ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCE

SCHOOL OF VETERINARY MEDICINE

VIROLOGICAL INVESTIGATION OF EMERGING CAMEL DISEASE IN ETHIOPIA

BY

DEJENE MILKESSA TUFA

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By

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Praise the Lord!

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ABBREVIATIONS

AdARD	Adenovirus associated acute respiratory disease
AFLP	Amplified fragment length polymorphisim
AGID	Agar gel immunodiffusion
APHIS	Animal and plant health inspection service
ATCC	American type culture collection
BHK	Baby hamster kidney
cDNA	Complementary deoxyribonucleic acid
CFT	Complement fixation test
CPE	Cytopathic effect
CPT	Cycling probe technology
CSA	Central statistical agency
CVRL	Central veterinary research laboratory-Dubai
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOP	Degenerate oligonucleotide primed
DTT	Dithiothreito
Dubca	Dubai camel
ECACC	European collection of cell culture
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immunoassay
EIs	Emerging infections
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FADDL	Foreign animal disease diagnostic laboratory
FAO	Food and agricultural organization
FCS	Foetal calf serum
FVM	Faculty of veterinary medicine
GMEM	Glasgow minimum essential medium
HAI	Heamagglutination inhibition
IFS	International foundation for science
ILAR	Institute of laboratory animal resource

LCR	Ligase chain reaction
MoARD	Ministry of agriculture and rural development
NAHDIC	National animal health diagnostic and investigation centre
NASBA	Nucleic acid sequence based amplification
NCBI	National center for biotechnology information
NVI	National veterinary institute
OIE	Office internationale des epizooties
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RIA	Radio immunoassay
RNA	Ribonucleic acid
Rpm	Revolution per minute
RT- PCR	Reverse transcriptase polymerase chain reaction
SARS	Severe acute respiratory syndrome
SBCDM	Soya bean casein digested medium
SDA	Strand displacement amplification
TAE	Tris base acetic acid
TMA	Transcription mediated amplification
UAE	United Arab Emirates
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VIDISCA	Virus discovery

ABSTRACT

The dromedary of East Africa particularly, Ethiopia has been suffering from different diseases for the past so many years. Of the many diseases of camels that are rampant in the region, the recently emerged camel disease with still unknown causes is the single most important disease. This camel disease is the leading problem inflicting high morbidity and mortality among the camel population. To this effect, isolation and characterization of the causative agent has of paramount importance. This study was undertaken in the National Veterinary Institute laboratory, Debre Zeit, Ethiopia from October 2009 – June 2010 to isolate and characterize the viral agent from camel postmortem tissues collected during the outbreak of 2007 in Ethiopia. The study was conducted on the basis of cell culture, degenerate oligonucleotide primed polymerase chain reaction (PCR) and laboratory animal assay. Virus isolation was attempted for virus in Vero and Dubca cells; four specimens yielded cytopathic virus when inoculated onto the Vero cell culture. The cytopathic effect (CPE) consisted of round floating cells, giant cells, rounded refractive cells, ballooning of cells, sloughing, and some syncytia formation were observed from day two to day seven post inoculation. The appearance of the CPE was looking uniform among different purified tissue samples used for cell infection. The growth of virus was confirmed by degenerate oligonucleotide primed PCR in which amplification was recorded particularly, where extracted RNA was amplified, both from tissue and cell culture. The growth of the virus after storage in -20 ^{0}C for days was lost while it can grow from +4 ^{0}C and -85 ^{0}C in Vero cells. The cell culture suspension was injected to laboratory guinea pigs in which the animals were observed to develop abnormal clinical signs that resulted in death. Although many scientists suspected different known viruses for the cause of outbreak, the isolated virus was confirmed to be out of adenovirus, morbillivirus, parainfluenza 1-3 and respiratory syncytial virus groups. However, although the above known viruses were excluded, a variant of the above viruses that can skip detection using previously known primers and other emerging viruses might not be excluded.

Key words: Camel, Cell culture, Emerging disease, Molecular diagnosis, Viral etiologies, Virus isolation.

1. INTRODUCTION

There are two species of Old World camels living in Africa and Asia-the Arabian and the Bactrian camels. The Arabian, dromedary camel, has one hump whereas the Bactrian camel has two humps. Camel belongs to the family *Camelidae* and the genus *Camelus*. The camel is an important animal because of its adaptation to adverse climatic conditions and shortage of forage and water. It is also an indicator of social prestige and wealth (Bekele, 1999).

Among the huge livestock resources, camels (*Camelus dromedarius*) are the important livestock species uniquely adapted to hot and arid environments and have been known to provide milk, meat, wool, hair, hides and serve for riding and is used as a draft animal for transport (Bekele, 1999; Schwartz and Dioli, 1992; Kadim *et al.*, 2008). 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; Yagil, 1985; Higgins *et al.*, 1992).

With an increasing human population pressure and declining per capita production of food in Africa, there is an urgent need to develop previously marginalized resources, such as the semi-arid and arid rangelands, and optimize their utilization through appropriate livestock production systems of which the camel is the most suitable one (Schwartz and Dioli, 1992). A well-known feature of camels is their ability to survive and produce in drought prone areas where other animals can hardly survive. And that is why there is a steady increase in the number of camels while the recurring drought is causing huge losses in other livestock species in the horn of Africa (Bekele, 1999).

About 11.5 million camels are living in the eastern part of Africa (Djibouti, Eritrea, Ethiopia, Kenya, Somalia and Sudan) in the arid and semi-arid range lands and this figure represents over 80% of the African and two thirds of the world population (Hartley, 1979; Bekele, 1999). These data also explained the importance of camels for the region and the camel herders as camels do also play a significant sociocultural role in the live of pastoralists (Hartley, 1979).

Ethiopia hosts about 2.3 million heads of camel, which are found in the arid and semi-arid regions of the country (FAO, 1990; CSA, 2007). The importance of camel in this country cannot

be overemphasized as camels serve as a means of survival particularly during drought for better milk production compared to other livestock species (Bekele, 1999).

Although camels seem to suffer from fewer diseases than other domestic animals and epidemics are rare, the major diseases which affect camels include trypanosomiasis, helmenthiasis, mange, and camel pox (Odeh *et al.*, 1999; Bekele, 2002). According to Yigezu *et al.* (1997) the agent of strangles, *Streptococcus equi* subspecies *equi* was also isolated from the camels found in Afar region, Ethiopia. Anthrax and Salmonellosis have public health importance, while the less common diseases include blackquarter, brucellosis, pneumonia, rabies and tetanus. 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; Azwai *et al.,* 1995; Eisa, 1998; Odeh *et al.*, 1999). In fact, the dromedary of East Africa, particularly Ethiopia, Kenya and Somalia, has 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).

Of the many diseases of camels that are rampant in the region, the recently emerged camel disease with still unknown causes is the single most important disease with huge mortality. The 1995 camel disease outbreak of Ethiopia with severe respiratory involvement, which drew attention of the nation and certain international agencies and has almost affected the whole camel population in less than a year, is worth mentioning. By then the disease had a highly contagious nature with high morbidity rate of over 90% and variable rate of mortality (Fekadu *et al.*, 2003; Roger *et al.,* 2001). Diseases are still flaring up in the region though the causative agents involved seemed to vary. However, researchers who made studies on camel respiratory diseases in Ethiopia agree that *Manhaemia haemolytica*, *Pasteurella multocida*, *Parainfluenza* virus, and *Morbilli*-like viruses are the major incriminated agents causing repeated outbreaks in the country (Bekele, 1999; Fekadu *et al.*, 2003; Roger *et al.*, 2001).

Various lower respiratory tract diseases have been reported in camels although the definitive etiology of most respiratory diseases is not determined. A variety of viruses have been associated with outbreaks of respiratory disease among camels. Adenovirus (AdV), IBR viruses, influenza viruses A and B, PIV 3 and RSV were identified so far in respiratory infections of

camels (Dioli and Stimmelmary, 1992; Intisar *et al.*, 2009, 2010a, 2010b). Respiratory infections caused by AdV, PIV or RSV may result in severe lower respiratory tract disease (Corne *et al.*, 1999; Intisar *et al.*, 2010a; Trei *et al.*, 2010).

Within the *Morbillivirus* genus and related viruses, several emerging diseases have been recently described. Accordingly, *Peste des petits ruminants* virus (PPRV) was suspected to be involved in the epizootic disease that affected one humped camels in Ethiopia in 1995-1996 (Roger *et* al., 2001). According to Chauhan *et al.* (2009), although no live virus was isolated, PPRV antigen and PPRV nucleic acid were detected in some pathological samples collected during that outbreak, and that has received a growing attention because of its wide spread, economic impacts (Lefevre and Diallo, 1990) and the role it plays in complication of the ongoing global eradication of rinderpest and epidemiological surveillance programs (Couacy-Hymann *et al.*, 2002).

An outbreak had occurred in Afar and East Shoa Zone in 2005 which caused sudden death of around 530 camels (Getachew *et al.*, 2005). The outbreak was investigated by a team from different laboratories including National Animal Health Diagnostic and Investigation Centre (NAHDIC-Sebata), National Veterinary Institute (NVI-Debre zeit), Central Veterinary Research Laboratory (CVRL-Dubai) and Foreign Animal Disease Diagnostic Laboratory (FADDL-APHIS-USA) came up with different findings which included trypanosomiasis, hydatid cyst disease, tapeworm, trichuris, sarcocyst, haemonchosis, gossypol toxicity, euphoria toxicity, clostridia toxicity and bacterial disease. However, the researchers pointed out that the above listed health problems may not be the primary cause of the problem but could be secondary or may not be associated to the problem. Accordingly, this team highly recommended that detail epidemiological survey and further laboratory investigation to be carried out to come up with the primary causes of the problem. Therefore, identification and characterization of the primary causative agents which is most probably of viral agent/s is highly required (Wernery *et al.*, 2006)

Similar disease occurred in 2007 in Somali regional state, Borena and Guji Zones of Oromia regional state of Ethiopia; the same disease was also observed in Somalia and Kenya in 2007. The disease was characterized by sudden death without any avert clinical signs in any parts of the body systems. As it was reported lactating and pregnant camels were more affected than

other female and male camels. It was also observed that herds with good body condition were more affected than poor ones (Daniel and Solomon, 2007). Very recently, Dawo (2010) also reported that the 2007 outbreak of sudden camel mortality in southern pastoral area of Ethiopia targeted productive age groups like pregnant, lactating and breeding male and female camels. But there was no report of zoonotic consequence of these diseases while the pastoralist used to eat meat and milk of these diseased and apparently healthy camels (Daniel and Solomon, 2007; Dawo, 2010).

The camel disease is still not known clearly and becoming mysterious for the herders, animal health and production professionals and policy makers. The disease seems to occur cyclically and lead many camels to death and threatening the livelihood of the East African pastoral community, in particular that of Ethiopia. It is the leading problem inflicting high mortality and morbidity among the camel population. Because of the unknown nature or etiology of the mortality, it has been difficult to take appropriate prevention and control measures at a national level. Hence, identification of the causes of the disease will have significant contribution to design and implement cost efficient control and/or preventive measures and will contribute to food self-sufficiency of the people inhabiting the affected areas. Therefore, the objective of this research were

- ✓ Isolation of the possible viral causative agents.
- ✓ Characterization of the agent.
- ✓ Determination of the pathogenicity of isolates in the laboratory animals.

2. LITRATURE REVIEW

2. 1. Camel and their viral diseases

2. 1. 1. The Camel

The Camel belongs to the family *Camelidae* and the genus *Camelus*. There are two species of Old World camels living in Africa and Asia-the Arabian and the Bactrian camels. The Arabian, dromedary camel, has one hump whereas the Bactrian camel has two humps. 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 (Yagil, 1985; Schwartz, 1992; Al-Eknah, 2000).

Camels mature late. The female camel usually reaches puberty at 3–4 years of age (Schwartz, 1992). However, females are not bred until they are 5–6 years old. Male camels reach puberty at about 3 years, but full reproductive activity is not developed until they are 6–7 years old. Inadequate body weight caused by lack of sufficient feed and occurrence of diseases appears to be the cause of delayed puberty in the camel thereby reproductive loss results (Al-Eknah, 2000; Tibary *et al.*, 2006).

Camels provide milk, meat, wool, hides, leather, and their dung is used for fires. They are used for riding and transport, they are a means of investment and long-term savings, and they are a source of prestige for their owners. Sales of surplus milk, livestock or livestock products are sources of cash income for pastoral families (Kaufmann, 2005). There is a large market for exportation of live camels, and people uses as an important sport and tourism resource in some of the Arabian Gulf countries. Camels are slaughtered for consumption and during ritual occasions (Wilson, 1984; Yagil, 1985; Higgins *et al.*, 1992).

2. 1. 2. Viral diseases related to camels

In their natural desert habitat, where camels are usually raised particularly during the long dry season, camels are subjected to severe stress conditions which render them susceptible to many diseases and ailments (Abbas *et al.*, 1993; Dirie and Abdurahman, 2003). Although camels were

considered in the past, and for a fairly long time, as resistant to many disease causing factors (Dalling *et al.*, 1988), it has been proved that camels are susceptible the same as other livestock or even more to the common disease causing pathogens affecting other animal species (Wilson *et al.*, 1982; Abbas and Agab, 2002; Dirie and Abdurahman, 2003). Camel diseases that are shared with other species of livestock are comparatively well-known, while other camel-specific diseases are still remaining a mystery to the scientific community (Dirie and Abdurahman, 2003).

A camel seems to suffer from fewer diseases than other domestic animals and epidemics are rare in this animal. However, different diseases of different viral etiologies can be found to affect camels (Table 1). Bluetongue, foot and mouth disease, vesicular stomatitis, rinderpest and rift valley fever could be among Office International des Epizooties (OIE) list A diseases which are known or suspected to infect camels. Among those viral diseases known or suspected to affect camels and listed under OIE list B, although they are many one can mention rabies and equine rhinitis (Brown, 2004).

2. 1. 3. Peste des petits ruminants virus infection in camel

PPRV belongs to a *Morbillivirus* genus in the *Paramyxoviridae* family. There is close antigenic relationship with rinderpest virus (RPV), canine distemper virus, measles virus, dolphin distemper virus, phocine distemper virus, porpoise distemper virus (Murphy *et al.*, 1995; Albayrak and Gur, 2010). PPR-virus isolates can be grouped into four distinct lineages on the basis of partial sequence analysis of the fusion (F) protein genes; lineage III is the lineage found in Eastern Africa (Ethiopia) (Abraham *et al.*, 2005; OIE 2008a).

Within the *Morbillivirus* genus and related viruses, several emerging diseases have been described (Govindarajan *et al.*, 1997; Parker, 1997). In that context, new diseases could result from an interspecies transfer of PPRV or RPV strains from cattle or goats and sheep to camel or through the emerging of a new morbilliviruses, serologically related to PPRV and/or RPV (Roger *et al.*, 2001; Albayrak and Gur, 2010).

Table 1: Viral diseases known or suspected to infect camel.

Disease	Causative agent	Reference
Abortion (equine rhinitis)	Equine rhinitis A virus	Wernery *et al.*, 2008
Bluetongue	Bluetongue virus	Wernery and Kaaden, 2002
Border disease (BD)	BD virus	Klopries *et al.*, 1995
Bovine Viral Diarrhea	BVD virus	Van Amstel and Kennedy, 2010
Camel calf diarrhea	Came type A rotavirus	Ali *et al.*, 2008
Camel Papillomatosis	Papilloma virus	Khalafalla, 1998
Camel pox	Orthopoxvirus (OPV)	Kinne *et al.*, 1998
Contagious ecthyma	Parapoxvirus (PPV)	Khalafalla *et al.*, 2005
Foot and mouth disease	FMD virus	Wernery and Kaaden, 2004
Infectious bovine rhinotracheitis	Bovine herpes virus1	Intisar *et al.*, 2009
PPR	PPR virus	Abraham *et al.*, 2005
Rabies	Rabies virus	Bloch and Diallo, 1995
Respiratory infections	Adenovirus	Intisar *et al.*, 2010b
	Influenza A and B	Intisar *et al.*, 2009
	Parainfluenza virus 3	Intisar *et al.*, 2010a
	Respiratory syncytial virus	Intisar *et al.*, 2010b
Rift Valley fever	RVF virus	Brown, 2004
Rinderpest	RP virus	Roger *et al.*, 2001
Vesicular stomatitis (VS)	VS virus	Brown, 2004

PPRV was suspected to be involved in the epizootic disease that affected one humped camels in Ethiopia in 1995-1996. According to Roger *et al.* (2001) considering the extremely rapid spread of the disease in Ethiopia, and in spite of the fact that the antibiotics had no effect, they presupposed that the disease observed was initiated by a virus. PPRV antigen and PPRV nucleic acid were detected in some pathological samples collected during that outbreak, but no live virus was isolated (Chauhan *et al.*, 2009). The outbreak has received a growing attention because of its wide spread economic impacts (Lefevre and Diallo, 1990) and the role it plays in complication of the ongoing global eradication of rinderpest and epidemiological surveillance programs (Roger *et al.*, 2001; Couacy-Hymann *et al.*, 2002; Abraham *et al.*, 2005; Chauhan, *et al.*, 2009).

2. 1. 4. Respiratory viral infections in camels

Various lower respiratory tract diseases have been reported in camels, but the definitive etiology of most respiratory diseases is not determined. A variety of viral, fungal, bacterial and parasitic microorganisms have been associated with outbreaks of respiratory disease among camels. Viruses encountered in respiratory infections in camels are AdV, influenza viruses A and B, PIV3, RSV and IBR (Dioli and Stimmelmary, 1992; Intisar *et al.*, 2009, 2010a, 2010b). Respiratory infections caused by RSV, PIV or AdV may result in severe lower respiratory tract disease requiring hospitalization (Corne *et al.*, 1999; Intisar *et al.*, 2010a; Trei *et al.*, 2010).

Respiratory syncytial virus (RSV) is one of the main viruses associated with respiratory infections in various animal species (Murphy *et al.*, 1999; E-Hakim, 2003; Intisar *et al.*, 2010b). Respiratory syncytial disease is caused by a virus belongs to genus *Pneumoviruses* of family *Paramyxoviridae*, subfamily *Pneumovirinae*. The virus is enveloped covered with peplomers and contains helically symmetrical nucleocapsid (Baltimore, 1972). It is single-stranded, negative-sense, non-segmented RNA (Andrew *et al.*, 2004). The virus differs from the rest of the *paramyxoviruses* in lacking neuraminidase (Stott and Geraldine, 1985).

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. They are RNA viruses containing a linear, non-segmented, negative-sense RNA strand of about 15,000 nucleotides (Corne *et al.*, 1999; Henrickson, 2003). 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). The PIV have also been implicated in non-respiratory disease, and such infection is a cause of mortality in the immunosupressed with death from disseminated infection (Wendt *et al.*, 1992; Corne *et al.*, 1999).

Adenoviridae has been subdivided into four genera in which genus *Mastadenovirus* and *Atadenovirus* contains viruses infecting mammals. Adenoviruses are medium sized, non enveloped icosahedral viruses with a single linear, double-stranded DNA molecule. They are highly resistant to inactivation and readily spread in closed populations (Zuckerman *et al.*, 2009). Adenovirus (AdV) associated acute respiratory disease (AdARD) epidemics have been

widely reported among human and animals worldwide. Most cases involved only mild, acute, febrile, respiratory illness. However, occasionally, infections with pneumonia and more severe sequelae may happen (Trei *et al.*, 2010).

2. 2. Emergence of viral infections

Generally, emerging infections (EIs) can be defined as "infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range". EIs have shaped the course of human history and have caused incalculable misery and death (Brown, 1997; NCBI, 2004). The reasons underlying the general increase in emerging diseases are multiple and often overlapping (Domingo, 2010). Physical movement to a susceptible population has resulted in epidemic scourges throughout history. The second factor responsible for an increase in emerging diseases is crossing of species boundaries (Trei *et al.*, 2010). The third factor responsible for an increase in emerging diseases in humans is considered to be lifestyle changes. In animal health, the term lifestyle changes could effectively be translated to husbandry changes. The fourth factor inherent in disease emergence is environmental disruption and the alteration of ecosystems, leading to an increase in new diseases (Brown, 1997; Nichol *et al.*, 2000; Domingo, 2010). The role of the laboratory in the creation of emerging diseases with a focus on the importance of genetic variation of viruses should also not be ignored (Hoelzer and Parrish, 2010; Maclachlan and Guthrie, 2010).

Emerging viral diseases are nowadays common both in humans as well as animals. The main reasons could be the relatively benign viruses can be converted in to highly virulent viruses via the introduction of genes of interest. Therefore, we have to expect more virulent or/and pathogenic viruses that we cannot readily detect using our current "state of the art" diagnostics (culture on common cell lines, PCR, microarray chips) (Clem *et al.*, 2007). Viruses with RNA as their genetic material can quickly adapt to and exploit the varying conditions because of the high error rates of the virus enzymes (polymerases) that replicate their genomes. It comes as no surprise, then, that several prominent recent examples of emerging or re-emerging diseases are caused by RNA viruses (Sampath *et al.*, 2005; Hoelzer and Parrish, 2010; Maclachlan and Guthrie, 2010). In addition to virus genetic variation such as mutation, recombination, and reassortment; environmental factors including ecological, social, health care, and behavioral influences can also play important roles. Such factors, coupled with enormous increases in the human and animal population with the advances in the speed and volume of global

transportation combines to create increased opportunity for emergence and re-emergence of viral diseases (Domingo, 2010).

For instance, within the genus of *Morbillivirus* and related viruses, several emerging diseases have been recently described. The existence of mild strains of rinderpest in domestic cattle has had devastating effects in African buffaloes, eland and lesser kudu in Africa (Parker, 1997; Kock *et al.*, 1999). PPRV was isolated during an outbreak of mid 1990s in Indian buffaloes (Govindarajan *et al.*, 1997). New morbilliviruses with devastating effects have been described in marine mammals, while canine distemper virus caused mortalities in lions of East Africa (Mah *et al.*, 1988; Roelke-Parker *et al.*, 1999). Viruses belonging to the *Paramyxoviridae* family, initially classified as *morbillivirus*, were recently described in diseases declared in horses, pigs and human (O'sullivan *et al.*, 1997; Chua *et al.*, 2000). The cause of the new disease of camel was also assumed to be morbillivirus which could result from an interspecies transfer of PPRV or RPV strains from cattle or goats and sheep to camel or through the emerging of a new variant *morbillivirus* serologically related to PRV and/or RPV (Rogers *et al.*, 2001).

Some other prominent examples of emerging and re-emerging RNA virus could be *influenza viruses, hantaviruses, coronavirus* (Sloots *et al.*, 2008), *parvovirus*, bluetounge virus, African horse sickness virus, Ebola virus, adenovirus, and Nipah virus (Hoelzer and Parrish, 2010; Maclachlan and Guthrie, 2010; Trei *et al.*, 2010).

2. 3. Diagnostic virology

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).

2. 3.1. Culture methods in virus diagnostics

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

Cell culture is the process by which cells are grown under controlled conditions. In practice the term "cell culture" has come to refer 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. Human diploid fibroblasts such as MRC-5 are the example of diploid cells. 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. The most commonly used cell lines derived from animals is presented in table (2) (Cann, 2005; Ali *et al.*, 2008; Dunham and Guthmiller, 2008).

Table 2: Descriptive lists of available animal origin cell lines.

Cell line	Organism	Origin tissue	Morphology	Link/Source
3T3 cells	mouse	embryonic fibroblast		ECACC
9L	rat	glioblastoma		
A20	murine	B lymphoma	B lymphocyte	
ALC	murine	bone marrow	stroma	PubMed
B35	rat	neuroblastoma		ATCC
bEnd.3	mouse	brain	endothelium	ATCC
BHK-21	hamster	kidney	fibroblast	ECACC/Olympus
C3H-10T1/2	mouse	embryonic	mesenchyma	ECACC
CHO	hamster	ovary	epithelium	ECACC/ICLC
COS-7	ape	kidney	fibroblast	ECACC/ATCC
CMT	dog	mammary gland	epithelium	
CT26	murine	colorectal carcinoma	colon	
D17	canine	osteosarcoma		ECACC
DH82	canine	histiocytosis	monocyte/macrophage	ECACC
Dubca	camel	dermal	fibroblast	
E-Derm	equine	dermal	fibroblasts	ECACC/ATCC
EMT6/AR1	mouse	breast	epithelial-like	ECACC
Hepa1c1c7	mouse	hepatoma	epithelial	ECACC/ATCC
MDBK	bovine	kidney	epithelium	ECACC/ATCC
MDCK II	dog	kidney	epithelium	ECACC/ATCC
MTD-1A	mouse		epithelium	
MyEnd	mouse	myocardium	endothelium	
NIH-3T3	mouse	breast	epithelial-like	ECACC
NIH-3T3	mouse	embryo	fibroblast	ECACC/ATCC
RenCa	mouse		renal carcinoma	
RIN-5F	mouse	pancreas		
RMA/RMAS	mouse		T cell tumor	
Vero cells	African green monkey	kidney epithelium		ECACC
X63	mouse	melanoma		
YAC-1	mouse	lymphoma		ECACC

Note that this list is a sample of available cell lines of nonhuman (animal) origin, and is not comprehensive.

Edited from: (http://en.wikipedia.org/wiki/Cell_culture), accessed on June 20, 2010

Isolation of cells and Maintaining in culture

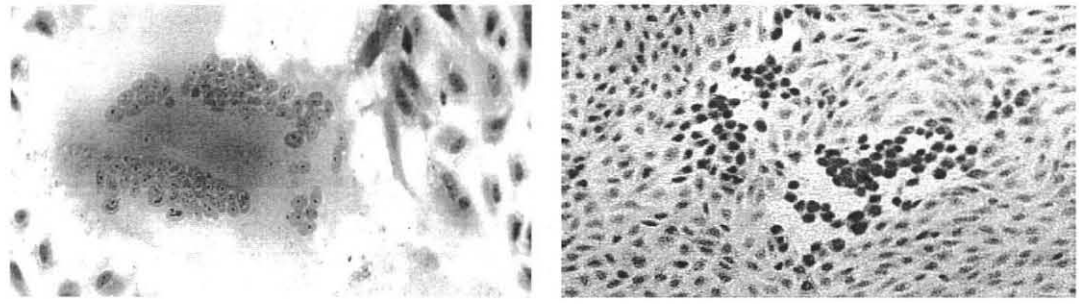
Cells can be isolated from tissues for *in vitro* culture in several ways. Cells can be easily purified from blood; however, only the white blood cells are capable of growth in culture (Masters, 2002). Mononuclear cells can be released from soft tissues by enzymatic digestion with enzymes such as collagenase, trypsin, or proteinase which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture and this method is known as explant culture. Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37 ^{0}C, 5% CO_2 for mammalian cells) in a cell incubator (Freshney, 2000). Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed. Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as calf serum (Burleson *et al.*, 1992; Williams *et al.*, 1994; Ali *et al.*, 2008).

Advantages and disadvantages of cell cultures over other techniques

Cell cultures vary greatly in their susceptibility to different viruses. It is of utmost importance that the most sensitive cell cultures are used for a particular suspected virus. Cell cultures are relatively lower in cost and easier to manipulate when compared to embryonated egg inoculation and animal assay. They are also relatively homogenous population cells, relatively free of immunological and hormonal influences (can be present in calf serum as additive) that might affect replication of the virus, broad spectrum and sensitive (Ogilvie, 2001; Zuckerman *et al.*, 2009). It is limited by the difficulty in maintaining cell cultures, variability of cell cultures and contamination by endogenous viral agents such as Simian virus 40 (SV40), mycoplasma and bacteria that may occur. Another problem in isolating certain viruses, especially myxovirus and paramyxovirus is the presence of inhibitory substances or antibodies in the calf serum used in the cell culture media (Cutrín *et al.*, 2009). Using fetal calf serum reduces this problem but adds to the expense. The large number of sequential operations involved in cell culture means that aseptic technique is of the utmost importance even in the presence of a blanket of antibiotics (Williams *et al.*, 1994; Carman, 2001).

Detection of virus replication on cell culture

The most important factors to be considered in studies involving viruses are choice of a cell culture method that can detect the effect of virus multiplication, choice of proper growth medium free of antibodies and non specific virus or cell inhibitors, and choice of environmental conditions best suited to maintain cells during the entire period of the experiment. 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, the appearance of cytoplasimic or nuclear inclusion bodies (Figure 1) (Burleson *et al.*, 1992; Cann, 2005; Zuckerman *et al.*, 2009). Other means including change in pH of the medium, change in heamadsorption property of cell membrane, serologic technique to detect viral antigen, the ability of noncythopatic 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).



A B

Figure 1: Cytopathic effects of virus in cell culture.

A: Measles viruses, note the formation of syncytia; B: Herpes simplex virus, note the ballooning of cells (http://virology-online.com/general/test1.htm#VIRUS_ ISOLATION), accessed on June 10, 2010.

Embryonated chicken eggs and explants 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

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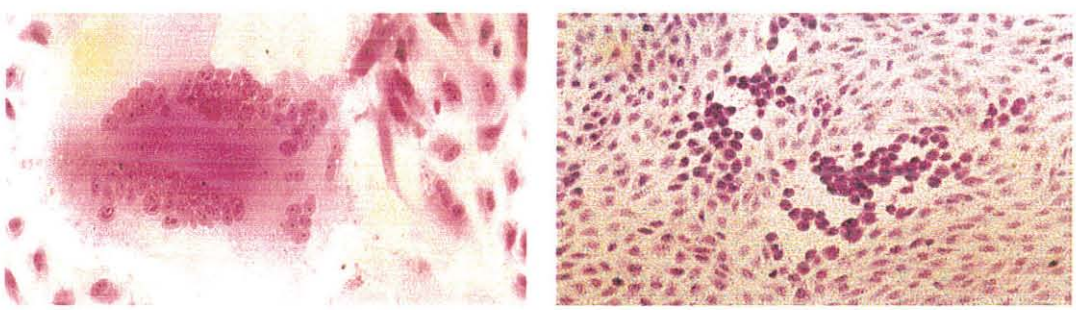

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Embryonated chicken eggs and explants culture

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

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 (Smith and Paturzo, 1988; Jacoby *et al.*, 1991).

Laboratory animals

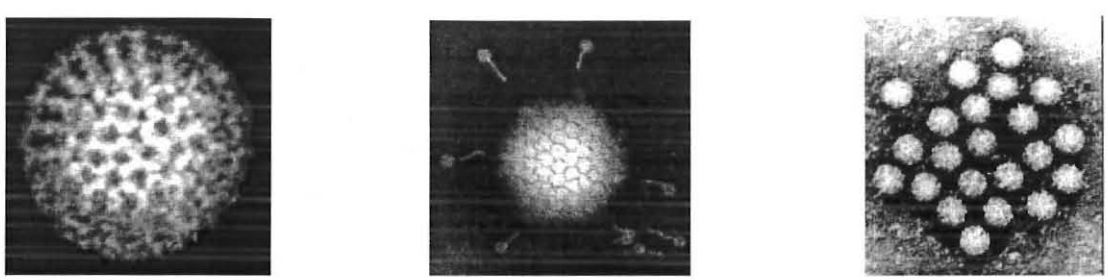
Some viruses cannot be propagated in either mammalian tissue culture systems or embryonated chicken eggs. An example is mouse thymic virus, which must be popagated *in vivo* in laboratory animals such as neonatal mice (Osborn, 1982). A number of additional diagnostic techniques have been developed and are now commonly used to identify potential pathogens in laboratory mice and rats (Chhabra *et al.*, 2007; 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).

2. 3. 2. Nonculture diagnostic techniques

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 (Cann, 2005; Cutrín *et al.*, 2009; Zuckerman *et al.*, 2009).

Electron Microscopy

Virus diagnosis by electron microscopy (EM) relies on the detection and identification of viruses on the basis of their characteristic morphology (Zuckerman *et al.*, 2009). A major advantage of virus diagnosis by EM is the ability to visualize the virus. By identifying the virus directly, it is possible to perform an examination without a preconceived concept of the aetiological agent, in contrast with those assays which require a specific viral probe (Renshaw *et al.*, 2000). Speed is another advantage of EM as the specimen can be processed within minutes of receipt and thus EM can be used as a rapid diagnostic method (Cann, 2005; Zuckerman *et al.*, 2009). On the other hand, the main disadvantage of EM is its inability to examine multiple specimens coincidentally. Secondly, there must be a minimum number of virus particles present (around 10^6 virus particles per ml of specimen) for detection. Some viruses may give a non-distinct morphological appearance which may make detection very difficult. Finally, EM is a very expensive service to provide and requires highly skilled personnel (Williams *et al.*, 1994; Cutrín *et al.*, 2009). EM has found a particular niche in the detection of fastidious gastroenteritis viruses. In addition EM may be used to confirm the results of virus isolation by cell culture such as for parainfluenza viruses (Pfeffer *et al.*, 1996; Weisbroth *et al.*, 1998; Ali *et al.*, 2008).



A B C

Figure 2: Electron micrographs of viruses commonly found in feacal or stool specimens.

A: rotavirus, B: adenoviruses, C: Norwalk-like viruses (Stannard, 1995).

Antigen detection

The detection of viral antigen from clinical sample has become an essential component of diagnostic virology. It can provide diagnostic information within few hours. 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 AdV and rotavirus; and blood viruses such as hepatitis virus and human immuno virus 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 EIA the same principles are used as in case of enzyme linked immunosorbent assay (ELISA) (discussed later) except it is antigen that is detected in this case (Williams *et al.*, 1994; Knipe and Holey, 2001).

Agar gel immunodiffusion

The agar gel immunodiffusion (AGID) tests may be conducted in petridishes or on glass microscope slides. In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm apart. For Petri dishes, the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer the wells are placed from each other, the shorter the reaction time. Using a small volume pipette, known hyperimmune rabbit serum should be placed in the central well. Similarly, control known positive antigen, should be placed in alternate peripheral wells (i.e. one, three and five). Negative control antigen is placed in well four. Test antigens are added to wells two and six (OIE, 2008b). Tests are best developed at 4°C or low ambient temperatures. The reaction area should be inspected from 2 hours onwards for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls (Durojaiye, 1982). Tests should be discarded after 24 hours if no result has been obtained. The result is not acceptable unless precipitation reactions are also obtained giving a line of identity with the control positive antigen preparation. Although the test is neither highly sensitive nor highly specific, it is robust and adaptable to field conditions. The lack of specificity could be because of cross reaction between different viruses such as in the

case of rinderpest virus and PPRV which requires further differentiation (Durojaiye *et al.*, 1983; Foreman *et al.*, 1983; OIE, 2008b; Zuckerman *et al.*, 2009).

Serology

The measurement of antiviral antibodies was one of the first methods used for the specific diagnosis of viral infections and remains an important tool in the diagnostic virology. The role of serology may be for the diagnosis of acute or current infections or for the determination of immune status to specific viruses. Serologic diagnosis is important for viruses that cannot be readily cultured or for which culture is slow or otherwise impractical. Haemagglutination Inhibition (HAI), EIA, radioimmunoassay (RIA), and Complement Fixation Test (CFT) are the commonly used serological tests (Azwai *et al.*, 1996; Weisbroth *et al.*, 1998; Knipe and Holey, 2001).

Haemagglutination Inhibition Test

A wide variety of different viruses possess the ability to agglutinate the erythrocytes of mammalian or avian species. The actual animal species whose erythrocytes could be agglutinated depends on the actual virus. Examples of viruses which could haemagglutinate include adenoviruses, alphaviruses, bunyaviruses, flaviviruses, influenza virus, parainfluenza viruses, some strains of picornaviruses and rubella virus. Antibodies against the viral protein responsible for haemagglutination can prevent haemagglutination; this is the basis behind the HAI (Burgemeister *et al.*, 1975; Intisar *et al.*, 2009). The specificity of the HAI test varies with different viruses. With some viruses such as influenza A, the haemagglutination antigen is the same as the antigen responsible for virus adsorption and thus virus neutralization, and therefore the HAI test is highly specific for the different strains of the virus. With other viruses, the HAI test is less specific. Example is Flavivirus, where HAI antibodies against one flavivirus may cross-react with other related flavivirus. HAI tests are more sensitive than complement fixation tests but are less sensitive than EIAs and RIAs (OIE, 2008a).

The HAI test is simple to perform and requires inexpensive equipment and reagents. Serial dilutions of patient's sera are allowed to react with a fixed dose of viral haemagglutinin, followed by the addition of agglutinable erythrocytes. In the presence of antibody, the ability of the virus to agglutinate the erythrocytes is inhibited (Assaf *et al.*, 1983). The HAI test may be

complicated by the presence of nonspecific inhibitors of viral haemagglutination and naturally occurring agglutinins of the erthrocytes. Therefore, the sera should be treated before use to avoid false positive or false negative results that may arise. HAI tests are widely used for the diagnosis of influenza virus, Newcastle disease virus and rubella virus infections (Assaf *et al.*, 1983).

Complement Fixation Test

The complement fixation test was extensively used in different virus as well as bacterial serology after being introduced by Wasserman in 1909. It took a number of decades before the CFT was adapted for routine use in virology. CFT meet the following criteria; it 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. Although CFT is considered to be a relatively simple test, it is a very exacting procedure because five variables are involved (Cann, 2005; Ali *et al.*, 2008). In essence the test consists of two antigen antibody reactions, one of which is the indicator system. The first reaction, between a known virus antigen and a specific antibody takes place in the presence of a predetermined amount of complement. The complement is removed or "fixed" by the antigen antibody complex. The second antigen antibody reaction consists of reacting sheep red blood cells (RBC) with haemolysin. When this indicator system is added to the reactants, the sensitized RBCs will only lyse in the presence of free complement. The antigens used for CFT tend to be group antigens rather than type specific antigens. In order for the CFT to be set up correctly, the optimal concentration of haemolytic serum, complement, and antigen should be determined by titration (Iman *et al.*, 1997; Ali *et al.*, 2008; OIE, 2008b; Zuckerman *et al.*, 2009).

Enzyme Linked Immunosorbant Assay

Enzyme linked immunosorbant assay (ELISA) was developed in early 1970's, became rapidly accepted and clinical virology in the 1980's was characterized by the widespread use of ELISA technology. Solid-phase systems for antigen detection are still used widely. EIA 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). EIA 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 lead 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).

Indirect assays

Viral antigen is immobilized onto a solid phase. Specific antibody in the patient serum sample binds to this antigen and, after a washing step, is detected by an enzyme labeled anti-antibody immunoglobulin. In this way, either specific IgG or IgM can be detected, depending on the indicator immunoglobulin. Detection of IgM species is dependent on the prevailing level of IgG, and a high level of specific IgG may reduce the sensitivity of an IgM assay for the same virus. If rheumatoid factor is present in the clinical sample it may lead to false positive IgM reactions (Xu *et al.*, 2007; OIE, 2008a).

Capture assays

These methods are used to detect antibodies of specific immunoglobulin subclasses. They are widely used for the diagnosis of acute infections by IgM detection. These assays may also be used for detecting IgG and IgA. Immunoglobulin subclasses are captured onto the solid phase by insolubilized anti- immunoglobulin, after which antigen and then labeled antibody is added (Johann and Czerny, 1993; Weisbroth *et al.*, 1998).

Competitive assays

In this case, an enzyme labeled antibody in the EIA system competes for binding to immobilized antigen with antibody in the clinical sample. The analyte can then be quantified by its ability to prevent the formation of the complex between the insolublized and the labeled reagent. This assay may improve the specificity of the assay diagnosis (Libeau *et al.*, 1995; Rogers *et al.*, 2001; Abraham *et al.*, 2005; Albayrak and Gur, 2009).

Nucleic acid detection

Diagnostic virology has been revolutionized by the application of nucleic acid detection techniques and molecular methods for virus detection become a routine diagnostic tool starting from the 1990s. These techniques detect specific nucleic acid sequences and can be applied to the detection of virtually many viruses. Depending on the target sequence, the assays can be specific for a single virus species or for a group of related virus (Renshaw *et al.*, 2000). 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). Nucleic acid amplification assays can be divided into those that amplify target nucleic acid, those that amplify the probe itself, and those that amplify the detection signal. Among the three, the target amplification, particularly PCR is the commonly used assay (Williams *et al.*, 1994; Knipe and Holey, 2001; Jarrett *et al.*, 2006).

Polymerase chain reaction

Conventional PCR methods are used frequently to identify or exclude the presence of a viral pathogen suspected to be present in a biological sample. The proven technology to rapidly detect and identify known humans and animal pathogens as potential causes of disease or as bioweapons is PCR (Espy *et al.*, 2002; Kim and Chae, 2004; OIE, 2008a). PCR allows the *in vitro* amplification of specific target DNA sequences by a factor of 10^6 and is thus an extremely sensitive and specific technique, 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).

PCR is based on an enzymatic reaction involving the use of synthetic oligonucleotides flanking the target nucleic sequence of interest. These oligonucleotides act as primers for the thermostable Taq polymerase (Kottaridi *et al.*, 2006; Cutrín *et al.*, 2009). Briefly, PCR comprises repetitive cycles of a three step amplification procedure (Figure 3). In the first step, nucleic acid isolated from a clinical specimen is denatured (mostly at 94 ^{0}C). In the second step, 2 short oligonucleotide primers complementary to a specific microorganism's genome are allowed to anneal (commonly at 55 ^{0}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 ^{0}C). These 3 steps are repeated 20 to 40 times, yielding a product of defined size in reactions containing the microorganism of interest. In contrast, product of the expected size should not be produced from specimens in whom the microorganism is not present (Espy *et al.*, 2002; Kim and Chae, 2004). Further sensitivity and specificity may be obtained by the nested PCR technique, whereby the DNA is amplified in two steps. In the first step, an initial pair of primers is used to generate a long sequence that contains the target DNA sequence. A small amount of this product is used in a second round of amplification, which employs primers to the final target DNA (OIE, 2008a). To detect microorganisms with RNA genomes, the RNA is first converted to the complementary DNA (cDNA) by the enzyme reverse transcriptase. The cDNA is then used as the template in PCRs as described above (Corne *et al.*, 1999; Saravanan, 2004; Carr *et al.*, 2009).

Detection of DNA sequence product of the PCR assay may be performed in several ways. The least sensitive and specific method is to size fractionate the reaction product on an agarose or acrylamide gel and stain the DNA with ethidium bromide. A more sensitive technique involves the attachment of DNA to a membrane through dot or slot blot techniques followed by hybridization with a labeled homologous oligonucleotide probe. Alternatively, the PCR product may be probed directly by liquid oligomeric hybridization (Kim and Chae, 2004). However, these techniques provide no information on size of the amplified product and thus could not exclude the possibility that the product originated from a region of the human genome which exhibits homology with the target viral sequence. The most sensitive and specific detection methods result from combining the size information of gel electrophoresis with the improved sensitivity and specificity of hybridization techniques. This may be achieved by gel

electrophoresis followed by Southern transfer and hybridization, or through liquid oligomeric hybridization followed by gel electrophoresis (Williams *et al.*, 1994; Jarrett *et al.*, 2006).

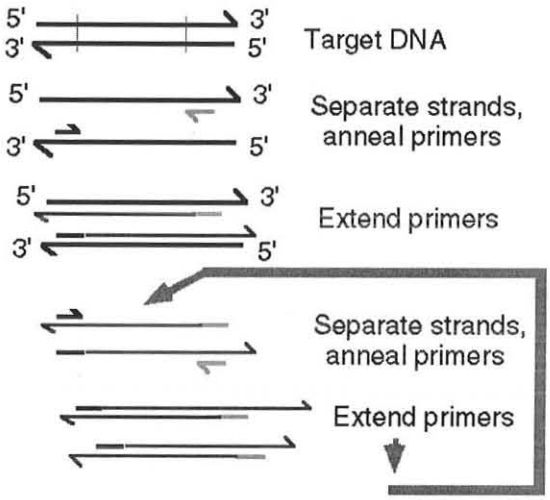


Figure 3: Schematic representation of polymerase chain reaction.
(http://virology-online.com/general/test10.htm), accessed on June 10, 2010.

PCR is advantageous because, first, it has extremely high sensitivity, may detect down to one viral genome per sample volume. Second, it is easy to set up and finally faster turnaround time. However, it has disadvantages in that it is extremely liable to contamination, requires high degree of operator skill, not easy to quantitate results and sometimes difficult to interpret a positive result, especially with latent viruses (Kottaridi *et al.*, 2006).

2. 4. Diagnosis of unknown viruses

To date, there are a variety of diseases with unknown etiologies. A viral origin has been suggested for many of these diseases, emphasizing the importance of a continuous search for new virus. Identification of previously unrecognized viral agents in patient samples is of great medical interest, but remaining a major technical challenge. Several problems were encountered when searching for new viruses. First, most of the unidentified viruses do not replicate *in vitro*, at least not in cells that are commonly used in viral diagnostics. Second, the molecular biology techniques previously employed to identify unknown viruses have their drawbacks. However, several techniques such as universal primer PCR, random priming based PCR, representational

difference analysis (RDA) and virus discovery cDNA- AFLP are in use for virus discovery (Allander *et al.*, 2001; Clem *et al.*, 2007; Nanda *et al.*, 2008).

2. 4. 1. Degenerate oligonucleotide primed polymerase chain reaction

The detection of unknown viruses is still one of the most challenging problems in diagnostic virology. However, in an era of emerging pathogens and bioterrorism (including the potential for bioterrorism agents genetically engineered to avoid detection using standard methods), improved methods for virus discovery and detection that are less dependent upon the availability of sequence information are needed (Allander *et al.*, 2001; Nanda *et al.*, 2008). PCR methods using primers based on consensus sequences shared among related viruses were used to detect PIV1-3 (Corne *et al.*, 1999), to identify and detect coronaviruses (Sampath *et al.*, 2005), to discover new herpes viruses (Van Devanter *et al.*, 1996) and to identify West Nile virus as the etiologic agent of human encephalitis in the New York outbreak (Briese *et al.*, 1999). These primers are designed to anneal to sequence regions highly conserved among members of a virus family, such as polymerase genes (Sampath *et al.*, 2005; Clem *et al.*, 2007; Nanda *et al.*, 2008).

Universal PCR primers should amplify new members of an already known virus family. But, this method has two major drawbacks (Adzhar *et al.*, 1996; Pyrc *et al.*, 2008). First, a choice for a specific virus family has to be made. This limits the possibility of identifying a member of an unsuspected family or the founding member of a totally new one. Second, the universal primers may simply not match the genome sequence of novel members of a virus family (Stewart., *et al.*, 1995; Stephensen *et al.*, 1999).

In order to identify viral genomes in any given biological sample, it is necessary to use methods that exclude cellular nucleic acids, which are the most abundant nucleic acids in most samples. The approach is based on physical separation of viral and cellular nucleic acids, after lysis of cellular material to release its content using a viral buffer (Nanda *et al.*, 2008). Encapsidation of viral nucleic acids is used as a basis for separation of viral nucleic acids from cellular nucleic acids. Viral capsids protect viral nucleic acids from digestion by nucleases, and also provide viral nucleic acids with a density that permits capsids to be separated from other cellular debris, including nucleic acids (Denniston *et al.*, 1981; Adzhar *et al.*, 1996; Nanda *et al.*, 2008).

2. 4. 2. Random Priming polymerase chain reaction

This technique uses nonspecific amplification of viral sequences in a random priming PCR at low annealing temperature. However, most ingredients of this assay contain contaminating DNA. For instance, the enzymes used may contain trace amount of DNA from bacteria in which they are produced. This contaminating DNA is also amplified and it is therefore not possible to determine at an early stage weather amplification represent a new virus or contaminating nucleic acids. This can be resolved only after excessive cloning and sequencing. Using this technique, high throughput screening of many clinical samples is impractical and the technique has been only successful with viruses that replicate *in vitro*, in which cell culture supernatant was used as input for the assay (Kleber *et al.*, 2008; Pyrc *et al.*, 2008).

2. 4. 3. Representational difference analysis

It is a subtractive hybridization technique that enriches for nucleic acid sequences that is present in one tissue but absent or present in lower concentration in an otherwise identical tissue sample. RDA utilizes PCR to generate sets of nucleic acid in a target and a tester sample (negative control). After subtractive hybridization, there is selective amplification of target enriched sequences. The method was developed for tissue material and not for non tissue samples such as serum/plasma or virus culture supernatant (Chang *et al.*, 1994). The fact that these liquid samples have low concentration of DNA and RNA in the tester sample may restrain the selective amplification of an unknown viral target. A disadvantage of this technique is that it requires a negative control tissue from the same person from whom the diseased tissue was obtained (Pyrc *et al.*, 2008).

2. 4. 4. Virus discovery by cDNA-AFLP

According to Pyrc *et al.* (2008) this method is a general, simple and easy to use method that allows large scale screening for any RNA or DNA viruses in samples such as serum/plasma or virus culture supernatant. Virus discovery cDNA-AFLP (also known as VIDISCA) is based on the cDNA-AFLP technique (Bachem *et al.*, 1996; Van der hoek *et al.*, 2004). VIDISCA begins with a treatment to selectively enrich for viral nucleic acids, which includes a centrifugation step to remove residual cells and mitochondria. In addition, a DNase treatment is used to remove chromosomal DNA and mitochondrial DNA from degraded cells, whereas RNase in the sample

will degrade RNA. During this step, the viral nucleic acid is specifically protected within the viral particle (Kleber *et al.*, 2008). After inactivation of nucleases, viral nucleic acids are extracted and cDNA is formed by reverse transcription. Second strand synthesis is performed to make double stranded DNA (from viral RNA/DNA), which will be digested with restriction enzymes to ligate the anchors onto the digested DNA. The target is subsequently PCR amplified with primers that anneal to the anchor sequences. The PCR fragments that are specific to the 'infected' clinical sample can then be cloned and sequenced. Because amplification is based on the presence of restriction sites, the PCR is reproducible (Kleber *et al.*, 2008; Pyrc *et al.*, 2008).

3. MATERIALS AND METHODS

This work was planned to be a part of the project 'investigation of the aetiologies of the emerging camel disease in pastoral areas of Ethiopia to design possible control measures' by the NVI. The project was designed to be a collaborative endeavor among various stakeholders having direct or indirect concern in the pastoralists' communities.

3. 1. Study sites

During last outbreaks of 2007, different tissue and blood samples were collected from different camel rearing areas including the Somali, Afar and part of Oromia region (Guji and East Shoa Zones) and preserved under -85 ^{0}C at NVI. Therefore, this study was based on use of the already collected samples and all investigations were done at the NVI laboratories for possible isolation and characterization of viral agents according to published procedures (OIE, 2008a).

3.2. Type of samples

Two lungs, one heart, one heart blood and one liver postmortem samples from different animals that were collected during 2007 camel disease outbreak from Negelle Borena areas were used for this study.

3. 3. Study methodology

3. 3. 1. Isolation of the viral agent on cell line

Suspensions that were obtained from tissue samples were inoculated onto susceptible cells for virus isolation (OIE, 2008a).

Cell culture and its preparation

Two appropriate commercial cell lines, African green monkey kidney (Vero) and camel cell lines (Dubca) were used for this study (Dunham and Guthmiller, 2008). Vero cell lines of different passage numbers was obtained from the NVI, while the Dubca cell line was obtained from the CVRL in Dubai by a kind donation of Professor U. Wernery.

The cell lines were propagated from a single 25 cm^2 or 75 cm^2 tissue culture flask (Cornings) to three flasks at a time to have a culture both for sample inoculation and for further reserve of cell lines. During propagation, split ratio method was used (Burleson *et al.*, 1992). The monolayer in the original flasks was treated with 1.25% trypsin to remove the attached cells from the flask and to get single independent cells. The flask containing trypsin were incubated at 37 ^{0}C for 5 minutes to facilitate the action of trypsin before stopping its action by addition of Glasgow Minimum Essential Media (GMEM) containing 10% fetal calf serum (FCS). Using the split ratio 1:3, the cell suspension was divided into three flasks adding 10 ml of GMEM growth media to each flask. The flasks were incubated at 37 ^{0}C for the following days until >70% confluence was attained, which is fit for inoculation of the samples. The flasks were thoroughly observed for any contaminant growth while the nature of growth or confluence was also observed thoroughly (Burleson *et al.*, 1992; Williams *et al.*, 1994; Masters, 2002; OIE, 2008b).

Sample processing and inoculation

The tissue samples were taken from ultra freezer, -85 ^{0}C and thawed, put under 4 ^{0}C for 30 minutes to prepare for further processing. Only one sample was processed at a time to avoid any cross contamination (Dunham and Guthmiller, 2008). All tissue processing steps were conducted inside the class II safety cabinet which has inbuilt ultra violate (UV) light to decontaminate the inside working environment after every laboratory works. One gram of tissue sample was taken from the original specimen for sample processing. This tissue was washed twice with 10 ml sterilized phosphate buffered saline (PBS) pH 7.2- 7.4 containing antibiotics and antifungal drugs on the petridish before drained and transferred to the mortar. The drained tissue in the mortar was sliced into pieces using scalpel blade. Nine ml of sterile PBS was added to the mortar containing the tissue pieces to grind it using pistil after addition of sterile sand to facilitate grinding. The grinded tissue with PBS was poured into the sterile test tubes to the level of three fourth for further centrifugation at 10,000x rpm for 10 minutes. The suspension was used for inoculation; hence, transferred to other sterile test tubes. Before inoculation, the supernatant was collected using sterilized syringe and filtered through Millipore filter (0.22 μm filter paper) and penicillin-streptomycin was also added to it at the dose of 100 IU /ml and 100 mg/ml for penicillin and streptomycin, respectively (Masters, 2002; OIE, 2008a).

The 25 cm^2 tissue culture flask monolayer was inoculated with 1ml of 10% tissue suspensions after removal of GMEM and incubated at 37 ^{0}C for an hour to facilitate virus cell attachment.

After attachment, the monolayer was washed with PBS and fresh GMEM with 2% FCS was added to it. The inoculated monolayer and growth of virus on the tissue culture was examined daily for appearance of cytopathic effect. Growth of virus on the tissue culture was detected by any evidence of CPE. The CPE that was expected to be produced by virus could develop within 5 days. However, in this case it was followed for 14 days to detect any evidences of CPE in due course. After 14 days, if no CPE was detected as CPE may take time to appear, the cells were blindly passaged at least twice (frozen and thawed, then inoculated to fresh cultures) before the samples were declared negative (Masters, 2002; OIE, 2008a).

3. 3. 2. Antigen detection by agar gel immunodiffusion

The agar gel immunodiffusion tests were conducted using 1% Nobel agar with a thickness of about 4 mm. Six peripheral wells around a single central well were cut in a hexagonal pattern by the help of agar gel tubular cutter. The wells had 4 mm diameter and the distance between wells was 3 mm. Using a small volume pipette, 15 µl AdV, African horse sickness (AHS), Bluetongue (BTV), FMD, PIV1-3, PPRV, RPV and RSV known hyperimmune serum was placed in the central well. Similarly, known positive control antigen was placed in alternate peripheral wells (i.e. one, three and five) (Durojaiye, 1982). Negative control antigen was placed in well four. Test antigen from cell culture suspension was added to wells two and six. The test was done at room temperature. The reaction area were inspected after 24 hours for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls. The result was not acceptable unless precipitation reactions were also obtained giving a line of identity with the positive control antigen preparation (Foreman *et al.*, 1983; OIE, 2008a).

3. 3. 3. Molecular techniques

Extraction and purification of viral nucleic acids

After the CPE was observed both on Vero and Dubca cells, to determine whether the isolated virus is DNA or RNA virus, special viral nucleic acid extraction technique was applied. The viral nucleic acid extraction method used during this study was mainly based on viral capsid purification techniques described previously with fewer modifications (Denniston *et al.*, 1981; Nanda *et al.*, 2008). Briefly, 1 ml of tissue or culture suspension sample was suspended in a locally prepared 1 ml viral buffer (30mM Tris/HCl pH 7.5, 3.6mM $CaCl_2$, 5mM Na Acetate, 125mM KCl and 0.5mM EDTA), sonicated at 800 speed and 40 mV using Vibro ^{m} cell 72434

ultrasonicator (Bioblock Scientific, Illkirch, France) and incubated at 37 ^{0}C for 1½ hrs to further facilitate cell and nuclear membranes disruption. Cellular nucleic acids were digested away by treatment of nucleases, 10 µl DNase I (100U/ml, Invitrogen) and 10 µl RNase ONE (100U/ml, Invitrogen). The encapsidated viral nucleic acids were recovered in the aqueous phase and viral nucleic acids (DNA and/or RNA) were extracted from the capsid suspended in viral buffer using commercial nucleic acid extraction kits (Qiagen) (Allanders *et al.*, 2001; Nanda *et al.*, 2008).

Total RNA was extracted from the tissue suspension and cell culture lysates by using RNeasy® mini kit (Qiagen) based on the manufacturer's protocols. Briefly, 460 µl of sample containing a capsid in viral buffer was taken and put into a 1.5 ml eppendorff tube, an equal volume of Lysis buffer (RLT) (containing 1% β-mercaptoethanol) was added to the sample and mixed by vortexing, and then 460 µl of 70% ethanol was added and mixed by vortexing. The mixture was transferred to RNeasy spin column containing a silica gel membrane (700 µl maximum loading volume) and spinned in a microfuge for 15 sec at 13,000 rpm. The flow-through was discarded and repeated with remaining volume. The RNA was washed with 700 µl washing buffer (RW1) (centrifuge for 15 sec at 13,000 rpm) and with 500 µl RPE buffer subsequently. After the flow-through was discarded, the column was centrifuged at 13,000 rpm speed for 2 min to dry the membrane. Then the RNA was eluted with 50 µl DEPC-H_2O into a new clean RNase free collection tube and stored at -20 ^{0}C for further experiments (Qiagen, 2006b; Witlox *et al.*, 2008).

Total DNA was extracted from the tissue suspension and cell culture lysates by using DNeasy® mini kit (Qiagen) according to recommended procedures. Brefiely, 200 µl of sample in viral buffer was taken and put into a 1.5 ml eppendorff tube; 180 µl of Lysis buffer (ATL) (containing 1% β-mercaptoethanol) and 20 µl of proteinase K were added to the sample and mixed by vortexing and incubated at 56 ^{0}C for an hour in water bath. Then 200 µl of AL buffer was added to it which was followed by addition of 100% ethanol and mixed by vortexing. The mixtures was transferred to DNeasy spin column (700 µl maximum loading volume) and spinned in a microcentrifuge for 1 minute at 10,000 rpm. The flow-through was discarded and spinning was repeated with remaining volume at the same speed and duration. The spin column was then changed to another new 2 ml collection tube between each wash and the DNA was washed with 500 µl washing buffer (AW1) (centrifuged for 1 minute at 10,000 rpm) and with 500 µl AW2 buffer (centrifuged at 13,400rpm for 3 minutes) subsequently. For elution the flow-through was discarded and the column was changed to a new 1.5 ml microcentrifuge tube and

100 µl AE buffer was added, followed by incubation at room temperature for 1 minute, centrifuged at 13,000 rpm speed for 1 minute. Then, the DNA was eluted with 50 µl DEPC-H_2O into a new clean DNase free collection tube, labeled and stored at -20 ^{0}C for further use (Qiagen, 2006a; Nanda *et al.*, 2008; Witlox *et al.*, 2008; Dalton *et al.*, 2009).

Complementary DNA synthesis

In order to detect viral RNA, RNA samples were subjected to reverse transcription (RT) using oligodT or random hexamer primers and Superscript™ III Reverse Transcriptase (Invitrogen) prior to DOP-PCR. The cDNA was synthesized based on the manufacturer's protocol (Invitrogen) in 20 µl reaction volume. Primarily, 1 µl 50µM oligodT primer or random hexamer, 1 µl 10mM dNTPs, 5 µl extracted RNA and DEPC-H_2O to 10 µl were added to 0.5 ml PCR tube and incubated at 65 ^{0}C for 5 minutes in thermal cycler (Applied Biosystems) and chilled on ice for 3 minutes. Then, 10x RT buffer, 25mM $MgCl_2$, 0.1M DTT, RNase OUT™ (40U/µl) and Superscript™ III RT (200U/µl) were added with the volume of 2 µl, 4 µl, 2 µl, 1 µl and 1 µl, respectively, and incubated at 25 ^{0}C for 10 minutes (only in cases of random hexamer primer) followed by 50 ^{0}C for 50 minutes and terminated at 85 ^{0}C for five minutes. Finally, 1 µl of RNase H was added to each tube and incubated at 37 ^{0}C for 20 minutes and the cDNA was stored at -20 ^{0}C until needed (Freymuth *et al.*, 1997; Corne *et al.*, 1999; Carr *et al.*, 2009; Intisar *et al.*, 2009a).

Degenerate oligonucleotide primed polymerase chain reaction

A method described previously for generating representational libraries of eukaryotic genomes, known as degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius *et al.*, 1992) was adapted to amplify nonspecifically portions of any viral genome. Five DOP-PCR primers designed and used before by Allanders *et al.* (2001) were used. The primers were designed with a short (four to six nucleotide) 3'-anchor sequence (which allows the primers to bind in consistent locations), preceded by a nonspecific degenerate sequence (of six to eight nucleotides in this study). Immediately upstream of the nonspecific degenerate sequence, each primer also contained a defined 5'-sequence of 10 nucleotides in length (Table 3). The sequence of the primer used in most of the experiments involving DOP-PCR primer populations was 5'-CCGACTCGAG*INNNNNN*TGTGG-3', where N refers to an equimolar representation of all four deoxyribonucleotides, I refer to inosine, the underlined nucleotides correspond to the 3'-

anchor sequence, and the italicized nucleotides correspond to the degenerate sequence. Because of the six Ns in each degenerate sequence, each reaction included a mixture of 4096 different primers (Clem *et al.*, 2007; Nanda *et al.*, 2008).

Table 3: Universal primers used in viral nucleic acid amplification.

Primer number	5'_specific sequence	Ambiguous sequence	3'_-anchor sequence	Detection limit
1	CCGACTCGAG	NNNNNN	ATGTGG	10^6
2	CCGACTCGAG	INNNNNN	TGTGG	10^2
3	CCGACTCGAG	IINNNIINNN	TGTG	$>10^6$
4	CCGACTCGAG	IINNNIINNNI	TG	$>10^6$
5	CCGACTCGAG	IINNNNNN	TTCT	$>10^6$

Source: Nanda *et al.* (2008).

The PCR reaction contained 2mM $MgCl_2$, 5.5mM KCl, 10mM Tris pH 8.4, 200µM dNTP, 4pM DOP primer and 2.5U dream Taq DNA polymerase (Fermentas). Primarily master mix containing 5 µl 10x PCR buffer (containing 20mM MgCl2, 55mM KCl, 100mM Tris pH 8.4), 1 µl 10mM dNTPs mix, 0.5 µl of 5U/µl dream Taq DNA polymerase and 38 µl DEPC-H_2O was prepared for each reaction. Then, 46 µl of the master mix, 2 µl of DOP primer of 100pM and 2 µl of the cDNA were added to each tube. The PCR cycling program used in these experiments was: Initial denaturation for 5 minutes at 95 ^{0}C followed by 5 cycles of incubations for 1 minute at 94 ^{0}C, 1.5 minute at 30 ^{0}C and 3 minutes extension at 72 ^{0}C. This was then followed by 35 cycles of incubations for 1 minute at 94 ^{0}C, 1 minute at 55 ^{0}C, and 2 minutes extension at 72 ^{0}C, with the addition of 14 sec per cycle to the extension step. Amplifications were performed on a thermal cycler (Applied Biosystems) (Nanda *et al.*, 2008).

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 (Freymuth *et al.*, 1997; Corne *et*

al., 1999). This assay permits detection of respiratory tract associated AdV, PIV1, PIV2, PIV3 and RSV in two step multiplex RT-PCR. The various virus types were 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).

Five sets of oligonucleotide primers were used which were designed for RT and amplification of the nucleocapsid genes of PIV1, PIV2, PIV3, RSV, and the hexon genes of adenovirus types 1 to 7 according to nucleotide sequences available from GenBank (Table 4). All primer sets were originally designed to have similar melting temperatures (65 to 70°C) and a higher G/C content to allow a higher annealing temperature to be used during amplification (Osiowy, 1998).

Table 4: Sequences of oligonucleotide primers used for detection of respiratory viruses.

Primer	Gene(N/NP)	Position	Sequence (5'-3')
RSVN3	N	426–451	GGGAGAGGTGGCTCCAGAATACAGGC
RSVN5	N	748–773	AGCATCACTTGCCCTGAACCATAGGC
PIV1PR3	NP	64–89	TCTGGCGGAGGAGCAATTATACCTGG
PIV1PR5	NP	122–147	ATCTGCATCATCTGTCACACTCGGGC
PIV2PR3	NP	360–385	AACTATGTCCAGAGGAGAGGTGCTGG
PIV2PR5	NP	498–523	CCATGCCTGCATAAGCACACTGTAGC
PIV3PR3	NP	416–441	ACCAGGAAACTATGCTGCAGAACGGC
PIV3PR5	NP	624–649	GATCCACTGTGTCACCGCTCAATACC
ADHEX3	Hexon	154–179	CCTACGCACGATGTGACCACAGACCG
ADHEX5	Hexon	343–368	GTGTTGTAGGCAGTGCCGGAGTAGGG

Position is relative to translation start site. Positions shown in hexon gene are according to the adenovirus type 5 hexon gene sequences. N is for nucleocapsid gene of respiratory syncytial virus while NP refers to nucleocapsid gene of parainfluenza viruses.

Source: Osiowy (1998)

For multiplex RT-PCR, a reaction tube contained the following in a final volume of 50 µl: 45 µl Platinum® PCR Supermix containing 22U/ml complexed recombinant Taq DNA polymerase with anti platinum Taq antibody, 22mM Tris HCl (pH 8.4), 55mM KCl, 1.65mM $MgCl_2$ and

220µM concentrations (each) of dATP, dCTP, dGTP, and dTTP (Invitrogen); 0.2mM concentrations (each) of RSV specific primers (RSVN3 and RSVN5) and PIV1 specific primers (PIV1PR3 and PIV1PR5); 0.1mM concentration (each) of PIV2, PIV3, and adenovirus specific primers (PIV2PR3 and PIV2PR5, PIV3PR3 and PIV3PR5, and ADHEX3 and ADHEX5, respectively); and 3 µl cDNA (DNA in case of adenovirus) from each suspected virus. RT-PCR was performed in thermal cycler under the following PCR reaction conditions: initial denaturation at 94 ^{0}C for 3 minutes and 35 cycles of 94 ^{0}C for 40 seconds, 68 ^{0}C for 30 seconds, and 72 ^{0}C for 45 seconds (with 5 seconds primer extension added per cycle). A final primer extension step of 5 minutes at 72 ^{0}C completed the cycle (Freymuth *et al.*, 1997; Osiowy, 1998; Corne *et al.*, 1999; Intisar *et al.*, 2009).

For PIV1, since the size of the amplicon is less than 100bp it was necessary to identify the primer dimer from the amplicon of PIV1. Therefore, conventional two step RT-PCR was conducted separately for PIV1. For this purpose, 45 µl Platinum® PCR Supermix was added to 1 µl of each forward and reverse PIV1 primer (PIV1PR3 and PIV1PR5 respectively) and 3 µl cDNA in to 50 µl volume of reaction. The same temperature cycle as in the case of multiplex RT PCR was utilized (Osiowy, 1998; Intisar *et al.*, 2010a). Positive controls for the RT-PCR assay included RNA extracted from PIV3-infected cells from NVI using PIV3 specific primers.

Test for Morbilliviruses

Ribonucleic acids from infected cultured Vero cells and tissue specimens were extracted by using RNeasy® mini kit (Qiagen) according to the manufacturer's suggested method (Witlox *et al.*, 2008) as well as the cDNA was also synthesized based on the manufacturer's protocol (Invitrogen) in 20 µl reaction volume using oligodT primer. The same protocols as in the case of detection of PIV1 was used for RT-PCR, but the primer used in this case was the one that is specific for the already known morbilliviruses; forward, NAD1 and reverse, NAD2 having sequences 5'-CAA GCC AAG GAT TGC AGA AAT GA-3' and 5'-AAT TGA GTT CTC TAG AAT CAC CAT-3' respectively. The protocol used in the thermal cycler in this case was initially denaturation at 95 ^{0}C for 3 minutes followed by 35 cycles of 95 ^{0}C for 1 minute, 60 ^{0}C for 30 seconds, and 72 ^{0}C for 1 minute and final extension at 72 ^{0}C for 7 minutes. For positive control the cDNA was synthesized from PPRV vaccine strain from NVI (Corne *et al.*, 1999).

Agarose gel electrophoresis

The PCR products were analyzed with 1.2% agarose gel (Sigma Aldrich) containing 0.5μg/ml of ethidium bromide. Briefly, 5 μl PCR products mixed with loading buffer (Invitrogen) and loaded to wells in pre-prepared gel and run at 100 volt for about 40 minutes in parallel with DNA 1,000 bp molecular weight marker (Invitrogen) in electrophoresis apparatus using 1x TAE buffer. The DNA band was visualized by UV illumination, documented and the size was determined using the DNA molecular weight marker standard (Osiowy, 1998; Nanda *et al.*, 2008; OIE, 2008a).

3. 3. 4. Experimental infection of laboratory animals

A trail for production of the disease in susceptible hosts for understanding of the progress of the disease under laboratory condition in laboratory animals was also attempted. For these purpose 20 mice of 3-4 weeks of age (body weight 12-14 g) and 20 adult guinea pigs were used (Iman *et al.*, 1997; Valera *et al.*, 2008).

Infection of laboratory mice

The tissue suspension from which cell culture were inoculated was directly injected using insulin syringe at 0.5 ml dose via intraperitoneal route. Six mice, three per group were challenged and four mice, two per each group were kept uninjected as a control. Both the two groups were kept in separate cages in two conventional animal rooms with food and water *adlibitum*. Animal handling and all experimental procedures were carried out in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals; National Research Council, Academy press, Washington" (ILAR, 1996). Both groups were followed for development of any clinical and postmortem changes for 21 days post challenging. Another group of laboratory mice were challenged with 0.5 ml cell culture suspensions after observation of any CPE on Vero cell. The same way, six mice were challenged intraperitoneal; three mice were injected with the suspension directly from incubator and another three mice after storage at +4 ^{0}C for 5 days and four mice, two per each groups were kept as a control. Both groups were kept in different cages and followed for any clinical sign and postmortem changes for three weeks after challenging (Chhabra *et al.*, 2007; Valera *et al.*, 2008).

Infection of laboratory guinea pig

The same protocol to the mouse was applied for guinea pigs. Using tissue suspension at the dose of 1 ml, three animals were challenged intramuscularly, three subcutaneously while four guinea pigs (two for each group) were kept uninjected as control. Both groups were kept in different cages and followed for development of any clinical and postmortem changes for three weeks after challenging (Chhabra *et al.*, 2007; Valera *et al.*, 2008). 1 ml culture suspension after observation of CPE on Vero cell was also inoculated to guinea pigs. Three guinea pigs were inoculated intramuscular while three of them injected subcutaneously. The first three were injected with suspected virus suspension directly from the incubator and the later three, after storage at +4 ^{0}C for 5 days. Both groups were again kept in different cages (ILAR, 1996) with two uninjected guinea pigs as a control for each groups and followed for development of any clinical signs and postmortem changes for 21 days post challenging (Iman *et al.*, 1997; Chhabra *et al.*, 2007; Valera *et al.*, 2008).

Isolation of the virus from laboratory guinea pig

For virus isolation the guinea pig was killed immediately after showing clinical sign. Heart, lung and liver tissues were aseptically collected in universal tube; processed and inoculated in Vero cells according to methods described under 'Isolation of the viral agent on cell line (Dunham and Guthmiller, 2008).

3. 4. Data collection and description

During the study period, the results of all laboratory works were thoroughly recorded. The results of isolation in cell culture; any CPE (appearance, type, character of the virus growing) was registered. Moreover, the results of molecular works (evident amplifications) were also carefully recorded. Any clinical and postmortem changes observed in laboratory animals during the trial were also recorded.

4. RESULTS

To understand etiological agent/s of emerging camel disease in Ethiopia, the findings of the viral investigation in cell culture, agar gel immunodiffusion, molecular techniques and laboratory animal assay were documented and presented below. Since the work was planned to isolate and identify candidate viral cause/s, every findings was thoroughly followed and documented without any underestimation that can be a lesson for ongoing investigation of emerging camel disease. Accordingly, every repeated success and all failures were presented as follows.

4. 1. Cell culture

Four of the five camel tissues that were processed and inoculated on Vero cells have shown visible CPE (Table 5). The CPE from heart, liver and heart blood tissues were observed relatively earlier that is one or two days post inoculation and revealed similar appearance. From passage 2 to 4, the CPE appeared on day 1 and reached approximately 80% on day 3 post inoculation. The CPE was invasive in character which invades all the neighboring cells after starting from certain focal cell groups. Very large amount of round floating cells, giant cells, rounded refractive cells, some syncytia formation (aggregates of cells) and ballooning of cells were the main features of the CPE. As time progresses, sloughing of cells, that is the monolayer cells were detached from the wall of the flask was seen. Generally, the cells were observed to be severely damaged within seven days post inoculation (PI) (Figure 4).

For one of the lung tissue, CPE was observed late after a week suggesting that relatively slower invasion and/or multiplication of the agent was observed in that case. The appearance of the CPE was similar to the others. However, it was possible in this case to observe the architecture of the monolayer even after 14 days PI which was impossible in the others. The second lung tissue shows no visible CPE (Table 5). The uninfected monolayer of Vero cells that were kept as a control have shown consistency in morphology due course except becoming older cells as time progresses, no CPE and the monolayer cells was available even after 14 days (Figure 4).

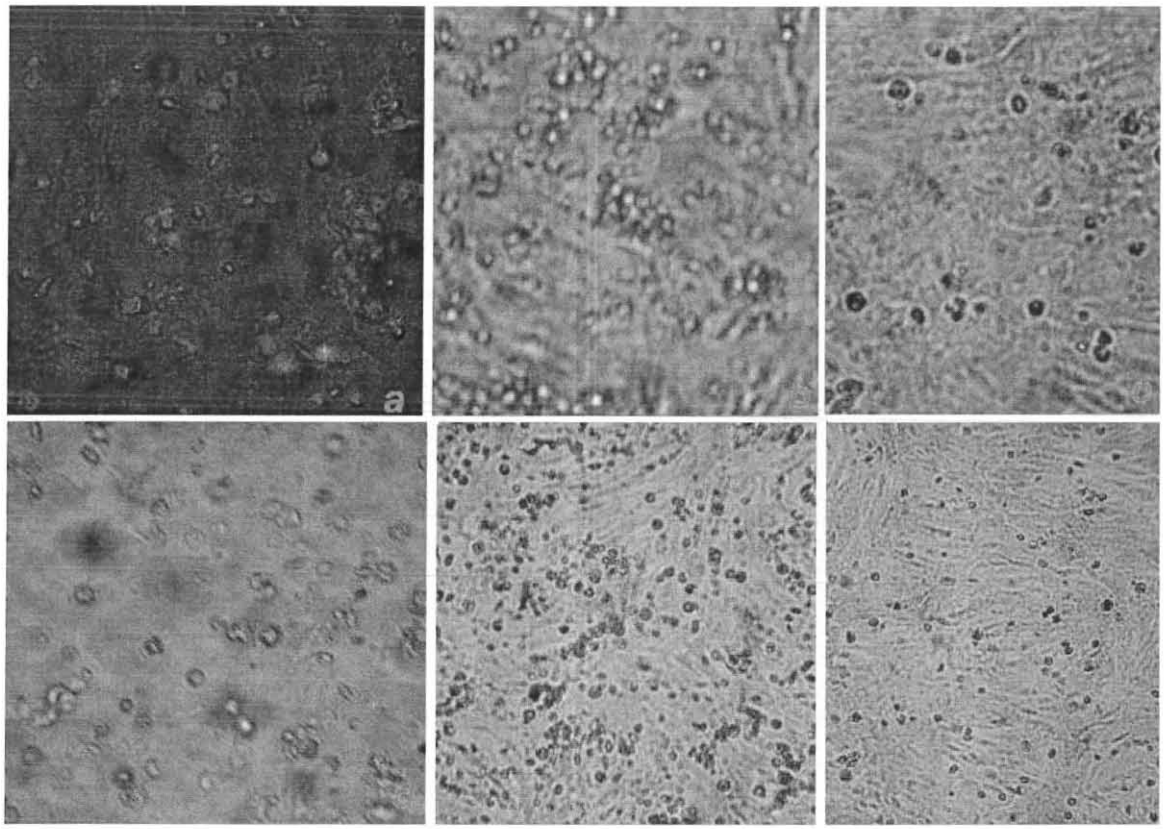
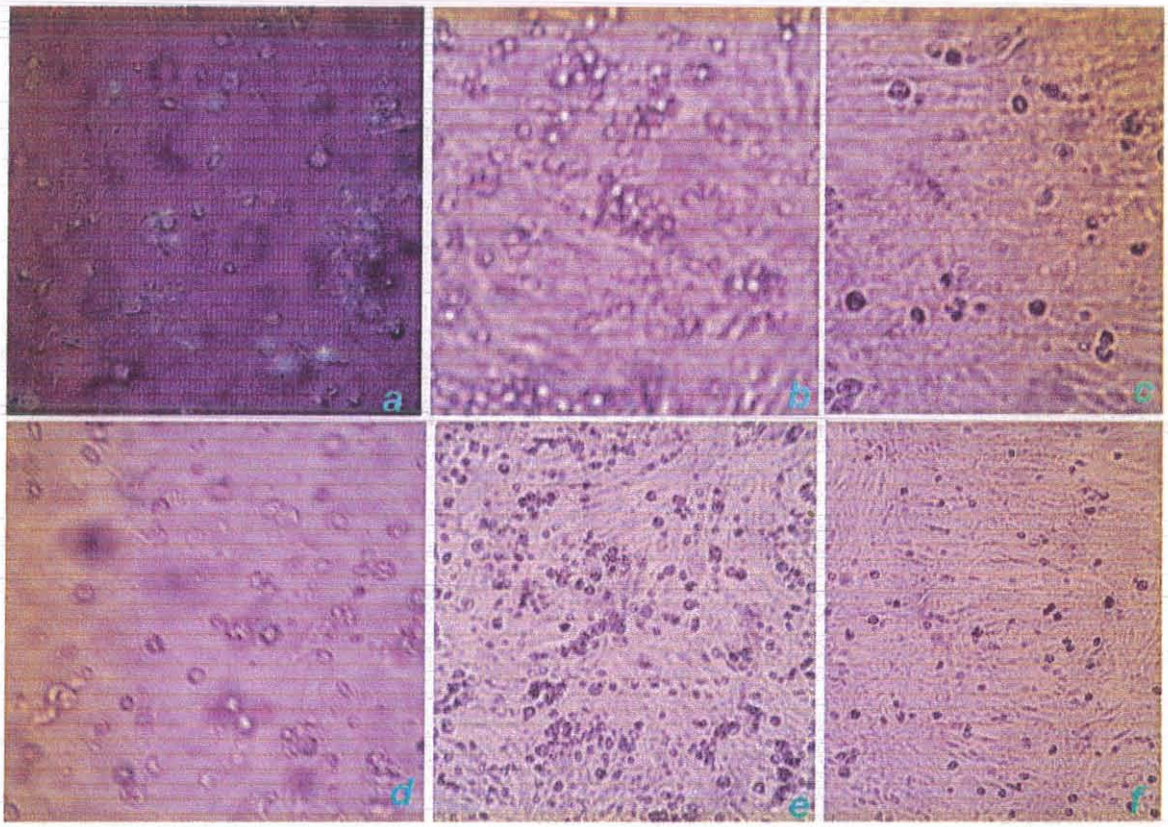


Figure 4: Cytopathic effect of unknown virus from different tissues of camel on Vero cells.

Note the round refractive floating cells and ballooning of cells (Figures a and b), sloughing of cells /detachment of monolayer (Figures a and d) and some syncytia formation (Figures b and e). Figure a: heart blood culture on Vero, 100x magnification after 7 days, Figure b: liver tissue culture on Vero, 100x magnification after 3 days; Figure d: lung tissue culture on Vero, 100x magnification after 7 days; Figure e: liver tissue culture on Vero, 40x magnification after 3 days; Figures c and f: a week old non inoculated Vero cells kept as a control, 100x and 40x magnification respectively. All pictures were taken by separate digital camera, Digital IXUS 200 IS (Canon, Japan).

The virus was also evaluated for its growing effect on cell line after storage under three sets of temperature; +4 ^{0}C, -20 ^{0}C and -85 ^{0}C. In all cases passaging directly from incubator without freezing and thawing produced more CPE rapidly. In addition, passaging from +4 ^{0}C or -85 ^{0}C to new monolayer cell indicated promising growth. Without freezing and thawing, as the number of passage increased the CPE was also observed to increase intensively. However, passaging after freezing under -20 ^{0}C and thawing showed no CPE or very rare CPE.

There was also growth observation on camel originated cell line (Dubca). In this case, the same as in Vero; round floating cells, giant cells, rounded refractive cells, some syncytia formation and detachment of monolayer were observed. However, on Dubca cells CPE was observed



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relatively earlier (24 hours after inoculation) in more extensive manner. The Dubca cell line was fast growing and form monolayer earlier and it became older very soon within five days. Hence, to avoid any confusion the old cells were carefully distinguished from proper CPE formed by comparing the inoculated Dubca cell with the uninoculated control Dubca cell.

4. 2. Antigen detection

The antigen from cell culture suspension was tested against known hyperimmune serum of AdV, AHSV, BTV, FMDV, PIV1-3, PPRV, RP and RSV viruses. The test was observed for 24 hours and the antigen did not show any line of precipitation between the wells that form a line of identity with the controls.

4. 3. Molecular methods

The results of all the molecular methods, namely, DOP-PCR and specific PCR tests for respiratory viruses and morbilliviruses were presented in this part. For all molecular tests, nucleic acids extracted from five tissues and their culture suspensions were tested.

4. 3. 1. Degenerate oligonucleotide primed PCR result

A DOP-PCR protocol developed originally for nonspecific amplification of large genomes, was adapted to amplify viral nucleic acids nonspecifically. The test utilized five different primers with different detection capacity to look for more efficient alternative. Primer 1, 2 and 3 had shown better amplification while the rest primers hardly amplified the template (Figure 5). In two steps of RT-PCR, the tissue samples and their cultures which produced CPEs indicated amplification (smear) in DOP-PCR (Table 5). The tissue (lung) that was negative on culture and its culture also did not show amplification (Figure 6).

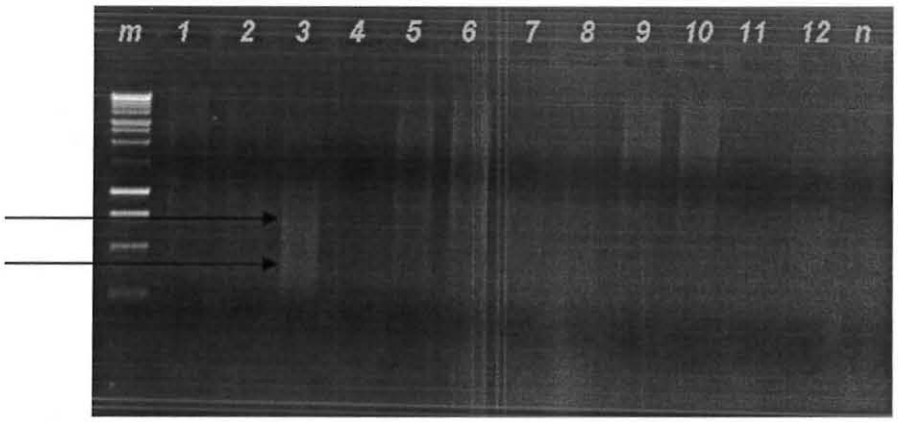


Figure 5: Two step RT-PCR for detection of RNA virus using 3 different universal primers.
Lanes: n, template-free negative control; 1, 2, 5, 6, 9 and 10 were cDNAs from tissue suspensions; 3, 4, 7, 8, 11 and 12 were cDNAs from their respective culture suspensions; m, 1,000 bp ladder DNA marker (Invitrogen). Lanes: 1-4, amplification using universal primer 1; Lanes 5-8, amplification using universal primer 2; Lanes 9-12, amplification using universal primer 3. Note the arrow indicating specific band within the smear.

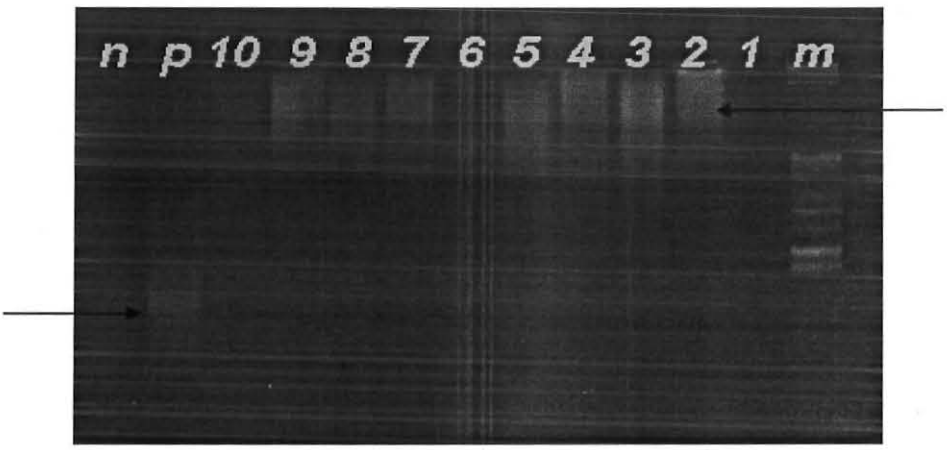


Figure 6: Two step RT-PCR for detection of RNA virus using universal primer 2.
Lane: n, template free negative control; Lane p, positive control cDNAs of PPRV from NVI; Lanes 1-5, cDNAs from lung, heart, liver, heart blood and lung suspensions respectively; Lanes 6-10, cDNAs from lung, heart, liver and heart blood culture suspensions respectively; Lane m, 1,000 bp ladder DNA marker (Invitrogen). Note the arrow indicating specific band within the smear.

DOP-PCR for extracted DNA of suspected DNA viruses show amplification only in tissue samples but not in culture suspensions which is different from the case for RNA viruses. Two lungs and liver tissues indicated amplification on DOP-PCR for DNA virus (Figure 7).

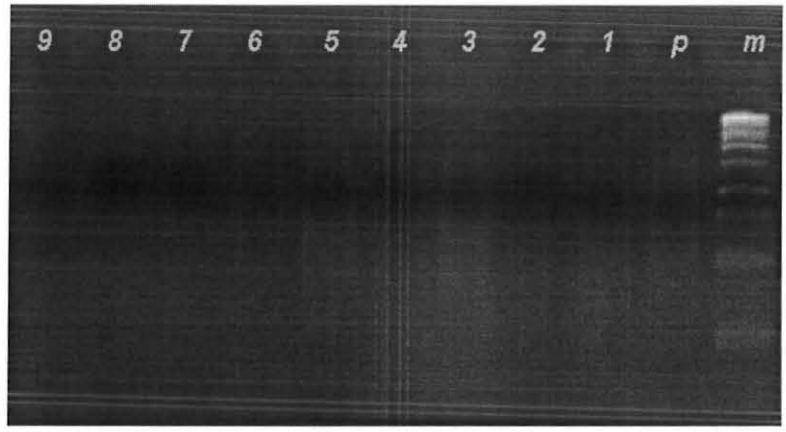


Figure 7: Conventional PCR for detection of DNA virus using universal primer 2.
Lanes 1-5, extracted DNA from lung, heart, liver, heart blood and lung tissue suspensions respectively; Lanes 6-9, extracted DNA from lung, heart, liver and heart blood culture suspensions respectively; Lane p, positive control DNA of sheep pox virus from NVI; Lane m, 1,000-bp ladder DNA marker (Invitrogen); Lane n, in figure 6 represents template free negative control (all reactions were run together).

Table 5: Growth of virus on two cell lines and amplification by DOP PCR.

Animal No	Tissue type	Growth on Vero	Growth on Dubca	Amplification by DOP PCR			
				DNA		RNA	
				tissue	culture	tissue	culture
1	lung	_	nt	+	_	_	_
2	heart	+	+	_	_	++	++
3	liver	+	+	+	_	++	++
4	heart blood	+	+	_	_	++	++
5	lung	+	nt	+	_	++	++

nt= no trial, += weak positive, ++= strong positive and _ = negative

Different optimization experiments were conducted inorder to get detectable amplification products. Increasing or decreasing the concentration of Taq DNA polymerase, KCl, $MgCl_2$, dNTP and primers; and optimization of annealing temperature were among the measures taken for PCR optimization. Optimization was also aimed to get a specific band within the smear-like result shown. However, as expected no specific band except the smear was observed in every trial of optimization (Figure 8).

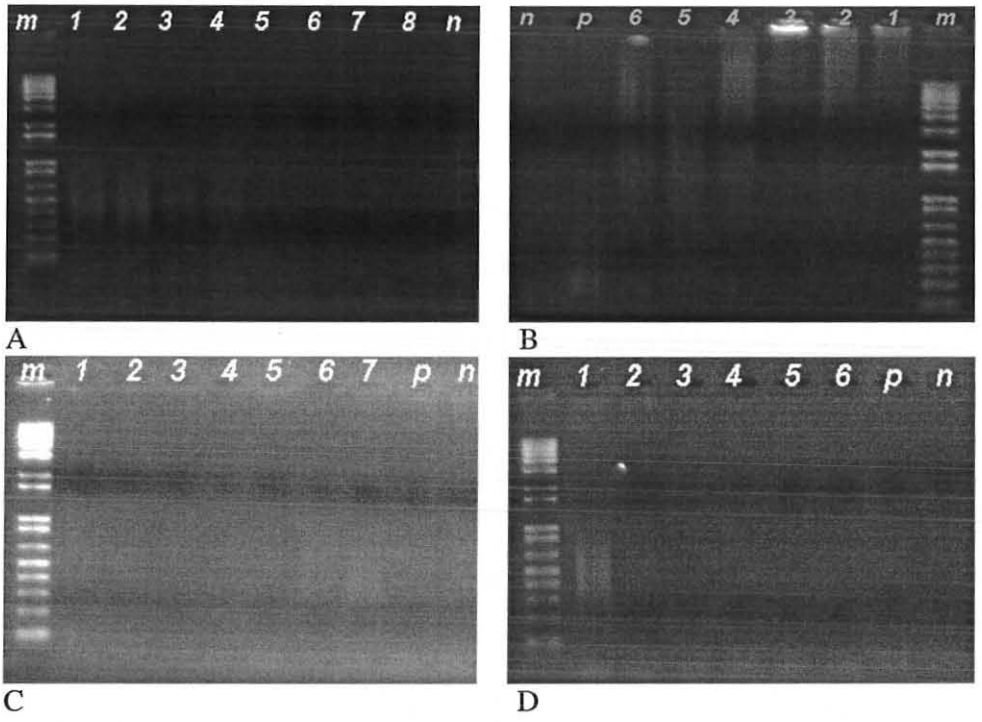


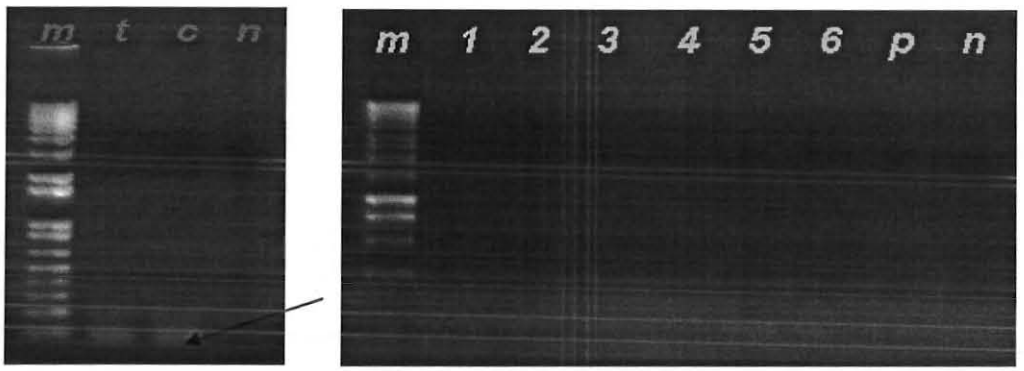
Figure 8: Optimization of DOP-PCR.

A, two sets of $MgCl_2$ concentration was used. Lanes 1-4, amplification using the same $MgCl_2$ concentration while in lanes 5-8, less $MgCl_2$ concentration (1.5 mM) was used, lane n was a template free negative control; B, DOP-PCR using the cDNA and amplicon. Lanes 1-3, optimized DOP-PCR amplification from cDNA synthesized from extracted RNA, lanes 4-6, optimized DOP-PCR amplification using PCR product as a template, lane p, was for positive control and lane n, was for a template free negative control; C, annealing temperature of other than 55, that is, 57 has shown no amplification (the same to when the annealing temperature was decreases to 53); D, different dNTP concentrations was added to the reaction, higher (lanes 3 and 4) and lower (lanes 5 and 6) concentration of dNTP than 200μM (lanes 1 and 2) have indicated lower or no amplifications.

4. 3. 2. Specific PCR for respiratory viruses

Multiplex RT-PCR based attempts were done to detect the presence of nucleic acid from five different respiratory viruses. RNAs extracted from the tissue suspension and their respective cell culture suspensions were reverse transcribed and amplified under the conditions described in materials and methods. The expected sizes of amplification were 84-, 164-, 215-, 234- and 348-bp for PIV1, PIV2, AdV1-7, PIV3 and RSV, respectively. However, no evident specific bands were observed during the test for samples originated from outbreak cases of disease in camels.

Primer dimmers were the only visible bands at the lower size (Figure 9a) which confuses with the size of PIV1 size. To remove this confusion, samples were tested individually for PIV1-3 using two steps RT-PCR. Similarly, no specific band, but primer dimers bands, was observed for all reactions (Figure 9b).



9a 9b

Figure 9a: Single-tube multiplex RT-PCR for detection of five respiratory virus types.
Lane n, template-free negative control; lane c, pooled cDNA from culture suspensions; lane t, pooled cDNA from tissue suspensions; lane m, 1,000 bp ladder DNA marker (Invitrogen). Note the arrow as primer dimmer.

Figure 9b: Two step RT-PCR for detection of PIV1-3.
Lanes n, template free negative control; p, positive control cDNA of suspected PIV3 from NVI showing no amplification;1, 3 and 5, pooled cDNAs from tissue suspensions tested for PIV1, PIV2 and PIV3 respectively; 2, 4 and 6 pooled cDNAs from culture suspensions tested for PIV1, PIV2 and PIV3 respectively; m, 1,000 bp ladder DNA marker (Invitrogen).

4. 3. 3. Specific PCR for Morbillivirus

The RNAs extracted from all the tissue suspensions and their cultures were checked for the presence of *morbillivirus* nucleic acids using a two step RT-PCR. The results of the amplification indicated no PCR amplification products for all samples. The cDNA from RNA of PPR virus vaccine strain of NVI was used as a positive control and it showed a thick specific band (Figure 10).

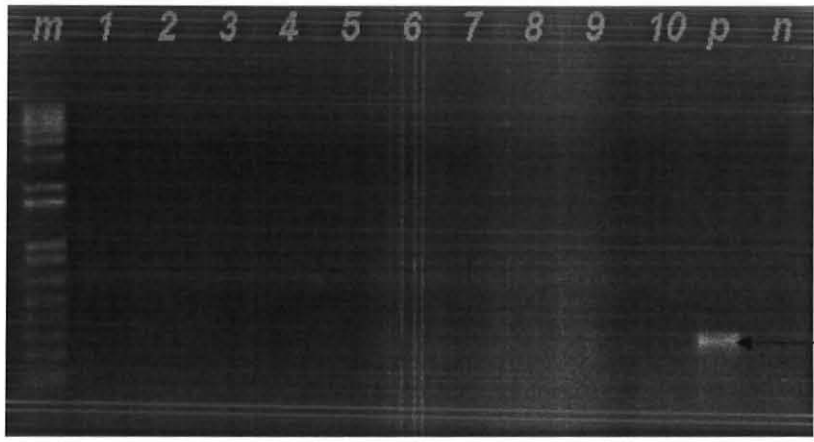


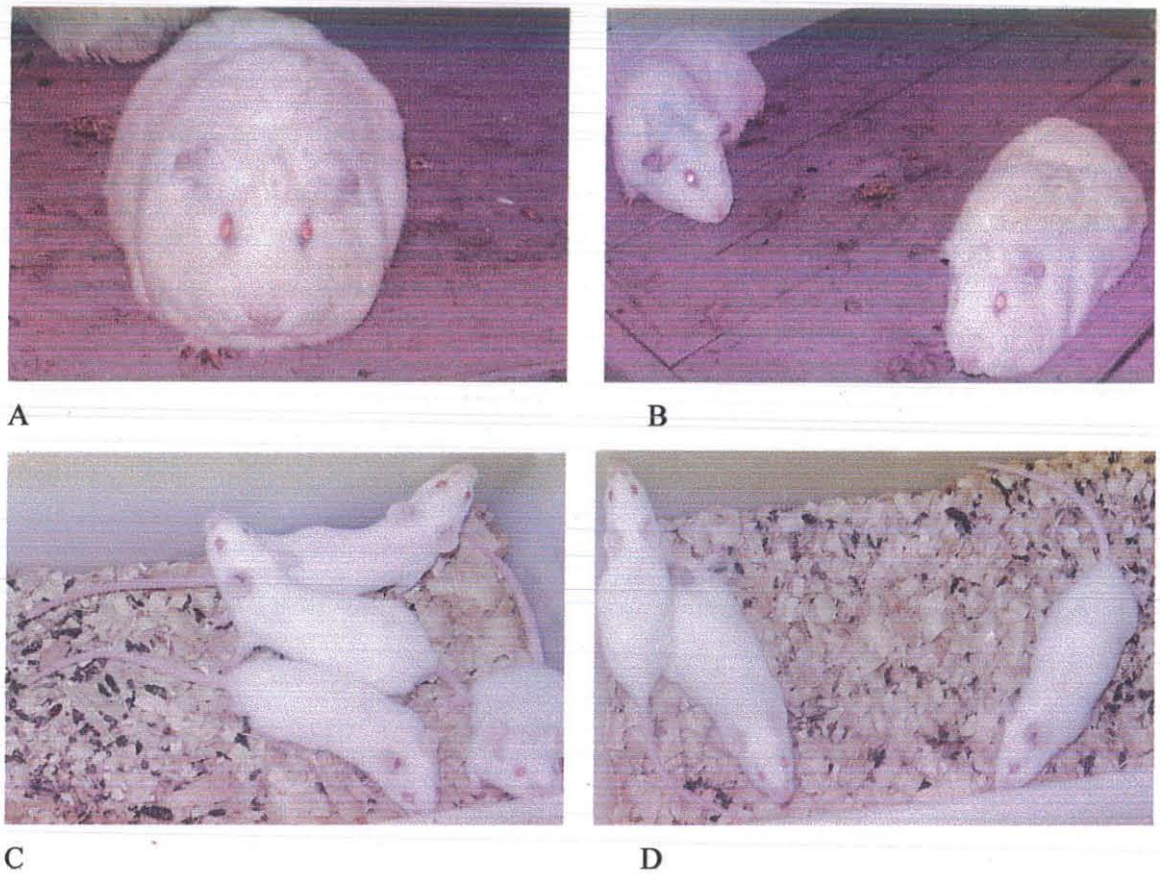
Figure 10: Two step RT-PCR for detection of Morbillivirus.

Lanes: n, template free negative control; p, positive control PPRV from NVI; 1-5, cDNA from tissue suspensions; 6-10, cDNA from culture suspensions; m, 1,000-bp ladder DNA marker (Invitrogen). Note the arrow indicating specific band between 200 bp and 300 bp for positive control.

4. 4. Laboratory animal assay

Inoculation of tissue suspensions into both mice and guinea pigs was resulted in no any desirable clinical changes. The animals were followed for three weeks and they were fast growing the same as their control groups and were shown good health conditions with no clinical problems recorded. They were finally killed to look for any postmortem findings but no differences were observed during postmortem examination.

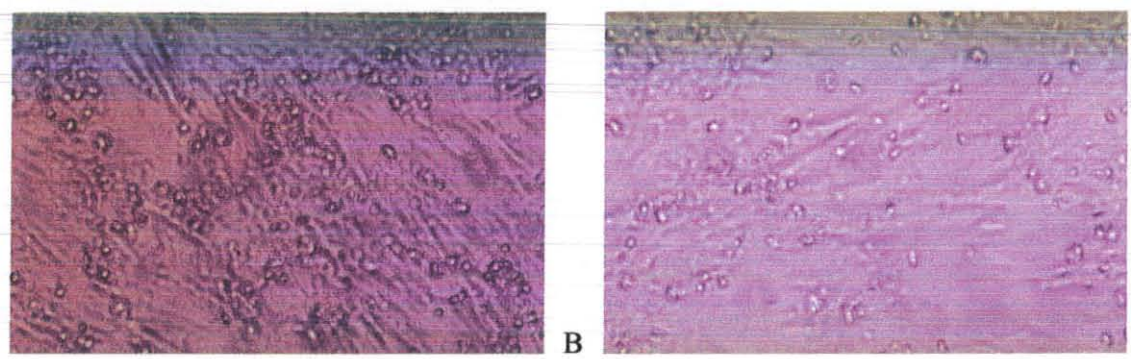
The other group of mice which were challenged with the cell culture suspension after CPE observation also showed no any clinical signs. However, the guinea pigs injected with the same culture suspension showed abnormalities. They were showed depression, weakness, inappetance, rising and rough hair, rise in body temperature as well as heart beat and fast and labored breathing starting from the third day of PI onwards. The assay was resulted in death of six guinea pigs after 20 days of PI. One group of animals was challenged with culture suspensions directly from incubator while the other group was injected with the same suspension after storage in +4 ^{0}C for days. In both groups they were showing the same clinical abnormalities that were mentioned earlier. The control animals were died between 29 to 32 days post contact without showing any clinical signs of disease (Figure 11).



A B C D

Figure 11: Pictures on results of two different laboratory animal assays.
A, challenged guinea pig showing clinical sign; B, challenged guinea pig showing clinical sign (right) compared with non injected and active one as a control (left); C, non challenged group of mice as a control; D, injected group of mice but showing no clinical sign.

Virus was isolated on Vero cell from guinea pig heart and liver suspensions that was obtained by killing immediately after observation of clinical sign. The CPE was recognized to be similar to the first (Figure 4) from which their suspensions were injected to guinea pigs (Figure 12).



A B

Figure 12: Virus isolation from guinea pig on Vero cell. A= heart and B= liver.

5. DISCUSSION

The isolation of different viruses from camel tissues in different types of cell cultures has been documented (Shaker, 2003). Viruses which have been shown to play a role in infections of camelids includes camelpox virus (CaPV), bovine herpesvirus-1 (BHV1), vesicular stomatitis virus (VSV) and border disease virus (BDV) that were propagated in Dubaca to determine the viral host range for the cell line (Klopries *et al.*, 1995). A camelpox virus from Dubai was isolated in Vero (MA104) and Dubaca cells (Pfeffer *et al.*, 1996) while the susceptibility of various cell lines [like BHK 21 (baby hamster kidney cells), E-Derm (equine dermal fibroblasts), HEP 2 (human epidennoid carcinoma cells), L929 (mouse fibroblasts), MDBK (Madin Darby bovine kidney cells) and Vero] was recorded for the same virus and found to be susceptible (Renner-Muller *et al.*, 1995). Pestiviruses, cytopathic genotype 2 bovine viral diarrhea virus was isolated in Egypt from camels and was propagated on MDBK cells (Hegazy *et al.*, 1995; Yousif *et al.*, 2004). Isolation of camel group A rotavirus in cell culture was also described; the virus was successfully propagated in MA104 cells (Ali *et al.*, 2008). Nawal *et al.* (2003) and recently Intisar *et al.* (2010a) also reported the isolation of PIV3 from camels lung using MDBK cells.

In the present study, the concentrated tissue suspensions from outbreak cases of camel were inoculated into Vero cells for virus isolation. Out of 5 samples, 4 showed a progressive CPE and 1 was negative. Observation of virus propagation from camel tissue on Vero cell agrees with previous works (Klopries *et al.*, 1995; Renner-Muller *et al.*, 1995; Ali *et al.*, 2008). However, this result may contradict with the report that detected no isolation of virus from pathological tissues of camel in Ethiopia from mid 1990's outbreak cases (Roger *et al.*, 2001; Chauhan *et al.*, 2009). Isolation of a virus from diseased and died camel tissue may indicate the virus as a causative agent for the death or it could also indicate the presence of latent infection in which the cause of the disease might be another agent. However, the former might be true as the isolate was observed to be killer in laboratory guinea pig, the role model of mammalian species that was challenged with virus culture suspensions. The death of control guinea pigs indicates that the disease could be transmitted through contact. Laboratory mice were not diseased or killed by the same culture suspension that was injected to guinea pigs. The reason might be because of the species selectivity of the virus under study (Chhabra *et al.*, 2007).

The typical CPE of the propagated virus observed included very large amount of round floating cells, giant cells, rounded refractive cells and sloughing with some syncytia formation. From passage 2 to 4, the CPE appeared on day 1 and reached approximately 80% on day 3 post inoculation. In all propagated isolates except in a lung tissue it was noticed that the cell sheet detached completely within a week and there was no difference in the CPE detected by all tested samples. However, it was observed that the CPE was seen 6 days later than the others and sloughing of monolayer was rare even after 14 days post inoculation in the lung tissue culture. This might indicate the existence of multiple viral infections in camel tissues. The normal Vero cells and the CPE from camel tissue were shown in Figure 3. The current observation is similar to that described by Intisar *et al.* (2010a). Ali *et al.* (2008) also reported almost similar CPE during their study on isolation of camel group A rotavirus in Vero cell culture. However, contrary to Ali *et al.* (2008) the CPE in this study do not show elongated cells, ring shaped cells and triangular cells but cell sheet detached/ sloughing was observed in this case.

Different approaches have been used for detection of new viruses non-specifically in the presence of substantial amounts of contaminating host DNA. Physical separation of viral nucleic acids from cellular nucleic acids and the non-specific amplification of the viral nucleic acids have been used (Allanders *et al.*, 2001; Nanda *et al.*, 2008). Previous studies reported viral detection methods based upon nuclease digestion of biological samples. A variety of DNA and RNA viruses were detected non-specifically in test samples designed to mimic detection problems that involve virus particles against a background of cellular nucleic acids (Allander *et al.*, 2001; Jones *et al.*, 2005; Nanda *et al.*, 2008). Purification of viral nucleic acids from viral capsids yields advantages and disadvantages. Because nonencapsidated viral nucleic acid is removed in the process of purification and thus not amplified, some potential sensitivity is lost. The ratio between infectious virions and total virus nucleic acid may vary for different viruses and is determined not only by viral but also by host factors. In samples with low plaque forming unit to nucleic acid ratios, capsid purification could substantially reduce the ability to detect viral nucleic acids. A significant advantage of the capsid purification is that any viral nucleic acid amplified from capsids is more likely to be derived from infectious virus. The elimination of cellular nucleic acids permits the concentration of viral nucleic acids to levels that would not be possible if cellular nucleic acids were retained (Nanda *et al.*, 2008).

Similar to this test, Nanda *et al.* (2008) has reported non-specific detection method (using nuclease digestion, ultracentrifugation and modified primer in DOP-PCR) to detect viruses. In

one of the previous studies (in which a new bovine parvovirus was detected in serum), PCR primers were ligated to purified viral nucleic acids after digestion with a restriction endonuclease (SISPA) (Allander *et al.*, 2001). The same scheme was also used to identify a new human parvovirus (Jones *et al.*, 2005). This approach is most suitable for detection of DNA viruses because digestion with a restriction endonuclease of cDNAs reverse-transcribed from RNA viruses would require second-strand synthesis from cDNA, which is known to be an inefficient process (Jones *et al.*, 2005).

The presence of amplification of viral genetic materials indicates the existence of viral nucleic acids in the camel tissues or their respective culture suspensions from Vero cells. As these tissues were sampled from camels died of outbreaks with unknown causes, virus/es might be the likely causative agent of the death. It could also be the normally occurring viruses as a latent infection in the camel tissue; whereby the main cause of the death was another agent. Although the actual virus needs to be identified, in this research it was ruled out that the etiological agent/s of sudden camel mortality that occurred in 2007 in Southern Ethiopia was not a member of the genus *Morbillivirus* and five respiratory viruses namely AdV, PIV1, PIV2, PIV3 and RSV that are detectable by existing primers. But a variant of these viruses that can escape the detection by use of known primers like that of Newcastle disease viruses (Kim *et al.*, 2007) might not be ruled out. Different viral nucleic acids were detected so far from camel tissues using specific primer amplification. The nucleic acids of many viruses like BHV1 (Intisar *et al.*, 2009), PIV3 (Intisar *et al.*, 2010a), RSV (Intisar *et al.*, 2010b), PPRV (Chauhan, *et al.*, 2009), Equine rhinitis A virus (ERVA) (Wernery *et al.*, 2008), Orthopoxvirus (OPV) (Kinne *et al.*, 1998) and Parapoxvirus (PPV) (Khalafalla *et al.*, 2005) were detected so far from camel samples. Therefore, the detection of viral nucleic acid in camel in the present study was not unexpected as they are expected to have some pathogens. However, we could expect more than the already detected viral nucleic acid types because there are many viruses for which their specific antibodies were detected in camel (Shaker, 2003; Intisar *et al.*, 2010b). On top of that, many camel infections are not well known and many disease causing viral pathogens are emerging (Dirie and Abdurahman, 2003; Domingo, 2010). Hence the probability of detecting new unidentified viruses might be occurring.

Figure (6) indicates higher amplification where the cDNAs from extracted RNA was used as template. This might show that the virus in the tissue belongs to RNA virus. This finding is similar to that of previous studies by Intisar *et al.* (2010a, 2010b), Wernery *et al.* (2008) and

Chauhan, *et al.* (2009) who detected PIV3, RSV, ERVA and PPRV nucleic acid, respectively. Surprisingly, no amplification was seen from culture when tested for DNA viruses. However, three DNA extract from the samples indicated the evidence of amplification which might indicate the presence of DNA viruses in the tissue but not in the culture. Although most of the nucleic acids detected from camel are of RNA type, the nucleic acid, DNA of OPV (Kinne *et al.*, 1998), PPV (Khalafalla *et al.*, 2005) and BHV1 (Intisar *et al.*, 2009) were also reported.

Evidence for a significant role of respiratory infections in camels had been reported. For example, Dioli and Stimmelmary (1992) reported that viruses that had been associated with respiratory infections in camels were AdV, BHV1, influenza viruses A and B, PIV3 and RSV. Seroprevalence of viruses causing respiratory diseases in camels reported in many countries with variable estimates: as higher as 99% in Chad (Maurice *et al.*, 1968). Intisar *et al.* (2010a) and Shaker (2003) reported the isolation of PIV3 from camel lung. In camels, RSV antibodies and its antigen were detected previously; for example Olaleye *et al.* (1989) detected RSV antibodies in apparently healthy camels in Nigeria. Based on RT-PCR, nucleic acids from respiratory viruses were also detected from fewer camel tissues (Intisar *et al.*, 2010a; 2010b).

The multiplex RT-PCR was capable of detecting five major respiratory virus types in a single pooled cDNA without requiring further hybridization steps for identification. Despite the presence of five primers sets within the reaction mixture, the RT-PCR assay was able to sensitively and specifically detect all virus types tested. All amplified products were presented and easily distinguishable following single-tube multiplex RT-PCR (Osiowy, 1998). However, in this study the absence of the nucleic acids of all five respiratory viruses was proved. The failure to detect nucleic acids of five respiratory viruses could be because the viruses were not the cause of the death of camels at that time. The infection caused by respiratory viruses generally runs a clinical course of 3–4 days with complete recovery. However, infection with some respiratory viruses appears to predispose the host to secondary bacterial infection and they have also been shown to play a role in shipping fever (Blood and Henderson, 1976; Murphy *et al.*, 1999). From the clinical observations during the outbreak of 2007, the disease was acute in nature involving no respiratory signs (Daniel and Solomon, 2007).

Different researches have been done to detect the presence of *morbillivirus* like PPRV and RPV infections in camels (Roger *et al.*, 2001). The role of PPRV in the production of disease in camel was hardly indicated; ''respiratory diseases'' in camel caused by PPRV should not be

ignored (Roger *et al.*, 2001; Abraham *et al.*, 2005). According to Abraham *et al.* (2005) 10% seroprevalence of PPRV antibodies was registered in camels from Afar, Ethiopia. Similarly, 4.2% (Ismail *et al.*, 1992) and 14% (Haroun *et al.*, 2002) seroprevalence of PPRV was determined in Egypt and Sudan, respectively. Roger *et al.* (2001) reported the PPRV antigen detection from samples collected during the 1995 outbreak of respiratory disease of camels in Ethiopia in mid 1990's. However, Albayrak and Gur (2010) from Turkey reported the absence of PPRV antibody from camel samples in their study.

The absence of *Morbillivirus* amplification in this study indicates the actual absence of *morbillivirus* nucleic acid in the sample collected during the outbreak and/or failure in detection limit of the existing primer because the currently used *morbillivirus* primers might not detect a variant of such viruses. This result might also indicate the reason of the death of the camel at the time could not be morbilliviruses that can be detected by existing primers. The output of this study disagrees with the report of outbreak investigation team during the year 2007 outbreak of camel in Ethiopia. In addition, the study does not coincide with the conclusion of Roger *et al.* (2001) which suspected PPR virus for the reason of the death of many camels; and Chauhan *et al.* (2009) which reported detection of PPR virus nucleic acid in some pathological samples collected during outbreak of the year 1995 provided that the outbreaks were caused by the same etiological agent. However, the present study shows similar indications with the seroprevalence study of Albayrak and Gur (2010) which recorded the absence of PPR virus antibody in camel of Turkey.

Different viral antigens were detected previously from camel (Olaleye *et al.*, 1989; Shaker, 2003). In the current study, the virus in question was detected for its antigen by different known serum. Apart from AdV, PIV1-3, PPRV, RPV and RSV which were also tested for their nucleic acids in the sample, AHS, BTV and FMD were tested for their antigens in the sample. In all cases, by AGID test no antigens were found to cross-react with any one of them. Although the test is not sensitive, the result could help as a clue to rule out that the virus under study might not belongs to these viral groups and this agrees with the nucleic acid test results (Intisar *et al.*, 2010a, 2010b).

6. CONCLUSION AND RECOMMENDATIONS

Ethiopia hosts about 2.3 million heads of camel, which are found in the arid and semiarid regions of the country. The importance of camel in this country cannot be underestimated as camels serve as a means of survival particularly during drought for better milk production compared to other livestock species. The country has been hosting different camel disease outbreaks that led to death of many camels. Of the many diseases of camels that are rampant in the region, the recently emerged camel disease with still unknown causes is the single most important disease with huge mortality. The disease seems to occur cyclically and no identified causative agents were fully determined yet. In the current study, virus was isolated from tissue of camels died of the unknown disease using cell culture technique and the nucleic acids of the unknown virus was identified using degenerate oligonucleotide primed polymerase chain reaction. The isolated virus belongs to RNA virus group and it was also characterized for its growth characters under different conditions. The isolated virus was confirmed to be out of the well known viruses of genus Morbillivirus and five respiratory viruses: adenovirus, parainfluenza virus 1-3, and respiratory syncytial virus. Although this virus is not yet specifically identified, it was confirmed for its pathogenicity on laboratory guinea pig but not pathogenic for laboratory mice. On the basis of this remark, the following points were recommended:

- The isolated virus should be further characterized and specifically identified.
- Pathogenicity of the isolates in camel should be determined to establish Koch's postulate if possible.
- Further research should be initiated towards creating possible preventive and/or control options such as the development of diagnostic tests and production of vaccine from the viral isolate.

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8. ANNEX

Annex 1: Agarose gel preparation protocol.

1. To make a 1.2% gel, add 1.40 gm agarose to 120ml 1XTAE buffer.
2. Microwave twice for 3 minutes, to completely dissolve the gel.
3. Let the gel cool to 45 ^{0}C under tap water and add 6 µl of Ethidium bromide.
4. Pour into comb fixed gel tray, let cool for 30 minutes at room temperature.
5. Use 5 µl of your PCR product and 2 µl loading dye to check amplifications on the gel.

Annex 2: Cell culture result registration sheet filled partially.

Sample 1, LUNG on Vero cell line				**Oct 26 2009**
Day	**contamination**	**confluence**	**CPE**	**Other remarks**
1	x	>95%	x	
2	x	confluent	x	
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14	x	few free spaces	suspicious	Older cells

Annex 3: DNA Extraction protocol using Qiagen DNeasy kit.

Note the number of spin columns used. The centrifuge can hold up to 12 samples at once.

Procedures:

1. Place 200 µl of a suitable tissue or /and culture suspension in a UV-crosslinked 1.5 ml tube.
2. Add 180 µl Buffer ATL and 20 µl Proteinase K and vortex.
3. Place in the 56°C incubator/water bath for 1 hours or overnight.
4. Remove from incubator, vortex, add 200 µl Buffer AL and vortex.
5. Place in heat block at 70 ^{0}C for 10 minutes.
6. Add 200 µl 100% Ethanol and vortex before transferring entire volume onto spin column.
7. Centrifuge at 10000 rpm for 1 minute; discard flow through and the collection tube. New collection tube changed.
8. Add 500 µl Buffer AW1 and centrifuge at 10000 rpm for 1 minute; discard flow through as well as the collection tube. Change the new collection tube.
9. Add 500 µl Buffer AW2 and centrifuge at 13400 rpm for 3 minutes; discard flow through.
10. Place spin column on UV-crosslinked 1.5 ml tube and add 50 µl buffer AE. Let sit for 1 minute at room temperature, then centrifuge at 10000 rpm for 1 minute.
11. Repeat step 10 with the flow through for a total volume of 50 µl DNA extracts.
12. Label the DNA with date, virus name, and name of the extractor and store it under -20 for further utilization.

Annex 4: Glasgow Minimum Essential Media (GMEM) preparation.

GMEM is the modification of Eagle's Minimum Essential Media and additionally contains 10% tryptose phosphate and two times vitamins and amino acids than Eagle's. It is hygroscopic in nature.

Composition of GMEM

Component	Amount in gm/l
CaCl2(anhydrous)	0.2
Ferric nitrate. 9H2O	0.0001
Mg2 SO4 (anhydrous)	0.09767
KCl	0.4
NaCl	6.4
Na2PO4	0.1078
L. Arginine. HCl	0.042
L. Cystine.2HCl	0.03129
L. Glutamine	0.0292
L. Histidine. HClH2O	0.021
L. Isoleucine	0.0524
L. Leucine	0.0524
L. Lysine.HCl	0.0731
L. Methionine	0.015
L. Phenylalanine	0.033
L. Thereonine	0.0476
L. Tryptophane	0.008
L. Tyrosine.2Na.2H2O	0.05219
L. Valine	0.0468
Choline Chloride	0.002
Folic Acid	0.002
Myo- Inositol	0.0036
Niacinamide	0.002
D-Pentothenic Acid(Hemicalcium)	0.002
Pyridoxal. HCl	0.002
Ribofilavin	0.0002
Thiamine.HCl	0.002
D-Glucose	4.5
Phenol red-Na	0.016

GMEM preparation protocol

1. Weigh the powdered GMEM, at the rate of 125 gm for 10 L fluid media preparation.
2. While stirring the water (should have temperature between 15 ^{0}C and 20 ^{0}C), 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 $NaHCO_3$ or 36.7 ml of $NaHCO_3$ 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 2 ^{0}C to 8 ^{0}C in dark.

Annex 5: Propagation of Cells by enzymatic Treatment of Cells; Trypsinization.

Materials and Preparation

Vero cells, phosphate buffer saline (PBS) without calcium and magnesium, 1.25% trypsin solution in PBS without calcium and magnesium, sterile centrifuge tubes, each sterile 25/75 cm^2 flasks, sterile pippets, GMEM 10/5%FCS, decontamination pans and 37 ^{0}C water bath.

Experimental protocol

1. Warm the trypsin solution, PBS, GMEM 10/5% FCS in a 37 ^{0}C water bath.
2. Check the Vero cells under the inverted microscope.
3. Decant the spent medium and wash the Vero cells 3 times with 5 ml of PBS without calcium or magnesium. All liquids should be decanted into the decontamination pan.
4. Add 5 ml of the 1.25%trypsin solution to the cell monolayer.

5. Place the flask in the incubator for 5 minutes.
6. Gently shake the flask and check under the microscope for cell detachment.
7. Use sterile pippet to transfer the contents of flask to a sterile plastic centrifuge tube and centrifuge at 300x g for 5 minutes.
8. Decant the supernatant in to the decontamination pan and resuspend the pellet in 5 ml of GMEM 10% FCS. Gently pippet up and down to break up cell clumps.
9. Add 10 ml of GEME 10% FCS to the 75 cm flask(s).
10. Do the appropriate cell splits using split ratio, transfer 1 ml for a 1:3 (confluent within 2 days).
11. Cap the flasks and tilt gently to distribute the cells.
12. Label flasks with date, passage number, type of cells and your name and place them in the incubator. Loosen the caps to allow CO_2 to enter.

Annex 6: Total RNA Extraction from Animal Tissues using the Qiagen RNeasy Mini Kit.

Important notes before starting

- Use appropriate amount of tissue and/or culture suspensions.
- β-Mercaptoethanol must be added to Buffer RLT. Add 10 µl β-ME per 1ml of Buffer RLT. The solution is stable for 1 month.

Procedures:

1. Add 460 µl 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 3 min at maximum speed in a microcentrifuge, and transfer the supernatant into a new eppendorf tube.
4. Add 460 µl of 70% ethanol to the cleared lysate, and mix well by vortexing.
5. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml 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 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow through after each centrifugation.

6. Add 700 µl 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 500 µl 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.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to buffer RPE before use.
8. Repeat the wash with 500µl buffer RPE onto RNeasy column, and this time centrifuge for 2 min at maximum speed (13400rpm) to dry the RNeasy membrane. Discard flow through and the collection tube.

   Note: Residual ethanol may interfere with downstream reactions. After centrifugation, remove the RNeasy column so that the column does not contact the flow through as this will result in carryover of ethanol.
9. (Optional): 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 1.5 ml eppendorf tube (remove the lid) and pippet 30-50 µl 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 >30 µg, repeat step 10 using another 30–50 µl RNase free water, or using elute from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10.

    Note: If using the eluate from step 10, the RNA yield will be 15–30% less than that obtained using a second volume of RNase free water, but the final RNA concentration will be higher.
12. Label the RNA with date, virus name, and name of the extractor and store it under -20 for further utilization.

Annex 7: Viral buffer preparation.

Chemicals required are Tris/HCl, $CaCl_2$, Mg acetate (Na acetate in this case), KCl, and EDTA.

Procedures:

1. All chemicals were obtained from NVI chemical store in the powder form.
2. 1M 0f 5 ml of each chemicals were prepared according to mole concept, a molecular weight (in gram) of a compound in a liter of solution.
    - 1M Tris/HCl contain molecular weight of the compound, 121 gm in a liter of solution. Therefore, to prepare 5 ml solution, 121 gm. 5 ml/1000 ml = 0.605gm in 4.4 ml distilled water.
    - 1M $CaCl_2$ contain molecular weight of the compound, 111 gm. Therefore, to prepare 5 ml solution, 111 gm. 5 ml/1000 ml =0.555 gm in 4.445 ml distilled water.
    - 1M Na-acetate contains molecular weight of the compound, 82.03 gm. Therefore, to prepare 5 ml solution, 82.03. 5 ml/1000 ml = 0.41015 gm in 4.59 ml distilled water.
    - 1M KCl contains molecular weight of the compound, 74 gm. Therefore to prepare 5 ml solution, 74 gm. 5 ml/1000 ml = 0.37gm in 4.63 ml distilled water.
    - 1M EDTA contains molecular weight of the compound, 372.2 gm. Therefore to prepare 5ml solution, 372.2 gm. 5 ml/1000 ml= 1.861 gm in 3.139 ml distilled water.
3. Stock solution of viral buffer containing 30mM Tris/HCl pH 7.5, 3.6mM $CaCl_2$, 125mM KCl, 0.5mM EDTA, and 5mM Na acetate was prepared in a universal tube.

    The amount was taken from each 1M solutions using the formula C1V1=C2V2, for example, to take 30mM Tris/HCl in 20 ml volume, using C1V1=C2V2; 1000mM.V1= 30Mm.20 ml
    - V1= 30mM.20 ml/1000mM = 0.6 ml
4. Autoclave the viral buffer for 40 minutes, the viral buffer were cooled and then used.

9. DECLARATION

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: Dejene Milkessa Tufa

Signature: ----------

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