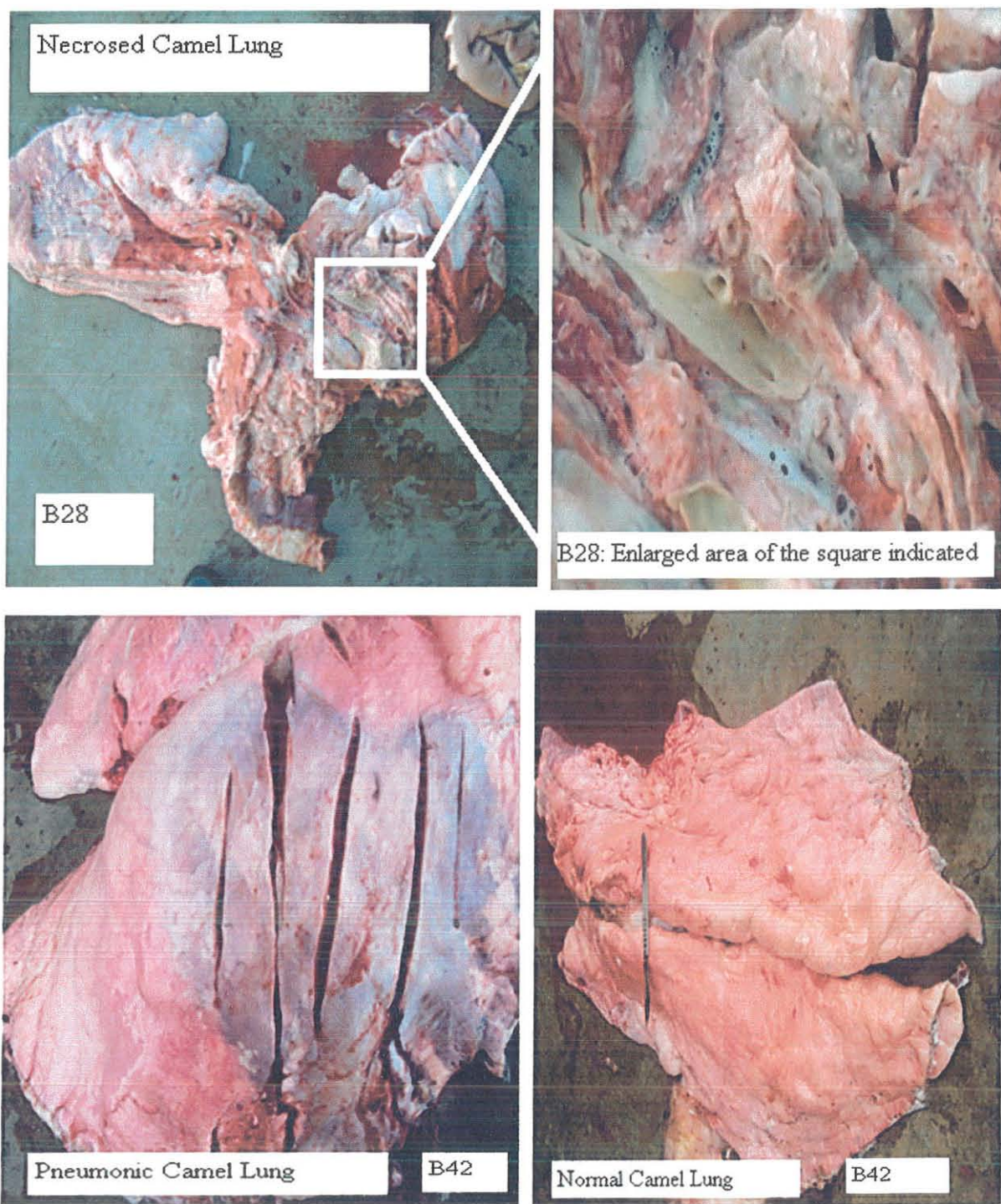
Annex-8: Electrophoresis gel preparation and gel reading

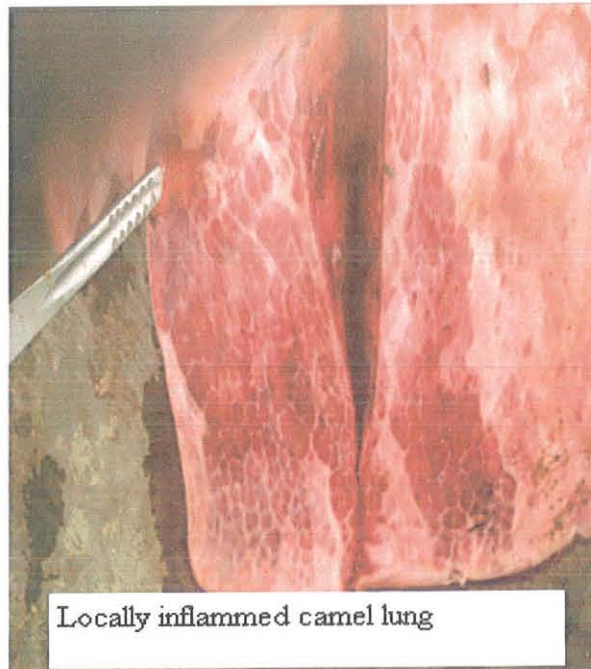
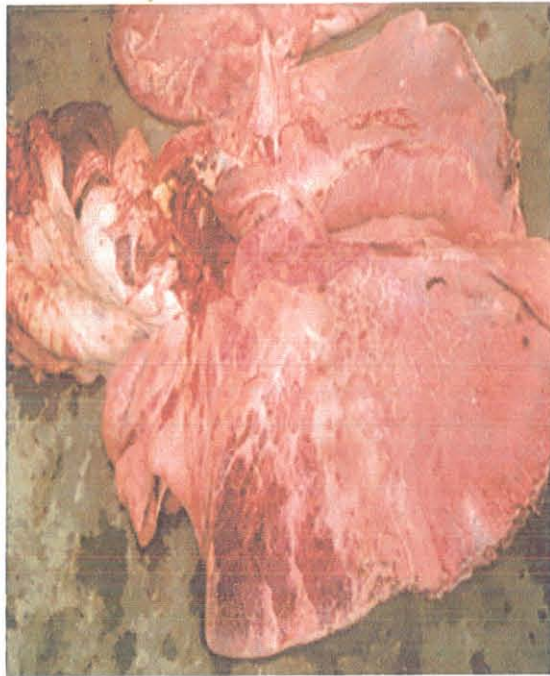
Procedure

1.5% agarose gel preparation and gel reading

- ❖ Weigh 1.8gms agarose powder
- ❖ 120ml 1xTAE buffer was added into a flask
- ❖ Boil at 800°C for 4 minutes, shake and repeat for the same time. Cool it to 50°C.
- ❖ Add 6µl ethidium bromide into the flask containing the liquid agarose.
- ❖ Pour on plate and put the comb on the gel so that it makes holes
- ❖ After drying of agarose, remove the comb
- ❖ Put the gel into electrophoresis dish
- ❖ Take 2µl of loading buffer and 10µl sample and load into electrophoresis gel wells.
- ❖ Switch on electrophoresis device/apparatus/ for 1 hour.
- ❖ Remove the gel and put on UV transilluminator
- ❖ Read the amplified product

Annex-9: Digital photographs of some pneumonic camel lungs sampled

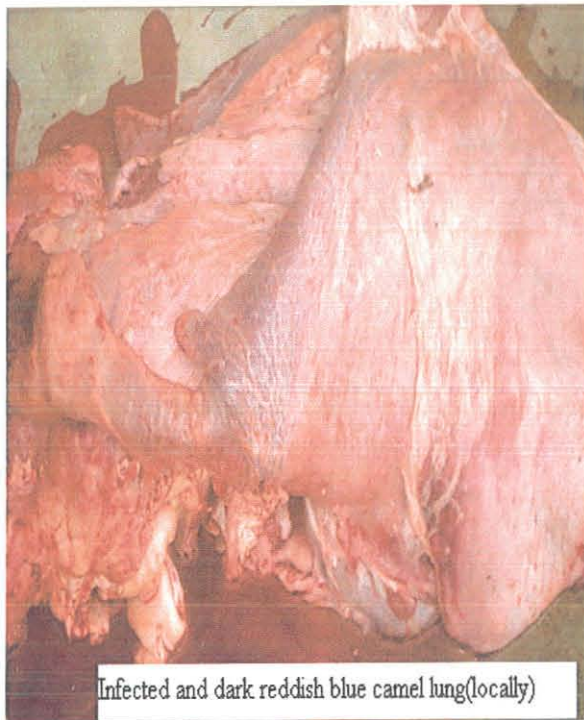




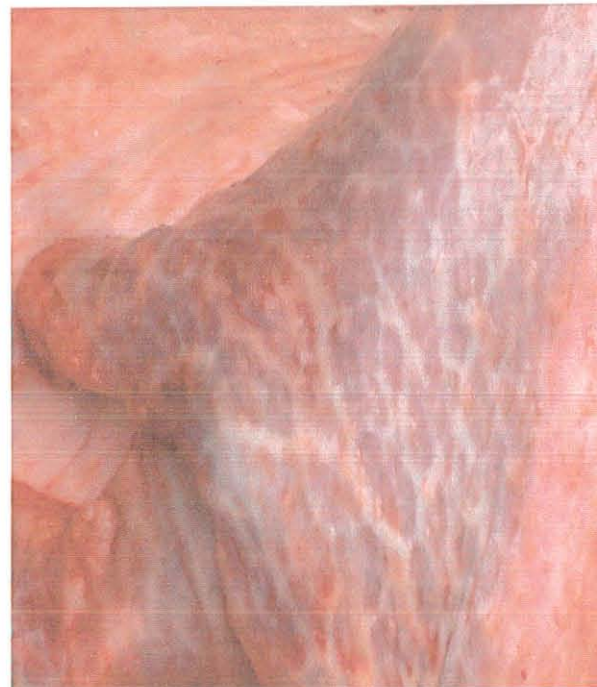
Locally inflammed camel lung



B46: inflammed camel lung



Infected and dark reddish blue camel lung(locally)



Dark reddish blue camel lung



Annex-10: Daily virus cultivation result registration sheet

- ☐ Date sample collected: -----
- ☐ Animal origin: -----
- ☐ Type of sample collected : -----
- ☐ Species of animal sampled: -----

Age: ----- Sex: -----

- ☐ Date of sample processing and inoculation: -----
- ☐ (Laboratory name), National Veterinary Institute(NVI), Debre Zeit, Ethiopia
- ☐ Type of test conducted: -----
- ☐ Cell line used: -----

Date Sample Processed: -----

Tissue processing procedure (general)

About 1gm of each suspected sample is first washed by PBS followed by grinding and homogenization, centrifugation and finally 0.5ml from each supernatant suspension was inoculated on Vero cells and incubated at 37°C.

Follow up of the viral samples inoculated

Date	Sample numbers (codes) and daily observation record		

CPE characterization



Annex-11: Pneumonic camel lung tissue sample collection format for camel virus investigation from Addis Ababa - Akaki Abattoir.

[illegible]

9. SIGNED DECLARATION SHEET

I declare that this thesis is my original work and has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

Name: Wossene Negash

Signature: -----

Date of submission: 26/07/2011

This thesis has been submitted for examination with our approval as academic advisors.

Academic Advisors:

Dr. Tesfaye Sisay (DVM, MSc., PhD, Assist. Prof) -----

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