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Camel viral disease outbreak investigation on Negele Borena zones of Oromia regional state of Ethiopia

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Abstract
Camel is an important domestic animal uniquely adapted to the hot and arid environment, but its value to Ethiopian pastoralists is unbalanced to its resource potential due to the presence of various infectious diseases in the area. Therefore this study was conducted to isolate and characterize the emerging suspected case samples collected in camel rearing areas in Borena Zone of Ethiopia in 2007at national veterinary institute (NVI) from December 2010 to April 2011. A total of eleven Negele Borena camel postmortem tissue samples (four lungs, four liver, two heart, and one heart blood) were processed by using cell culture techniques,AGID test and molecular techniques. The suspected viral pathogen was isolated from all of the samples processed and inoculated on Vero cell cultures with a variable amount of detectable CPE and growth character within 5 to 8 days. The supernatant samples exhibiting CPE were taken and tested for presence of both DNA and RNA viruses, using universal degenerate oligonucleo prim– polymerase chain reaction (DOP-PCR) and conventional polymerase chain reaction (PCR) techniques. All of the samples tested by DOP-PCR were positive for presence of RNA virus, but negative for DNA virus and Peste Des Petits Ruminants Virus (PPRV). Furthermore the immune diffusion test result on an isolated virus conducted by using known PPR antibodies was negative. Therefore, the actual virus in this study was ruled out that the etiological agent/s of sudden camel mortality occurred in 2007 in Borena Zone of Ethiopia were an RNA virus and also it was not a member of the genus PPR virus that are detectable by existing primers. On the bases of this remark, the viral causative agent of the disease was further molecularly characterized by using specific primed PCR, optimization of techniques and sequencing machines.

Keywords: Agar gel immune diffusion test, camel, cell culture, Ethiopia, PCR, viral diseases

1. Introduction
Human population and production of food in Africa an urgent need to develop previously marginal resources, such as the semi-arid and arid rangelands and optimize their utilization through appropriate livestock production systems, of which camel production is certainly the most suitable one (Schwartz and Dioli, 1992) [14]. This is because Camels (*Camelus dromedarius*) are vital domestic animal species that are best adapted to harsh environments and fluctuating nutritional conditions of arid and extreme arid zones and inhospitable to other domestic animals and an important asset in the capacity of humans to survive in and make use of these drier regions (Dirie and Abdurahman, 2003) [4].
In Ethiopia Camels are subset of huge livestock resources, mostly kept by pastoralists in subsistence production systems, which are found in Southern, Eastern, and North Eastern arid and semi raid areas of the country, namely Afar, Amhara (North wolo), Oromia Argoba special Woreda, Oromia West Harereghe, Bale, Borena, Guji, Somale: shinele, Jijiga, Libenand Dire Dawa. Camels are the principal source of income and food for millions of pastoralists with the population estimated to be over one million (CSA, 2010) [3]. This number ranks the country third in Africa after Somalia and Sudan and then followed by Mauritania and Kenya in that order (FAO, 2008) [6] and fourth in the world.
Camel is comparatively hardy animal and less susceptible to many of the diseases that affect other livestock species in the same areas (Dirie and Abdurahman, 2003) [4]. In fact the dromedary of Ethiopia has been suffering from different diseases for the past so many years and conventional intervention measures seemed to difficult due to the various reasons that the animals are reared in marginal areas where the veterinary services are not available or very limited (Tefera, 2004) [15]. The production is in migratory system in remote areas with harsh living conditions and poor infrastructure, the animals are presumed to inaccessible for research. This affects the depth of our knowledge on the general aspects of camels (Schwartz and Dioli, 1992) [14]. Since little is known about the health problem of Ethiopian camels.

Besides management, nutritional factors, and with poor supply of veterinary services, infectious and parasitic diseases have numerous negative impacts on productivity of given large livestock population and distribution in the country.

In 2005/2006 in Somali region, Afar region, and Borena, Guji and Keryuarea of Oromia region, there was a huge camel mortality due to respiratory disease outbreak which was characterized sudden death (Wernery *et al*., 2006) [16]. Researchers from National Veterinary Institute(NVI), National Animal Health Diagnostic and Investigation Center (NAHDIC), and other regional laboratories of the country have been doing disease investigation following the occurrence of the outbreak, but the extent etiological agents of the disease has never been identified yet (Asegedech, 2009) [2].

Additionally in 2007 similar disease outbreak was reported from Negele Borena zones of Oromia region. 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 pastoral community. 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. Based on the background information and clinical signs observed viral diseases were suspected. Therefore the objectives of this study were investigation and identification of the viral causative agents of the camel disease.

2. Materials and Methods

2.1 Study area

The study was carried out from December 2010 to April 2011at National Veterinary Institute (NVI), Bishoftu town which is situated 47 km south east of the capital city, Addis Ababa, at 90º N latitude, 40ºE longitudes and at an altitude of 1850m above sea level. Although the city receives 866mm annual rainfall (NMSA, 2010) [11].

2.2 Study design

The study design was field outbreak sample disease investigation.Camel disease outbreak was reported from Borena zones of Oromia regional state in to NVI. A veterinary team was then travelled from NVI to those outbreak areas to investigate the disease. A general and clinical examination of the affected and post mortem examinations of clinically dead camels' were performed. The disease was characterized by sudden death of huge population of camels.

2.3 Sample collection and transportation

Tissue samples were collected from clinically dead camels after post mortem examination without discrimination of their sex, age, body condition, coat color for further investigation. Immediately after slaughter clinically dead camels, the tissue samples with a size of greater than 10cm x 10cm were collected from the most promising lesions of tissues (heart, lung, liver and heart blood), using sterile forceps, scissors and scalpel blade aseptically, wrapped separately and placed each piece of tissue in a separated sterile screwed capped universal bottle, fully labeled with the date, type of tissue and transported to National Veterinary Institute Laboratory with in ice box, glycerol, phosphate buffer saline and antibiotic. Samples selected for this study were four lungs, two hearts, one heart blood and four livers from different animals of outbreak area (OIE, 2013) [12].

2.4 Laboratory analysis

2.4.1 Sample storage and processing

In the laboratory, the samples were stored at -85 ºC in tightly sealed sample bottles to avoid the deleterious effects of CO_2 on the viability of any virus until processed. The standard viral antigen was prepared from the representative tissue samples of (liver, lung, heart and one heart blood) which are camel viral disease suspected as followed: the preserved tissue samples was taken from ultra-freezer, -85ºc and placed in to -20 ºC then waited for 1 hour and then again taken out from -20 ºC, put under + 4 ºC and thawed at room temperature for 20 minutes to avoid the temperature shock and to make ready for further processing. Only one sample was processed at a time to avoid any cross contaminations (Dunham and Guthmiller, 2008).All tissue processing steps were conducted inside the class II safety cabinet which has its own ultra-violate (UV) light to decontaminate the inside working environment after every laboratory works. One gram of tissue samples was taken from the original specimen for sample processing. This tissue was washed twice or until the fatty or the debris of the tissue should have been avoided with 10 ml sterilized phosphate buffered saline (PBS) pH 7.2- 7.4 containing antibiotics and antifungal drugs on the Petridis before drained and transferred to the mortar. The drained tissue was transferred in to the mortar and then sliced, chopped with sterile scissor and scalpel blade. 9 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 in to the sterile test tubes to the level of three fourth for further centrifugation at 3,000 rpm for 10 minutes. The suspension was used for inoculation; hence, before inoculation, the supernatant was transferred to other sterile test tubes by sterilized pipette 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. Each of processed and filtered tissue samples was stored in aliquots at -20 ºC until virus isolation and characterization (Masters, 2002; OIE, 2013) [8, 12].

2.4.2 Virus isolation on cell culture techniques

The 25 cm^2 tissue culture flask of Freshly confluent monolayer cultures of African green monkey kidney (VERO) cell was washed with 37 ºC pre-warmed PBS and inoculated with 1ml of 10% tissue suspensions (previously prepared standard suspension of suspected materials) of heart, lung, liver tissue and heart blood separately and waited for 1 hour at 37 ºC for adsorbation (to facilitate the virus attachment to the cell). After virus cell attachments washed with PBS and added 10ml fresh 2% GMEM and one 25cm^2 TC flask confluent Vero cell was kept as a control, placed at 37 ºC incubator (OIE, 2013) [12]. The inoculated monolayer cells were examined daily for evidence of

cytopathic effect (CPE) and also the presence of any contamination. Examination was continued until 14 days after inoculation and the cultures were harvested when CPE was observed in 75% of the cells and kept at -85 ^{0}C until they were processed by immunodiffusion test and molecular techniques. However, if no CPE was detected after 15 days, the cells were blindly passaged at least twice (frozen and thawed, then inoculated to fresh cultures) before the samples were declared negative.

2.4.3 Extraction of viral nucleic acids

After the CPE was observed on Vero cells, to determine whether the isolated virus is DNA or RNA virus, special nucleic acid extraction technique was applied. The viral nucleic acid extraction methods used during this study was mainly based on viral capsid purification techniques with fewer modifications (Allander *et al.*, 2001; Nanda *et al.*, 2008) [1, 10]. Briefly, 1ml of tissue or culture suspension samples were suspended in a locally prepared 1 ml viral buffer (30mM Tris/HCL pH 7.2, 3.6 mM CaCl2, 5mM Na acetate, 125mM Kcl and 0.5mMEDTA), and homogenized at 800 speed sonicated and incubated at 37 ^{0}C for 1½ hrs to further facilitate cell and nuclear membranes disruption. The same time, 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 the nucleic acid extraction kit (Qiagen) (Allander *et al.*, 2001; Nanda *et al*., 2008) [1, 10].

2.4.3.1 RNA extraction

The total RNA was extracted from the cell culture isolates according to RNeasy® mini kit. 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 was added to the sample and mixed by vortexing and then 460µl of 70% ethanol was added and mixed by vortexing. The mixtures was transferred to RNeasy spin column (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 50µl DEPC-H2O into a new clean collection tube and stored at -20 ^{0}C as needed (Qiagen, 2006a; Nanda *et al*., 2008) [13, 10].

2.4.3.2 DNA extraction

The total DNA was extracted from the cell culture isolates according to DNeasy® mini kit. Briefly, 200 µl of sample in viral buffer was taken and put into a 1.5 ml eppendorff tube; 180 µl of Lysis buffer ATL and 20 µl of proteinase K was 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 micro centrifuge 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 500µl 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 micro centrifuge tube and100 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-H2Ointo a new clean collection tube, labeled and stored at -20 ^{0}C as needed (Qiagen, 2006a; Nanda *et al*., 2008) [13, 10].

2.4.3.4 Complementary DNA synthesis

In order to detect viral RNA, samples were subjected to reverse transcription using oligodT or Random hexamer primers and Superscript ™ III Reverse Transcriptase (Invitrogen) prior to DOP-PCR. The cDNA was synthesized according to the manufacturer protocol (Invitrogen) in 20 µl reaction volume. Primarily, 1 µl 50µlM oligodT primer or random hexamer, 1 µl 10mM dNTPs, 5 µl extracted RNA and 3µl DEPC treated H2O total of 10 µl were added in to 0.5 ml capacity PCR tube and incubated at 65 ^{0}C for 5 minutes in thermal cycler (Applied Biosystems) and chilled on ice for 3 minutes. Then, 2µl 10x RT buffer, 4 µl 25mM MgCl2, 2 µl 0.1M DTT, 1 µl RNase OUTTM (40U/µl) and 1µl Superscript™ III RT (200U/µl) were added 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 and incubated at 37 ^{0}C for 20 minutes and the cDNA was stored at -20 ^{0}C until needed (Intisar *et al*., 2009) [7].

2.4.3.5 Primers used in Degenerate oligonucleotied prime polymerase chain reaction (DOP-PCR)

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, primer also contained a defined 5_-sequence of 10 nucleotides in length (Table 1).

Table 1: Universal primers used in viral nucleic acid amplification

Primer Number	Specific sequence	Ambiguous sequence	anchor sequence	Detection limit
5	CCGACTCGAG	IINNNNNN	TTCT	>10^6

Source: Nanda *et al*. (2008) [10]

2.4.3.6 Polymerase chain reaction

The PCR was performed according to (advantage®cDNA PCR, Novagen) kit. Primarily master mix containing 5 µl 10x PCR buffer (containing 20mM $MgCl_2$, 55mM Kcl, 100mM Tris PH8.4), 1µl 10mM dNTPs mix, 0.5 µl of 5U/ µl dream Taq DNA polymerase and 25.4 µl DEPC-H_2O was prepared for each reaction. Then, 45 µl of the master mix, and 5 µl of the cDNA were added to each tube for RNA virus detection or 5µl DNA template for DNA virus detection. Finally run on a thermal cycler (Applied Biosystems) cycle PCR program of initial denaturation for 5 minutes at 95 ^{0}C followed by 5 cycles of 1 minute at 94 ^{0}C, 1 minute at 50 ^{0}C and 2 minutes extension at 72 ^{0}C. This

was then followed by 35 cycles of 1 minute denaturation at 94 ^{0}C, 1 minute annealing at 55 ^{0}C, and 2 minutes elongation at 72 ^{0}C, with the addition of final elongation at 72 ^{o}C for 7 minutes.

2.4.3.7 Agarose gel Electrophoresis

The PCR products were analyzed with 2% agarose gel containing 0.5µg/ml of ethidium bromide. Briefly, 5µl PCR products mixed with 2µl loading buffer (Invitrogen) and loaded to wells in pre-prepared gel and run at 100 volt for about 50 minutes in parallel with DNA 1,000 bp molecular weight marked (promega) in electrophoresis apparatus using 1x TAE buffer. The DNA and RNA band was visualized by UV illumination, documented and the size was determined against the DNA molecular weight marker standard (Nanda *et al*., 2008; OIE, 2013) [10, 12].

2.4.4 Test for detection of PPR virus

The same protocols as in the case of detection of RNA virus was used for RT-PCR, but the primer used in this case was the one that is specific for the already known PPR viruses; 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.

2.4.4.1 Antigen detection by agar gel immunodiffusion test

The agar gel immunodiffusion (AGID) tests were conducted by using 1% agar to a depth of about 4 mm. Wells were cut in a hexagonal pattern of six peripheral wells around a single central well 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 PPRV, known hyper immune serum was placed in the central well, Similarly, known positive control antigen (PPR vaccine) was placed in alternate peripheral wells (i.e. one,), Negative control antigen was placed in well two. Test antigens from cell culture suspension was added to wells three, four and five 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.

3. Results and Discussion

3.1 Results of virus isolation on cell culture

The virus was isolated from all of the samples that were processed and inoculated on Vero cell culture. The CPE of the propagated viruses were observed within 5 to 8 days which was characterized by large amount of rounding up of cells which become more retractile, aggregation and clumping in syncytia formation and final total destruction of the cell sheet (70% - 90% CPE), FIG 1, 2, 3 & 4 respectively with control cells at the beginning and final date of incubation, Fig.5 and 6. The isolated virus were showed aggregation and clumping of cells in all types of samples but were delayed on lung tissue samples and also fast, severely affected and syncytia formation in liver, heart and heart blood samples.

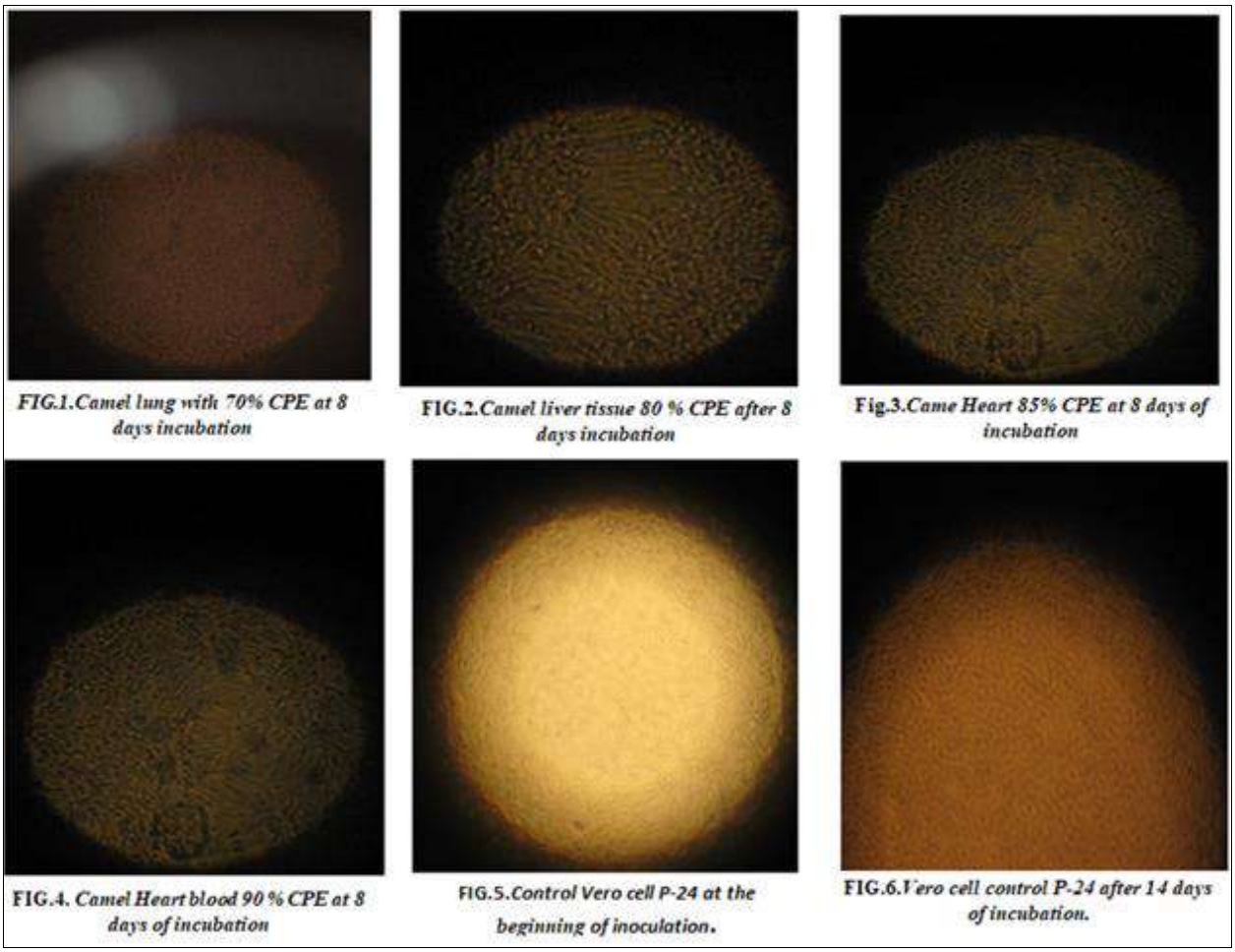

FIG.1.Camel lung with 70% CPE at 8 days incubation

FIG.2.Camel liver tissue 80 % CPE after 8 days incubation

Fig.3.Came Heart 85% CPE at 8 days of incubation

FIG.4. Camel Heart blood 90 % CPE at 8 days of incubation

FIG.5.Control Vero cell P-24 at the beginning of inoculation.

FIG.6.Vero cell control P-24 after 14 days of incubation.

3.2. Polymerase chain reaction results

% CPE at 8

Physical separation of viral nucleic acids from cellular nucleic acids and the non-specific amplification of the viral nucleic acids have been used for detection of new viruses. RNA was amplified from all of the isolated viruses with universal primed RT-PCR. The test adjustment were: tested samples placed from lane two to thirteen and lane seventeen which have been an amplification band, with three control lanes of which RNase free water for extraction control (lane -14and15) and Negative control without template(lane-16) and also two marker lans (lane 1and lane eighteen) (Figure.7). However, the universal primed conventional PCR had not been amplified DNA (Figure.8).

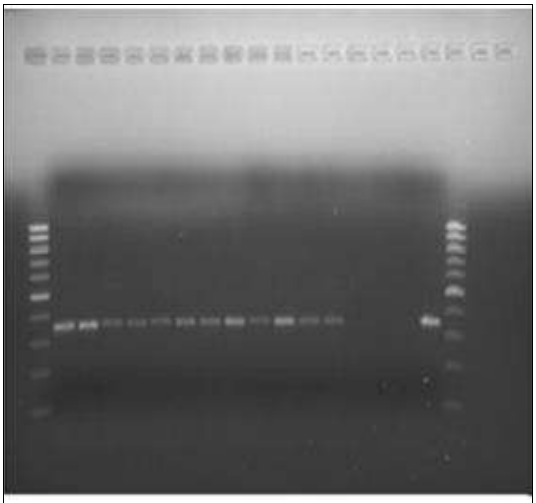

Figure 7. Two step TR-PCR for detection of RNA virus using universal primer NO.5

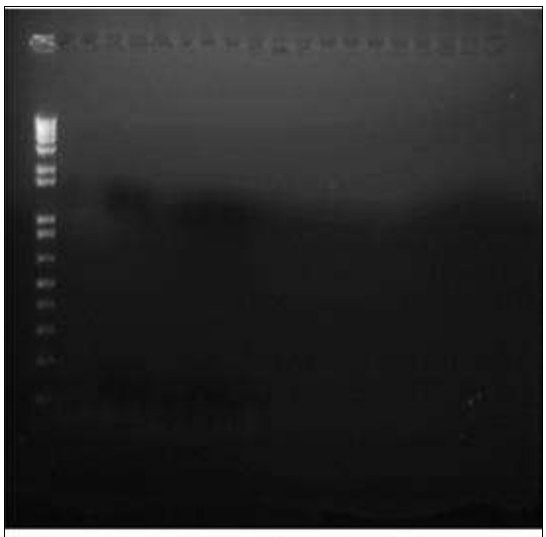

Figure 8. Conventional PCR for detection of DNA virus using Universal primer No.5

Additionally, the virus in question were tested with PPR virus specific primed RT-PCR (isothermal amplification protocol) with NVI PPR vaccine as a positive control, also there had not been any amplification on the test samples (Figure 9).

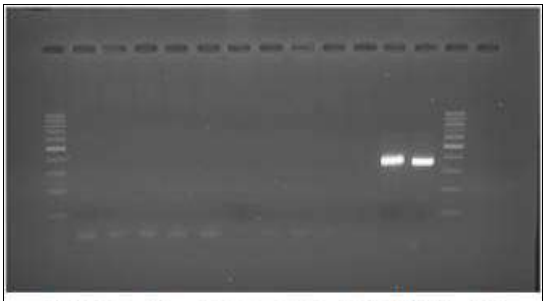

Figure 9. Two step RT-PCR for detection of PPR virus using specific primer. Lane 1 and 14: Marker; Lane 2 to 10: test samples, Lane 11: negative control; Lane 12 and 13 positive control (NVI PPR Vaccine)

3.3 Results of agar gel Immunodiffusion test of virus isolates with PPR specific antibodies

The isolated viruses were also tested for the detection of cross-reacted virus by agar gel immune diffusion test (AGID) with known PPR antibodies and NVI PPR vaccine as a positive control. No white line of precipitation was observed between the virus isolates and PPR antibodies. However, the positive control, NVI PPR vaccine had formed a line of precipitation. This result indicated that the virus isolates could not react with PPR antibodies.

To sum up, the results of virus isolation, PCR and immunodiffusion tests revealed that all of the virus isolates in question were RNA viruses and they were not PPR viruses and able to answer the previous researchers questions. However, further analyses have to be done to determine the genus, species and strain of the virus isolates by using specific primed PCR, optimization of techniques and sequencing machines.

Although this study finding result was matched with the hypothetically suspected disease, which is viral disease and able to answered to the previously researchers question but also contradict to those Researchers from National Veterinary Institute(NVI), National Animal Health Diagnostic and Investigation Center(NAHDIC), and other regional laboratories of the country have been doing disease investigation following the occurrence of the outbreak, but the extent etiological agents of the disease has never been identified yet (Asegedech, 2009) [2].

4. Conclusion and Recommendations

In this study, virus was isolated from tissue of clinically died camels of the unknown disease using cell culture technique, agar gel immune diffusion and molecular techniques. The nucleic acids of the unknown virus were identified by using degenerate oligonucleotied primed polymerase chain reaction on directly processed tissue samples suspension and cell cultured virus suspected tissue samples extracts. The isolated virus in this study were belongs to RNA virus group excluding PPR virus and it was also characterized for its growth characters on Vero cells. The country has been hosting different camel disease outbreaks that led to death of many camels at different time.

Even though the many diseases of camels, the recently emerged camel disease with still specifically unknown causes were the single most important disease with huge mortality. The disease seems to occur cyclically and also the causative agents were not specifically identified or fully determined yet. Therefore, the following points were recommended:

- The isolated virus should be further characterized and specifically identified by using updated specific primer and different optimization techniques.
- The isolated virus should be further characterized by sequencing machine for specific identification and
- Strong collaboration among governmental organization, nongovernmental organization, veterinarians (researchers) and pastoralists should be made in order to study active diseases of camels and initiated towards creating possible preventive and/or control options such as the production of vaccine from the viral isolate.

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