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Investigation of respiratory viruses in camel slaughtered at Addis Ababa Akaki Abattoir, Ethiopia

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Camel is an important domestic animal uniquely adapted to the hot and arid environment, but its contribution to Ethiopian pastoralists is disproportionate to its resource potential due to the presence of various infectious and parasitic diseases in the area. Therefore this study was conducted with an attempt to isolate and characterize respiratory viruses from infected lungs. Accordingly, camel lung tissues were collected from Akaki abattoir and transported to National Veterinary Institute virology laboratory and stored temporarily at -85°C. The samples were processed and inoculated on confluent VERO cells, incubated at 37°C and examined for the development of cytopathogenic effect (CPE) for 4 to 15 days. From thirty five examined samples twenty seven were CPE positive. Ten supernatant samples exhibiting CPE were taken and tested for presence of both DNA and RNA viruses, using universal degenerate oligonucleotide primed– polymerase chain reaction (DOP-PCR) and conventional polymerase chain reaction (PCR) techniques. Five out of six samples tested by DOP-PCR were positive for presence of RNA virus, while only one sample was positive for DNA virus. Nine of ten samples were positive for, *Respiratory syncytial virus* (RSV) and two of six were positive for *Adenovirus*, but all tested samples were negative for Peste Des Petits Ruminants Virus (PPRV) and *Parainfluenza viruses* 1-3.

Key words: Abattoir, camel, pneumonia, respiratory viruses, polymerase chain reaction.

INTRODUCTION

Camels are the most capable animal species in utilizing marginal areas and in survival and production under harsh environmental conditions (Abbas and Tilley, 1990; Schwartz, 1992). The camel is well adapted to the climatic extremes and is well appreciated for its significance in the pastoral economy (Raziq and Younas, 2006). Camels can live in areas that are inhospitable to other domestic animals and therefore have important feature in the capacity of humans to survive and using these drier regions (Dirie and Abdurahman, 2003).

According to FAO statistics (Global Livestock Production and Health Atlas - GLIPHA, 2006), the world population of camels is about 20 million animals, mainly in arid zones, of which 15 million camels live in Africa. Ethiopia is estimated to have the third largest camel herd in the world after Somalia and Sudan, followed by Mauritania and Kenya (FAO, 2008). The camel seems to be spared from the devastating epidemic infections which threaten other livestock species, the animal is however, affected by many other diseases, some of which are unknown to date. Camel diseases that are common with other species of livestock are comparatively well-known, while camel-specific diseases still remain a mystery to the scientific community (Dirie and Abdurahman, 2003). Trypanosomiasis, camel pox, contagious skin necrosis, pneumonia, mange mite infections and internal parasites are major health problems previously reported in camels in different rearing areas (Richard, 1979; Demeke, 1998; Odeh et al., 1999; Bekele, 1999).

Among the numerous diseases, respiratory disorders

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are the major threats to the camel population of Ethiopia (Roger et al., 2000;Roger et al., 2001). However, in Ethiopia, few studies were conducted on the extent of respiratory problems of camels compared with other species of livestock animals (Bekele, 1999). Respiratory diseases are among the emerging problems of camels that are causing considerable loss in production and death (Zubair et al., 2004; Kane et al., 2005). However, etiology of most respiratory diseases of camels have not been determined yet; variety of viruses, fungi, bacteria and parasites have potential to be the possible causes of respiratory outbreaks among camels (Schwartz and Dioli, 1992). These agents may represent risk to camels, other livestock and even human population (Bardonnet et al., 2002; Teshome et al., 2003). Most of the studies on camels were about parasitic infections (Al-Rawashdeh et al., 1999). Al-Tarazi (2001) and other authors had isolated and characterized many bacterial agents from pneumonic camel lungs. However, there is little or no information on possible viral etiologies of camel pneumonia or viral prevalence in camels. Thus, this study was designed to investigate camel respiratory problems associated with viral agents.

MATERIALS AND METHODS

Study population

The study animals were the local camels (*Camelus dromedarius/*Old world camels/one humped camels), slaughtered at the Addis Ababa Akaki abattoir. The camels originated from Borena and Bale zones, Fentale district of East Shoa zone and Meiso district of West Hararghe zone of Oromia regional state, Ethiopia. The camels were mostly adult females and few males whose ages were estimated above 15 years. Lorries were used to bring the camels from their local markets to the abattoir. Totally 389 camels were examined during the period of investigation.

Sampling method and sample collection

The non-random sampling method was used for sampling. At the Addis Ababa-Akaki abattoir average 8 apparently healthy camels were slaughtered each day. Before slaughter, camels were examined for disease symptoms. Following slaughter, lungs with pathological lesions suspected on viral infections were sampled, excluding lesions due to parasitic, pyogenic bacteria, foreign body and tuberculoid cases as described by Shiferaw et al. (2011). Postmortem examinations were assisted by palpation and incision. All carcasses were examined and about 6 g of camel lung tissue samples from lungs suspected on viral infection were aseptically/carefully sliced and taken using sterile scalpel blade and gloves. Each sample was then put into labeled sterile capped universal bottles and the labeled bottles placed in an ice box containing ice packs and transported to the National Veterinary Institute (NVI) virology laboratory for sample processing and cultivation. Fresh tissues were freezed at -85°C until testing.

Virus isolation

Thirty five lung tissue samples collected from the abattoir were processed and cultured on confluent grown VERO cell monolayer. Briefly, 1 g of each sample of lung tissue was washed three times using sterile phosphate buffered saline (PBS) on Petri dish, and then washed tissue was transferred to mortar and cut into small pieces using scissor and minced by sterile scalpel blade. The minced tissues were ground and homogenized using pestle. Nine milliliter of PBS was added to the prepared lung tissues and well mixed. The homogenized tissues were transferred to test tube and centrifuged at 3400 rpm for 10 min and 0.5 ml of the supernatant was inoculated on the confluent VERO cells and incubated at 37°C for 1 h. Following incubation, the inoculated cell lines were washed using PBS and 10ml complete Glasgow Minimum Essential Medium (GMEM) was added and incubated at 37°C to follow-up the development of cytopathogenic effect (CPE).

Molecular characterization methods

Viral DNA/RNA extraction

Samples that revealed CPE was processed using viral nucleic acid extraction technique to determine whether the virus is DNA or RNA. 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 samples were suspended in a locally prepared 1 ml viral buffer (30 mM Tris/HCl pH 7.5, 3.6 mM $CaCl_2$, 5mMNa Acetate, 125 mMKCl and 0.5 mM EDTA), sonicated at 800 speed and 40 mV using Vibro^m cell 72434 ultrasonicator (Bioblock Scientific, Illkirch, France) and incubated at 37°C for 1½ h to further facilitate cell and nuclear membranes disruption. As same time, cellular nucleic acids were digested by treatment with nucleases 10 µlDNase I (100 U/ml, Invitrogen) and 10 µlRNase ONE (100 U/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 the nucleic acid extraction kit (Qiagen) (Allander et al., 2001; Nanda et al., 2008).

RNA extraction

RNA was extracted using QiagenRNeasy mini spin column kit. Accordingly, 460 µl lysis buffer RLT was added to a 1.5 ml eppendorf tube containing 460 µl of infected cultured VERO cell suspension. Four hundred and sixty microliter 70% ethanol was applied to precipitate nucleic acids released from disrupted host VERO cells due to lysis buffer RLT followed by homogenization. The homogenized suspension was transferred to RNeasy spin column and centrifuged where viral nucleic acids released bound to the silica membrane; the fluid part passed through the membrane to the collection tube and the flow through discarded. The nucleic acids bound to the membrane was washed using 700 µl wash buffer RW1 followed by addition of 500 µl RPE buffer and centrifuged to dry the membrane and the flow through discarded. Finally, the nucleic acids bound to the silica membrane were eluted into eppendorf tube using 100 µl RNase free water and the eluted RNA was used for further procedures.

DNA extraction

The viral DNA was extracted from the tissue suspension and cell culture isolates by using DNeasy® mini kit (Qiagen) according to recommended procedures. Briefly, 180 µl of infected cultured cell suspension and 180 µl ATL lysis buffer were added to a 1.5 ml reaction tube and vortexed. Proteinase K was added and incubated at 56°C for 1 ½ h in water bath. 200 µl of each AL buffer and ethyl

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

Primer	Gene[a]	Position[b]	Sequence[c]
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[d]	CCTACGCACGATGTGACCACAGACCG
ADHEX5	Hexon	343–368[d]	GTGTTGTAGGCAGTGCCGGAGTAGGG

Source: Osiowy (1998). [a]N, nucleocapsid gene of RSV; NP, nucleocapsid gene of PIV, [b]Relative to the translation start site. [c]Sequences are shown 5′ to 3′, [d]Positions shown are according to the adenovirus type 5 hexon gene sequences.

alcohol were added and the mixture was transferred to QIAamp DNA mini Spin column. The DNeasy Spin column was placed into a 2 ml collection tube and centrifuged at 8000 rmp for 1 min where the flow through in the collection tube was discarded. The DNA sample bound to the silica gel membrane was washed and the membrane dried using 500 µl of each wash buffers AW1 and AW2 by centrifugation. Finally, the DNA sample bound to membrane was eluted into a 1.5 ml eppendorf tube using 500 µl elution buffer AE, the eluted DNA was stored at -20°C till further processing.

Complementary DNA synthesis

cDNA was synthesized using cDNA synthesizing kit (Invitrogen). The cDNA was synthesized based on the manufacturer protocol (Invitrogen) in 20 µl reaction volume. Primarily, 1 µl 50 µMoligodT primer or random hexamer, 1 µl 10mMdNTPs, 5 µl extracted RNA and DEPC-H_2O to 10 µl were added to 0.5 ml PCR tube and incubated at 65°C for 5 min in thermal cycler (Applied Biosystems) and chilled on ice for 3 min. Then, 10x RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase OUT™ (40U/µl) and Superscript™III RT (200U/µl) were added with the rate of 2, 4, 2, 1 and 1 µl, respectively, and incubated at 25°C for 10 min (only in cases of random hexamer primer) followed by 50°C for 50 min and terminated at 85°C for five minutes. Finally, 1 µl of RNase H was added to each and incubated at 37°C for 20 min and the cDNA was stored at -80°C until analysed.

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

A master mix containing 35.1µl RNase free water, 5 µl of 10X Dream Taq buffer, 1.5 µl of 25 mM $MgCl_2$, 1 µl of 10 X mMdNTPs, 1 µl of universal primer (5'-CTCGAG*NNNNNN*ATGTGG-3') and 0.5 µl Taq DNA polymerase was prepared for each reaction. Then 45 µl of the master mix and 5 µl of the cDNA sample were added to an eppendorf tube. Once all the reagents were mixed, the reaction tube was placed in the PCR machine. The amplification was carried out according to the following programme: A cycle of initial denaturation at 95°C for 5 min, 5 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min with final extension at 72°C for 7 min. 10 µl of PCR products and 2 µl loading dye were mixed and loaded into the agarose gel wells (1.5%) and then electrophoresis and analyzed by UV trans illuminator gel documentation system.

Conventional PCR

Conventional PCR was performed for *Parainfluenza virus* 1, *Parainfluenza virus* 2, *Parainfluenza virus* 3, *Respiratory syncytial virus* (RSV) and *Adenovirus* using specific primers (Table 1). *Peste des petitsruminants virus* (PPRV) was also investigated using PPRV specific forward and reverse primers sequences: NP4 (5'-CCTCCTCCTGGTCCTCCAGAATCT-3') and NP3 (3'-TCTCGGAAATCGCCTCACAGACTG-5'), respectively. PCR reaction was carried out in a total volume of 50µl in a 0.2 ml reaction tube containing 32.6 µl RNase free water, 5µl of 10X Dream Taq buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mMdNTPs,1 µl of each forward and reverse primers, 0.4 µl of dream Taq DNA polymerase enzyme and 10 µl of the cDNA/DNA.

PCR product analysis by 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 and the size was determined by the DNA molecular weight marker standard (Osiowy, 1998).

RESULTS

Pathological findings

During the study period, 389 apparently healthy camels were examined before slaughter in the local plant. Thirty five of them were found with symptoms that justify the suspicion on viral etiology of pneumonia. The nature of most frequently encountered observed pathological lesions were acutely and moderately inflamed lungs and a few of them included chronically lesion, interstitially pneumonic, hemorrhagic, hepatized, atelectatic and adhesive lung lesions.

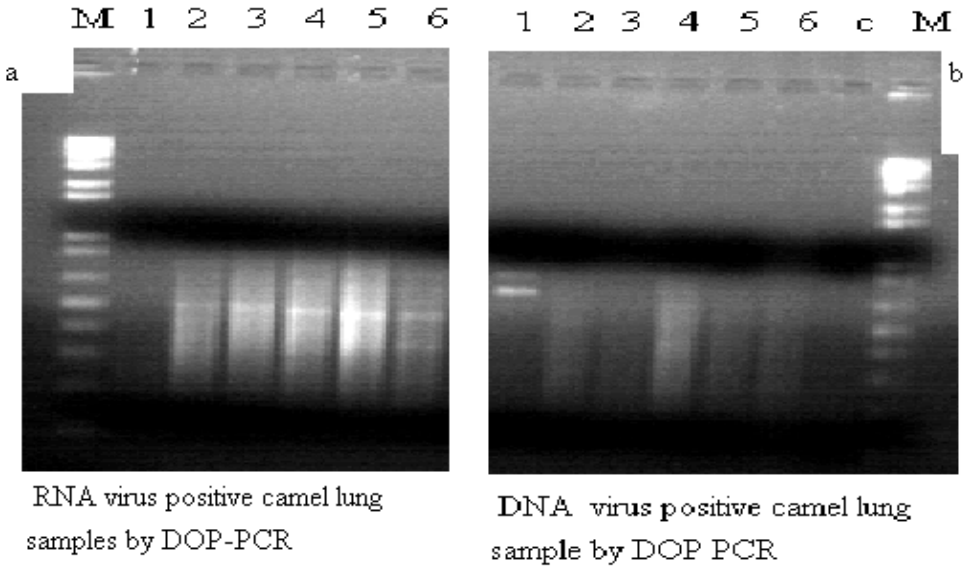

Figure 1. Digital photography ofethidium bromide (EthBr) stained agarose gel electrophoresed DOP- PCR amplified DNA and RNA productsanalyzed by UV illumination. (a) Lane 2-6 were positive for RNA virus, (b) lane 4 was positive for DNA virus. M= 1000bp Molecular weight Marker and C=Control (Negative).

Morphologic alterations on VERO cell monolayer

Of the 35 examined lungs tissue samples 27 samples exhibited morphologic alterations (CPE) on VERO cell monolayer. The presence of virus in the pneumonic samples was evidenced by initial swelling and rounding of infected VERO cells. The most predominant and frequently observed type of CPE was the aggregation of infected cells with syncytia formation.

Molecular characterization methods

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

Total of six samples were tested for presence of viral RNA and DNA with DOP-PCR revealed presence of unspecified viral RNA in five samples (Figure 1). Lane 4 (Figure 1a and b) revealed that the sample was positive on both sides (DNA and RNA extracted samples) implying that the sampled animal was infected by both DNA and RNA viruses.

Conventional PCR

Samples were tested for the presence of *Respiratory syncytial virus*, *Adenovirus*, *Pest des petitisruminants virus* and *Parainfluenza virus* 1, 2 and 3 genome. All samples tested were negative for PPRV and *Parainfluenza viruses* 1, 2 and 3. Nine of ten samples tested were positive for *Respiratory syncytial virus* and two of six samples tested were positive for *Adenovirus* (Figures 2 to 4).

DISCUSSION

The study showed that the overall prevalence of virally suspected pneumonia was 8.99% (n=35/389). The study conducted by Shiferaw et al. (2011) in Ethiopia to assess the type and frequency of gross andmicroscopic lung lesions encountered on camels showed that the occurrence of pulmonary lesions was recorded in77.5%.

From the total of examined camels 30.5% were positive for acute and chronically interstitial pneumonia, which indicates on involvement of viral infections. The duration of CPE development for all samples ranged from 4-14 days. Few CPE were characterized by larger swelling and rounding up, detaching and floating of singled, paired and clumped infected VERO cells. The majority of CPE were characterized by initial rounding, elongation and syncytium formation.

Two out of six samples tested for *Adenovirus* gave visible positive PCR results forming smears which showed that *Adenoviruses* plays a role in camel respiratory disease syndrome (Table 2). The finding of the present study is the first report of *Adenovirus* in Ethiopian camels. Hadia et al. (2001) detected adenovirus type 3 antibodies in 35.8% of camel sera collected from slaughter houses in Egypt and Similarly,

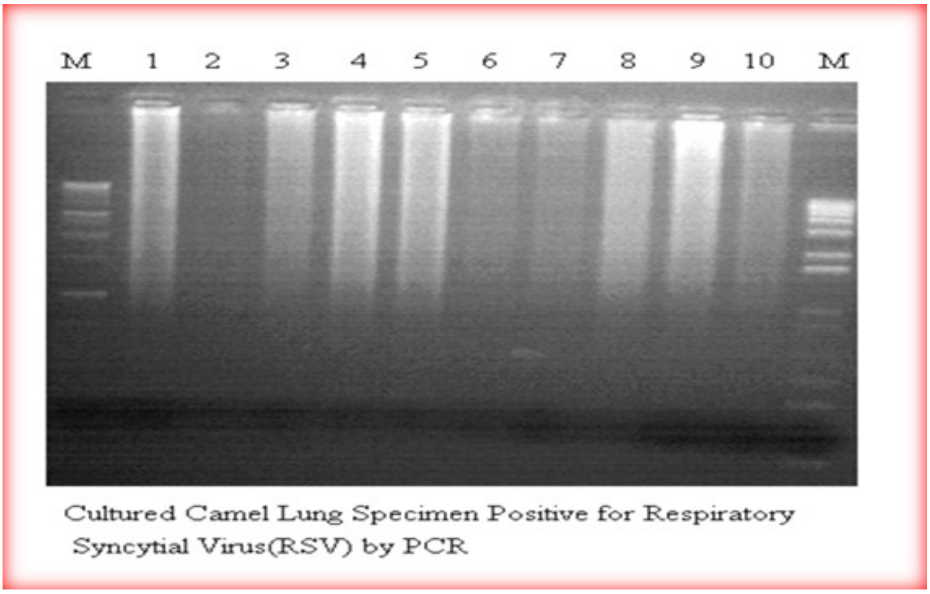

Figure 2. Digital photography of EthBr stained agarose gel electrophoresed PCR amplified RSV products of ten samples analyzed by UV.M=Molecular weight marker.

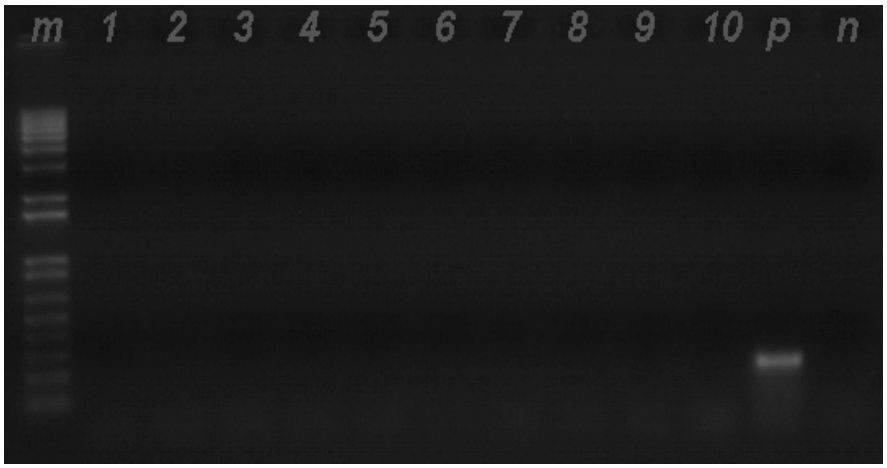

Figure 3. Digital photography of electrophoresedagarose gel analyzed by UVillumination of viral RNA samples using PPRV specific primer amplifiedPCR products; lane 1-10 camel samples, P- PPR virus positive control, n- negative control andM=Molecular weight marker.

Intisar et al. (2010b) reported a prevalence of 90% using indirect enzyme linked immunosorbant assay (ELISA) in camel sera collected from abattoir in Sudan. Also in Sudan, Adenovirus type 3 antigens were detected in 1.3% of 239 tested samples of camel lungs by the use of sandwich ELISA (Intisar et al., 2010b).

The camel was not seems like as a possible host to PPRV until report of Ismail et al. (1992) which detected the infection by serological studies in Sudanese camels. Despite of PPRV serological confirmation from camels reported by Roger et al. (2001), which caused high mortalities and morbidities on camels, the virus is not yet isolated and molecularly characterized in Ethiopia. This study was thus designed to investigate PPR virus thus six CPE positive camel lung specimens were tested using RT-PCR and all tested samples were negative for PPRV. The result showed that PPRV may not cause respiratory infection in apparently healthy camels.

Respiratory syncytial virus is one of the well-known causes of respiratory infection in human and various animal species (Murphy et al., 1999). In Ethiopia, there is no information regarding *Respiratory syncytial virus*

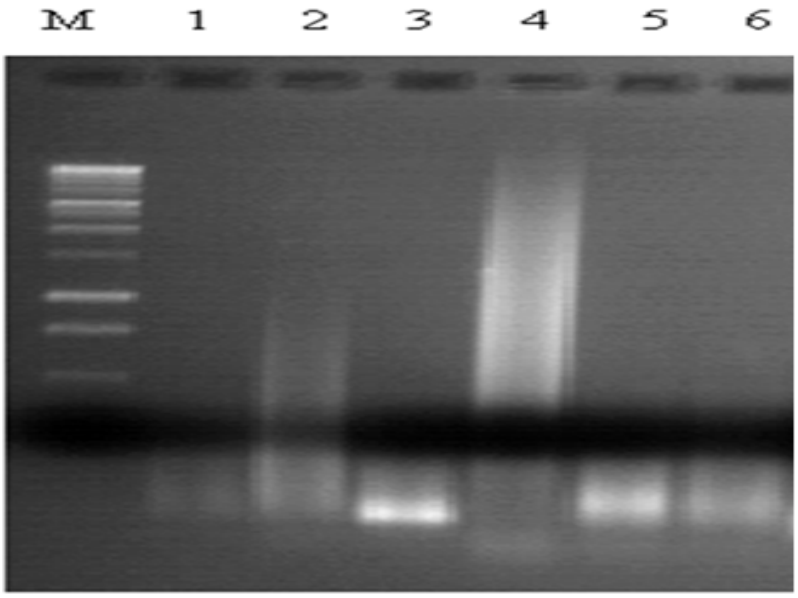

Figure 4. Digital photography of EthBr stained electrophoresed gel analyzed by UV illumination of six PCR amplified DNA products using*Adenovirus* specific primer.M=molecular weight marker.

Table 2. Summary of conventional PCR results.

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

respiratory infections in animals especially in camels. This study was also concerned with the investigation of camel *Respiratory syncytial virus* and accordingly, ten CPE positive samples characterized by initial rounding of infected VERO cells, elongation and aggregation (syncytia formation) were tested for RSV. Nine of them revealed PCR positive results. This is the first attempt to isolate RSV from Ethiopian camels based on RSV characteristic CPEs observed during virus cultivation and PCR positive results. This finding agrees with the findings of Intisar et al. (2010a) in Sudan. The established finding of this study also agreed with other serologic findings of Dioli and Stimmelmary (1992). Antibodies to RSV were detected in 0.6% of apparently healthy camels in Nigeria (Olaleye et al., 1989). In Egypt, camel sera tested for RSV were found positive and indicated the prevalence rate of 9.8% (Shaker, 2003). Furthermore, Intisar et al. (2010a) confirmed RSV infection from the lung specimens of Sudan camels by cell culture, RT-PCR and serological methods.

Parainfluenza virus 3 is one of the viruses known to cause respiratory infection. According to Kebede and Gelaye (2010), *Parainfluenza*-3 was found as primary responsible agent of the camel respiratory disease outbreak in Ethiopia. The detection and isolation of PIV3 using haemo-agglutination inhibition (HI) method from imported Djiboutian camels in Egypt were reported for the first time by Nawal et al. (2003). Similarly, Shaker (2003) reported about isolation of PIV3 from camel lungs in Egypt. Intisar et al. (2009) isolated PIV3 from camel lung specimens in MDBK cell cultures observing the typical CPE of PIV3: rounded retractile cells, cell elongation and

sloughing with some syncytia formation, which was similar early described by Henrickson (2003). In the present study, six CPE positive samples characterized by initial rounding of VERO cells, elongation and syncytia formation of infected cells were PCR amplified using PIV3 specific primers, but no amplified PCR product was detected. The absence of a PCR product could be due to the use of VERO cell lines for virus culture in this study insteadof the commonly used MDBK cell by other researchers which is more susceptible for *parainfluenza viruses*.

This study had also attempted to investigate the involvement of *parainfluenza viruses* 1 and 2 viruses in camel respiratory infection but none of them were positive. This finding is in contradiction with the serological findings reported by Olaleye et al. (1989) in Nigeria and Schwartz (1992) indicating on the common occurrence of *Parainfluenza* 1 and 2 virus infection in camel rearing areas.

In conclusion though the causative etiologies of camel respiratory diseases are still remained mysterious, this study had found out the involvement of *Respiratory syncytial virus* and *Adenovirus* as causative agents for camel respiratory disease in Ethiopia. Thus the study indicated that a continuous and detailed molecular epidemiologic investigation is needed to determine the viral species prevailing in Ethiopian dromedary camels.

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