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Detection and characterization of Malignant Catarrhal Fever virus in Cattle, Sheep and Deer using Real Time PCR

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Malignant Catarrhal Fever (MCF) is one of the most important viral diseases of deer and bison, also cattle and goat can be infected. The MCF is caused by gammaherpes viruses, genus rhadinovirus; which includes a group of viruses including ovine herpesvirus-2 (OvHV-2), caprine herpesvirus 2 (CpHV-2) and white tailed dear strain (MCFV-WTD). Malignant catarrhal fever virus infection characterized by sporadic occurrence, fever and high mortality. In this study two real time PCR assays had been used to detect the MCF virus strains in both cattle and deer. The virus was detected in cattle samples by Ovine MCF RT-PCR while was detected in deer samples by a newly developed assay for deer MCF. Prevalence of ovine MCF was 18.5% in cattle and 58% in sheep. Nucleotide sequencing analysis of selected cases from cattle and deer showed that the similarities among the detected positive samples and published one ranged between 98-100% for both. The high prevalence of MCF in cattle and deer shows the significant economic burden of the disease in these animal species.

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INTRODUCTION

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of cattle and many other species of order Artiodactyla. The virus was classified as a gammaherpesvirus by (Plowright, 1965). It has a significant source of economic loss in several ruminant species, such as cattle, deer and, in particular, confined bison (Crawford et al., 1999). Gammaherpesviruses are enveloped, icosahedral, double stranded, linear and negative sense DNA viruses. The diameter of the viral particles ranges from 150 to 200 nm, and the genome size range from 120 to 200 kbp. These viruses are members of an emerging subfamily among the Herpesviridae. Two genera are discriminated: (i) lymphocryptovirus, including its type species Epstein-Barr virus (EBV, agent of infectious mononucleosis and Burkitt's lymphoma) and (ii) rhadinovirus, including a great number of viruses of interest for medicine, veterinary medicine, and biomedical research. Herpesviruses are highly disseminated in nature and most mammalian species carry at least one herpesvirus. Many more herpesviruses, particularly members of subfamily Gammaherpesvirinea, have been recognized recently by using newer molecular technology in a variety of species (Ehlers & Lowden, 2004). Gammaherpesviruses are characteristically become latent in lymphocytes and their reactivation has evolved to depend on cellular transcription factors that relate to those cell types (Thonur et al., 2006). The gammaherpesviruses have evolved to actively protect their latently infected cells from being destroyed by immune functions of their native host. These reservoir hosts have evolved to become infected and transmit the virus without overt disease symptoms (Ackermann, 2006).

Latency and reactivation are key features of herpesvirus biology in relation to pathogenesis.

The alcelaphine herpesvirus-1 (AlHV-1) was first identified in 1960 (Plowright et al., 1960). Disease caused by AlHV-1 is restricted to Africa where wildebeest are present and to zoological collections elsewhere and so it has been referred to as wildebeest-derived MCF. The OvHV-2 form of the disease occurs world-wide and has been described as sheep-associated (SA) MCF in Europe and the United States of America (Ackermann, 2006). A highly virulent herpesvirus causing MCF in white-tailed deer (WTD-MCF) was recognized (Li et al., 2000). Herpesvirus causing MCF was identified in goat causing MCF in goats and named caprine herpesvirus-2, CpHV-2 (Chmielewicz et al., 2001; Li et al., 2001b) Moreover, the ovine herpesvirus-2 was shown to be endemic in goats using both serological and PCR-survey data (Li et al., 2001a).

Worldwide, most cases of MCF are caused by ovine herpesvirus 2 (OvHV-2), which exists as a ubiquitous subclinical infection in domestic sheep. Research progress historically has been constrained by the inability to propagate OvHV-2 in vitro or to readily reproduce the disease experimentally and by a lack of reliable detection methods for the virus. Technologies, such as competitive enzyme-linked immunosorbent assay (cELISA) and PCR (Baxter et al., 1993; Li et al., 1994; Li et al., 1995b) and quantitative PCR (Kim et al., 2003), have dramatically accelerated the understanding of the epidemiology of OvHV-2 in recent years, in both clinically susceptible species and in its own natural host, the domestic sheep. A number of studies have confirmed that under natural flock conditions the majority of lambs are not

infected until after 2 months of age. The disease was observed as a disease of cattle in Europe where, although no cause could be identified, circumstantial evidence implicated sheep as a source of infection and it was thus designated 'sheep-associated' malignant catarrhal fever. Subsequently the disease was observed in Africa where it became evident that a herpesvirus which normally infects wildebeest was the cause. It is now apparent that deer are highly susceptible to both forms of the disease, the sheep-associated form being a serious problem in farmed deer (Hart et al., 2007; Reid et al., 1984).

This study describes the development and optimization of a real-time PCR assay for detecting MCF virus in deer beside the present one for OvHv-2 in host species. We have been performed Real-Time PCR for each sample. The results of the commercially available CI-ELISA test and the results of real time PCR assay were compared.

MATERIAL AND METHODS

Blood samples. A total of 521 blood samples were collected, 433 for cattle and 88 for ovine samples. obtained in BD Vacutainer tubes with anticoagulant substance (Sodium heparin). The samples were centrifuged and the plasma samples were transferred to new tubes to be tested. The CI-ELISA positive samples submitted to be tested by real-time PCR test.

CI-ELISA for detection of MCFVs. Competitive inhibition ELISA was performed as previously described (Li et al., 2001c; Li et al., 1994). This assay uses a monoclonal antibody (15A) that binds to a conserved epitope of a glycoprotein complex common to MCFVs.

DNA extraction. For DNA extraction, both blood and tissue samples (lymph nodes)

were digested with proteinase K (QIAGEN Inc., Valencia, CA, USA) in buffer ATL (QIAGEN Inc.) for a minimum of 12 h at 55 °C for tissue samples. DNA extractions were performed using the QIAGEN DNeasy tissue kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer instructions. For each of the DNA extraction steps, strict protocols were followed to avoid cross-contamination of samples. Samples were stored at -80 °C until used as templates for amplification.

Conventional PCR. The conventional PCR was performed using the published primers and protocol designed by (Hussy et al., 2001) forward primer OvF TGGTAGGAGCAGGCTACC GT and the reverse primer OvR ATCATGCTGAC CCCTTGCAG` are targeting a 131 bp size of the genome. The PCR mixture was reacted in a thermal cycler initiated by denaturation at 95°C for 90 seconds, followed by 35 three-step cycles consisting of annealing at 55°C for 30 seconds, extension at 68°C for two minute and denaturation at 94°C for 30 seconds. At the end of last cycle, the reaction was held for 30 seconds at 55 °C followed by 5 minutes at 68 °C.

Agarose gel electrophoresis. The PCR products were electrophoresed in 1.4% agarose gel (Sigma-Aldrich Co. Saint Louis, MO, USA) prepared by melting 1.4 gram of agarose in 100 ml 1X TBE buffer (89 mM Tris, 89 mM Boric acid and 2mM EDTA, pH 8.3) obtained from Fisher Scientific International Inc. (Fair lawn, New Jersey, USA). Ethidium bromide was added to the gel at a concentration of 0.5 ug/ml. Electrophoresis was conducted at 100 volt (5V/cm) for 20 minutes. The PCR products were visualized by the UV illumination using the UVP's Bioimaging system.

Real time PCR. The oligonucleotide primers and

FAM probes were obtained from IDT (Integrated DNA Technologies, Inc, IA, USA). All primers were purified by standard desalting method developed by Integrated DNA Technologies INC. FAM probes contained 6-carboxyfluorescein (emission wavelength, 552 nm) fluorescent reporter dye at the 5` of the probe and a quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end of the probe.

The primers used for the deer strain was designed based on published sequences. The most conserved areas in the DNA polymerase genes were chosen as primer binding sites. The ovine herpesvirus-2 primers and probe were those used by (Hussy et al., 2001).

Table (1): Real time PCR primers and probes used in this study:

Primer & Probe		Sequence 5' to 3'	Amplicon and Reference
Deer strain	F	ACCATGCTGGAAAAGACCAA	226 bp, DNA polymerase gene, This Study.
	R	ATAGGAGGCTGCTTGTGTGG	
	probe	FAM-CAGCAAATATGCCCAACCCAGATT-TAMRA	
Ovine strain (OvHV-2)	F	TGGTAGGAGCAGGCTACCGT	131 bp, tegument gene, (Hussy et al., 2001).
	R	ATCATGCTGACCCCTTGCAG`	
	Probe	FAM-TCCACGCCGTCCGCACTGTAAGA-TAMRA	

The DNA of the OVHV-2 was amplified and quantified by the real-time (TaqMan®) PCR procedure as described by

(Hussy et al., 2001) targeting the conserved area of the DNA polymerase gene, sized as 131 bp. Real time PCRs for the deer strain was performed using the primers designed in the conserved areas of the DNA polymerase genes for deer strain. The targeted area size was to be 226 bp.

Briefly, the 25 µl PCR mixture for one reaction contained 20 µl of TaqMan Universal Master Mix, (Omnimix HS for Cepheid Smart Cycler Syster (containing 1.5U TaKaRa hot start Taq polymerase, 200 µl deoxynucleotide triphosphates with dUTP, 4mM MgCl2, and 25mM HEPES pH 8.0 ± 0.1), and 240 nM forward primer , 600nM reverse primer, 80 nM probe, and 5 µl of template DNA. The thermal cycle protocol used was as follows: 95°C for 2 minutes to denature the DNA and activate the DNA polymerase, and then 45 cycles at 95 °C for 15 seconds, 60 °C for 60 seconds and 72 °C for 30 seconds. Amplifications were performed in Cephid SmartCycler II System (Sunnyvale, CA, USA). The data were analyzed using Smartcycler software (version 2.0) provided by the company. This protocol was used for all strains of the virus.

Molecular Cloning of PCR products. Purification of the PCR product was performed using Montage PCR centrifugal filter kit (Millipore corporation, Bedford, MA, USA) following the manufacturer's instructions. So cloning of the DNA to cloning vector (2.1 vector): using TA cloning kit (Invetrogen life technologies Carlsbad, CA, USA) following the manufacturer instructions. Plasmid isolation was performed using Wizard Plus SV Minipreps DNA purification system (Promega Corporation 2800 Woods Hollow Road, Madison, WI USA), the purification was performed according to the protocol.

DNA sequencing. The PCR products were purified either

by using Montage PCR centrifugal filter kit (Millipore Corporation, Bedford, MA, USA) or by gel extraction, as The products were electrophoresed in 1.4% agarose gel in 1X TBE buffer. The band representing the expected size was purified from the gel using QIA quick Gel extraction kit (QIAGEN Inc., Valencia, CA, USA). The DNA was kept at -20 ºC.

Sequencing of the purified DNA was done at the molecular Biology Resources Service (University of Tennessee, Knoxville, TN). DNA sequence analysis and sequence alignment were carried out using the software packages DNAMAN version 5.2.9 (Lynnon BioSoft Corporation, Canada) and ClustalX (1.81) as described by Thompson et al (1997).

RESULTS

Amplification of DNA sequences from the ovine isolates of MCFV. The PCR primers and PCR conditions used to amplify a 131 bp DNA sequence out of the tegument gene were those previousely published by (Hussy et al., 2001) (Figure 1A). The amplification of 131 bp DNA fragment from ovine MCF viral isolates and positive control is shown in Figure (1B). The size of the PCR product was in agreement with the expected size according to the published sequence.

To confirm the authenticity of the amplified products, the DNA band corresponding to the 131 bp amplicon was excised from the gel and purified using a commercially available gel extraction kit as described under Materials and Methods. The PCR product was cloned into a T-A Topo plasmid vector to produce the recombinant plasmid pSOV1 (Figure 2) and subjected to automated DNA sequencing by using the forward and reverse pUC/M13 primers as shown in Figure 2. Sequence alignment and BLAST analysis indicated that the PCR product was

amplified out of an authentic ovine DNA sequences. Sequence identity approached 95% with only 2% gaps (Figure 3).

In this study, a fluorogenic real-time PCRs that was previously developed by (Hussy et al., 2001) was used for the quantitation of viral DNA from the ovine isolate of MCFV. The assay makes use of a dually labeled fluorogenic probe, which is designed to hybridize to the sequence between the primers. The fluorescent emission of a reporter dye, covalently attached to the 5' end of the probe, is quenched due to the physical proximity of a quencher dye at the 3' end. The 5' to 3' exonuclease activity of the DNA polymerase causes cleavage of the probe in the course of amplification, which leads to dislocation of both the reporter and the quencher. This results in an increase of the reporter dye's fluorescent emission, which is, at each time point measured, directly proportional to the amount of amplified target DNA. A dually labeled real time PCR probe was used in TaqMan® format in order to quantitate SA-MCFV in blood or tissue samples. Results of real-time PCR performed on ovine isolates of MCFV. The CT value is 25 for the positive control and ranged between (25-28) for positive samples and positive control (pSOV1 plasmid). The program time is one hour, 39 minutes. The fluorescent material, FAM, was detected at wave length 552 nm.

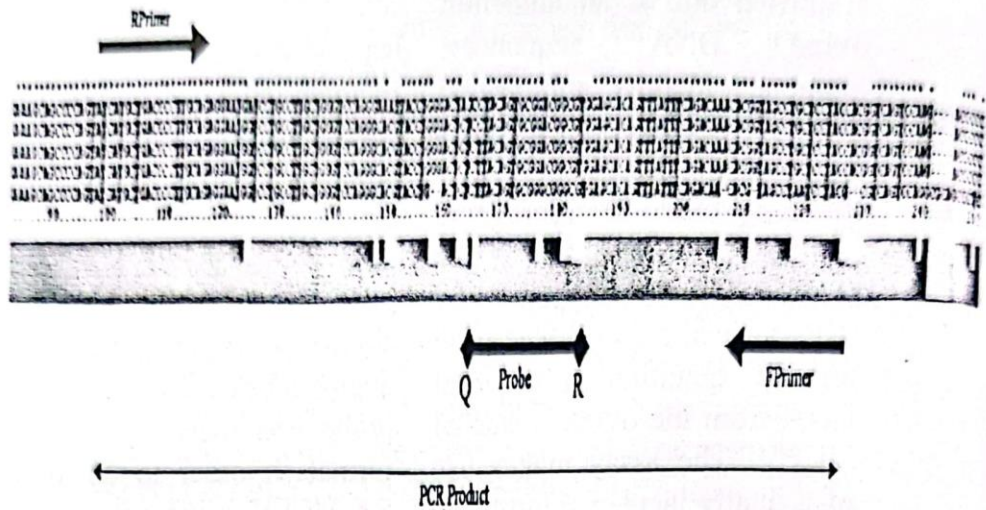

Figure (1A) The location of the oligonucleotide primers used for PCR. Sequences from GenBank were aligned using Clustal 1.81 Software. Accession numbers from top to bottom are DQ229939, DQ229938, S64565, DQ882652, and DQ875146.

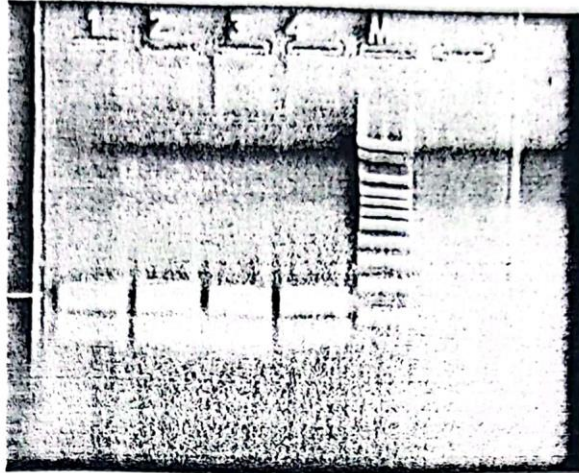

Figure (1B) Agarose gel electrophoresis showing the amplification of 130 bp DNA fragment (arrow) from ovine isolates. Lanes (1, 2, and 3) show the PCR products amplified from BSE 1125, BSE 713, and BSE 928 isolates. Lane (4) shows the PCR product amplified from the positive control (2µl of recombinant plasmid). Lane (M) is the molecular weight markers (100 bp ladder). Electrophoresis was conducted onto 1.4% agarose gel containing 0.5 ug/ml ethidium bromide at 5V/cm.

```
> gb|DQ198083.1|   Ovine herpesvirus 2, complete genome
Length=131621

 Score =  154 bits (170),  Expect = 4e-35
 Identities = 95/100 (95%), Gaps = 2/100 (2%)
 Strand=Plus/Plus

Query  7       AAANTGTGCTCC-CGCNGTCCGCACTGTAAGAGAGTCCCAGGTACGTGCCCTGGACCCAG  65
               ||| |||||||| ||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  121381  AAACTGTGCTCCACGCCGTCCGCACTGTAAGAGAGTCCCAGGTACGTGCCCTGGACCCAG  121440

Query  66      CAAGGTCAGGACCATTCCTCTGCAAGGGGTCAACATGATA  105
               ||||| |||||||||||||||||||||||||| |||||||
Sbjct  121441  CAAGG-CAGGACCATTCCTCTGCAAGGGGTCAGCATGATA  121479
```

Figure (1): Results of BLAST analysis indicating the authenticity of the ovine amplicon.

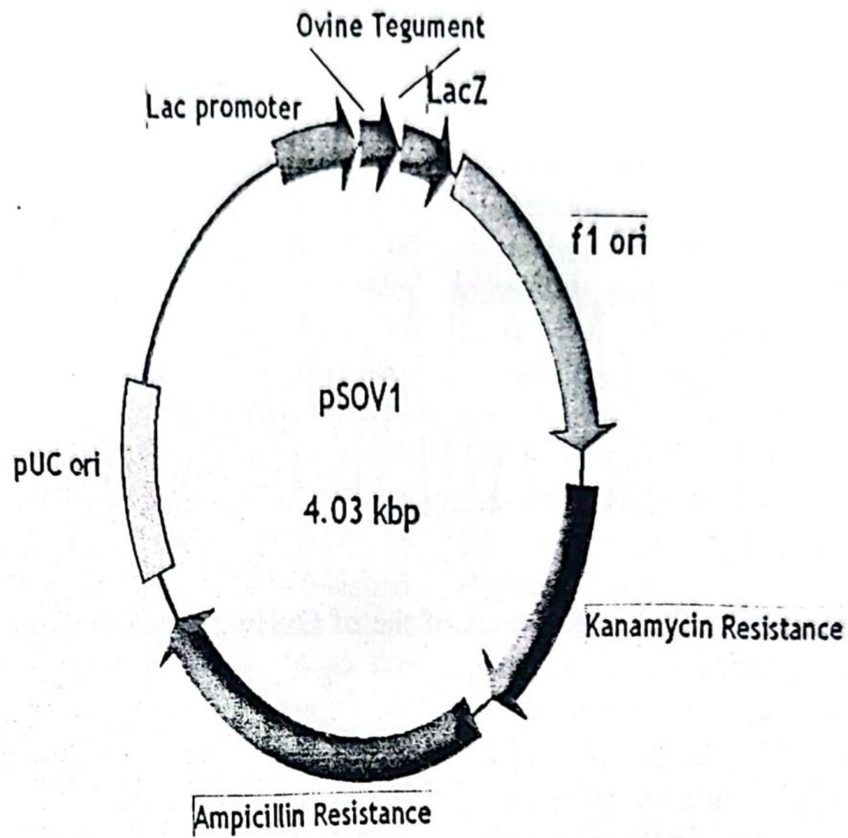

Figure (2A): The recombinant plasmid pSOV1 derived from TA-Topo vector.

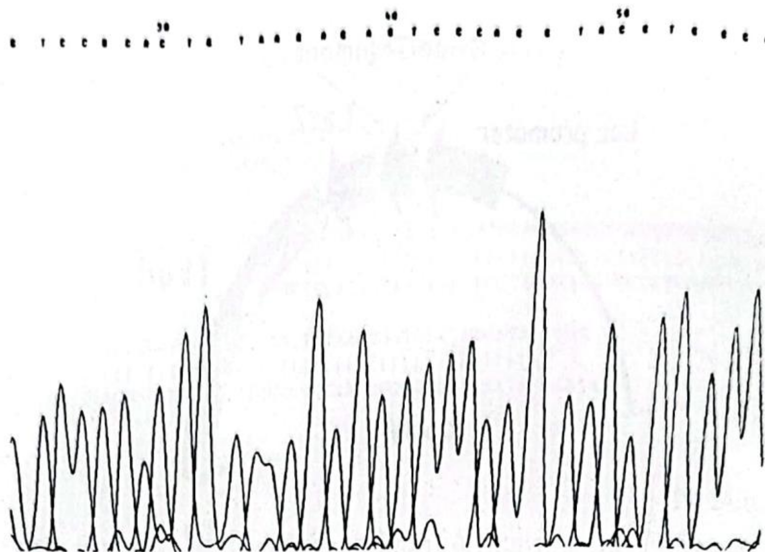

Figure (2B) Partial sequence of the of OvHv-2 DNA (tegument gene).

Amplification of DNA sequences from the deer isolate of MCFV: Two PCR primers were designed in the conserved sequences of the DNA polymerase genes of previously published deer isolates of MCFV. The sequences of the primers and probe had a perfect match with the sequence of deer isolate of MCFV (Figure 5A). All sequences were aligned by using the CLUSTAL1.81 Software and primers were selected by using Oligo 4.0 Primer Design Software.

To optimize the PCR reaction, the concentration of MgCl2 was varied from 1 mM to 5 mM in the reaction mix. The intensity of the amplified DNA bands was optimum at 5 mM MgCl2 concentration. Therefore, MgCl2 concentration of 5 mM was used throughout all conventional PCR experiments. Different amounts of genomic DNA were tried (50, 100, 150 and 200 ng per 25µl reaction mix) and 150 ng was found optimum. The stringency of amplification can be controlled by adjusting the annealing temperature. Minimizing the incubation during the annealing and extension steps limit the opportunities for mispriming by molecules DNA. The temperature at which annealing is done depends on the length and the GC content of the primers. Higher temperature may also be necessary to increase primer specificity. Of the different primer annealing temperatures tried (53°C to 58°C), 55°C was found to be optimal. Taq DNA polymerase (0.6U per 25 ul) resulted in good amplification of MCF DNA as compared to 0.3 U. The amplification of 226 bp DNA fragment from deer isolate of MCFV and positive control is shown in Figure (5B). The size of the PCR product was in agreement with the expected size calculated according to the published DNA sequence.

To confirm the authenticity of the amplified products The PCR product was cloned into Topo TA cloning vector to produce the recombinant plasmid pSDE1 (Figure 6) and subjected to automated DNA sequencing using the universal forward and reverse primers (Figure 6). Sequence alignment and a BLAST search performed with the GenBank database indicated that the PCR product was amplified out of an authentic DNA that belongs to the deer isolates of MCFV. The DNA sequence identity was 99% with 0% gaps when compared with deer MCFV polymerase gene. Sequence identity was only 82% when compared with the OvHv-2 DNA polymerase gene (Figure 7). Amplification of DNA from organisms other than deer strain of MCFV would be very unlikely. Therefore, this fluorogenic PCR was regarded as specific for the deer isolate of MCFV DNA.

In this study, a TaqMan® real time PCR technique was developed to detect and quantitate the deer isolate of MCFV. A dually labeled real time PCR probe was also designed and used. $MgCl_2$ concentration of 4 mM was found optimal throughout all real-time PCR experiments. The amplification of MCFV from ovine strains was performed at different annealing temperatures and the optimum temperature for real-time PCR was 60 °C.

The recombinant plasmid pSDE1 served as positive control, while DNA-less and negative samples were used as reagent and negative controls, respectively (Figure 8). The absolute quantitation of viral DNA is accomplished by generating a standard curve with serial dilutions of pSDE1 and then comparing the signals obtained from samples with unknown amounts of DNA to the standard curve. As expected, the cycle number at which a positive signal could

be discriminated from background values (threshold cycle or CT value) decreased in a linear manner with exponentially increasing numbers of DNA templates. The standard curve (logarithm of input pSDE1 copy number versus CT value) shown in Figure (8) indicates that detection MCFV would be possible up to a dilution of 1 ng (2.2 x 105 copies). However, diluting the standard DNA (pSDE1) a hundred folds further still gave a positive result. Therefore, a dynamic range for detection of MCFV would be possible from 10 pg (2.2 x 103 copies) to 100 ng (2.2 x 107 copies). The absolute MCFV copy number in each sample was calculated based on the molecular weight of the pSDE1 molecule.

To determine the efficiency of the real-time PCR, the absolute slope value was calculated. The absolute value of the slope of the standard curve is regarded as a measure of the efficiency of PCR. A slope of 3.632 was determined for the amplification of a fragment of the polymerase gene from the deer isolate of MCFV. A PCR efficiency of 88.5% was calculated based on this slope. The quantitative real-time PCR was used to measure the amplification of the polymerase gene fragment from the deer isolates of MCFV. A specific MCF negative deer was used as a negative control along with the DNA-less control. The MCFV copy number in different samples (viral titer) ranged from 106 to 107 copies per ml (Figure 9).

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>gb|AF387516.1| Deer malignant catarrhal fever virus DNA polymerase gene, partial
cds
Length=388

 Score = 338 bits (374),  Expect = 4e-90
 Identities = 191/192 (99%), Gaps = 1/192 (0%)
 Strand=Plus/Plus

Query  8    ACGTAGAC-TTCAGTACCTGCAGCAAATATGCCCAACCCAGATTATAAAGAGTCAATCGC  66
            |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93   ACGTAGACATTCAGTACCTGCAGCAAATATGCCCAACCCAGATTATAAAGAGTCAATCGC  152

Query  67   CCCACACTAACCCGAGATTCACAGTTGTGTATGGGGACACAGATTCTCTGTTTATTAATT  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153  CCCACACTAACCCGAGATTCACAGTTGTGTATGGGGACACAGATTCTCTGTTTATTAATT  212

Query  127  GCGAGGGTTTTGGCATAGACACAGTTCTAGCATTCAGCGACAAGCTAGCCTCCCACACAA  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  GCGAGGGTTTTGGCATAGACACAGTTCTAGCATTCAGCGACAAGCTAGCCTCCCACACAA  272

Query  187  GCAGCCTCCTAT  198
            ||||||||||||
Sbjct  273  GCAGCCTCCTAT  284
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Figure (2): Results of BLAST analysis indicating the authenticity of the deer amplicon.

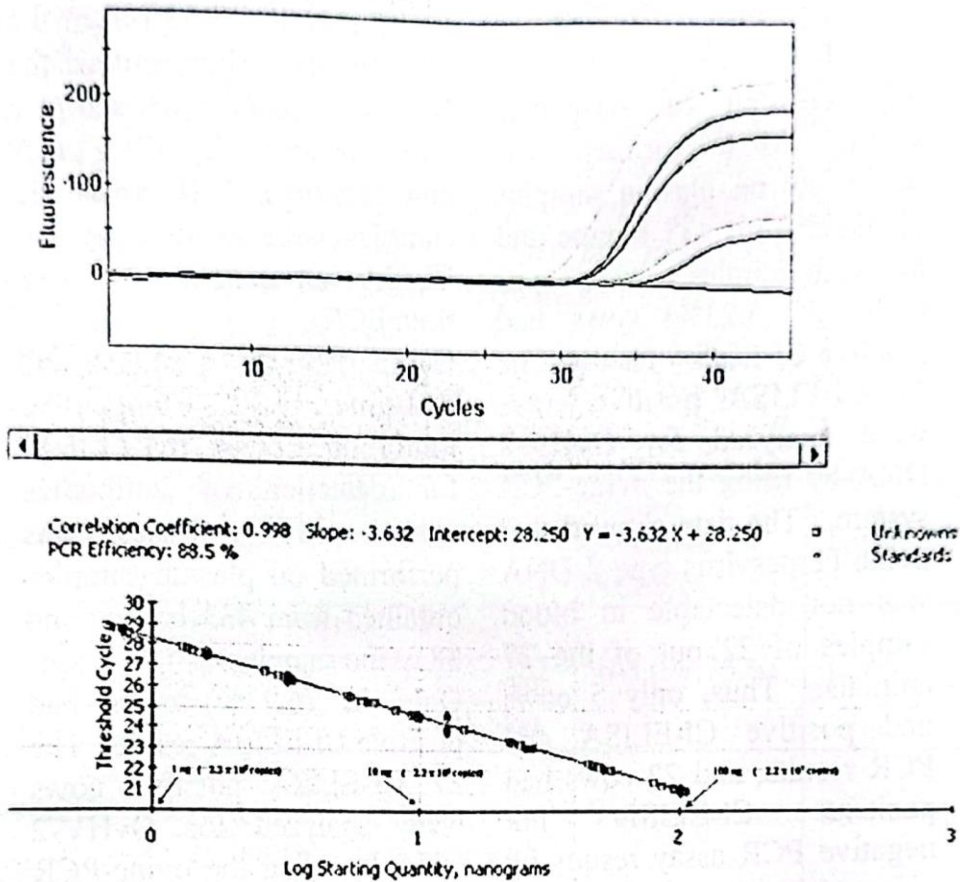

Figure (7): Quantitation of the deer isolate of MCFV by real-time PCR. DNA sequences were amplified by real-time PCR and a standard curve was established by plotting the threshold cyclt (Ct) against the log of the starting quantity.

Comparison of CI-ELISA and real-time PCR. Competitive inhibition ELISA (CI-ELISA) for detection of antibodies against MCF viruses was performed on plasma samples obtained from 433 bovine and 88 ovine samples.

Only 27 (6.23%) cows had positive CI-ELISA results. The 27 CI-ELISA positive cows were analyzed for OvHV-2 DNA by using the ovine-PCR system. The data showed that ovine herpesvirus type 2 DNA was not detectable in blood samples of 22 out of the 27 animals. Thus, only 5 cows had positive CI-ELISA and PCR results, and 22 cows had positive CI-ELISA but negative PCR assay results by using the ovine-PCR system (Table 3). On the other hand, 79 (89.7%) of the ovine samples had positive CI-ELISA results. The 79 CI-ELISA positive sheep samples were analyzed for OvHV-2 DNA by using the ovine-PCR system. The data indicated that ovine herpesvirus type 2 DNA was not detectable in blood samples of 33 out of the 79 animals. Thus, out of the 88 sheep samples, 46 samples were positive by CI-ELISA and real-time PCR, while 33 samples were positive by CI-ELISA but negative by real-time PCR

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Animal Species	ELISA Positive Samples*		ELISA Negative Samples	Total
Bovine	27 (6.23%)		406 (93.76%)	433
	PCR +ve	PCR -ve		
	5 (18.5%)	22 (81.5%)		
Ovine	79 (89.7%)		9 (10.3%)	88
	PCR +ve	PCR -ve		
	46 (58.2%)	33 (41.8%)		
Total	106 (20.3%)		415 (79.7%)	521

(Table 3). Table (1): Results of CI-ELISA and real-time PCR performed on blood samples.

Ovine Samples

ELISA

\+ -

PCR

\+ 46

\- 33

9

Bovine Samples

ELISA

\+ -

PCR

\+ 5

\- 22

406

DISCUSSION

The objective of this study was to develop a sensitive PCR-based testing for the detection and quantification of MCF in different host species. To this end, conventional PCR was used to amplify viral genomic segments from ovine and deer blood and tissue samples.A competitive PCR assay was previously developed for quantitating SA-MCF viral DNA in clinical samples (Hua et al., 1999). The assay was a one step, 40-cycle coamplification of target and competitor. The minimum threshold of sensitivity was 30 copies of the viral genomic DNA. Increasing the number of cycles beyond 40 yielded no practical benefit in sensitivity. Nested (2-step) PCR yielded almost the same sensitivity as compared to the one-step protocol. In competitive PCR assays, the differences in the amplification efficiency due to variation in length and base composition between target and competitor can be a source of significant error (Clementi et al., 1995; Mayerat et al., 1996).

In this study, a new PCR assay was developed for the detection and quantitation of the deer strain of MCFV. Conventional PCR was used in a one-step PCR format. The PCR primers used to detect the ovine herpesvirus 2 (OvHV-2) DNA were those published by (Hussy et al., 2001). The data of the present study indicated that an amplicon of 131 bp was amplified from the ovine stain of MCFV by using this PCR system.The PCR primers used to detect the deer strain was designed and amplification was optimized in the current study. The optimization of the PCR assay (deer-specific) PCR systems involved the concentration of MgCl2, amount of genomic DNA, and annealing temperatures. The amplification 226 bp DNA sequences from the deer isolates of MCFV was in agreement with the expected

size calculated according to the published DNA sequence. Several nested PCR assays for AlHv-1, OvHv-2, CpHv-2 (Chmielewicz et al., 2001) and WTD-MCF (Li et al., 2000) have been described. Conventional quantitative PCRs are time-consuming and carry a high risk of false-positive results due to contamination, especially when a high sample throughput is required. Conventional PCR assays have been described for bovine gammaherpesviruses and WTD-MCF virus, but they have had limited use in diagnostic labs (Fabian & Egyed, 2004; Li et al., 2000). Nucleotide sequence analysis of the 226-bp for deer amplification product demonstrated that the viruses detected was identical to that from white-tailed deer with lesions characteristic of MCF reported by (Li et al., 2000). This virus is closely related to the virus known to cause MCF in ruminants, AlHv-1 and OvHv-2; the deer MCFV is 82% identity to OvHv-2, 71% to AlHv-1 (Li et al., 2000; Li et al., 2001b).

In this study, a TaqMan® real time PCR technique was developed to detect and quantitate the deer isolates of MCFV. The assay makes use of a dually labeled fluorogenic probe, which is designed to hybridize to the sequence between the primers. The fluorescent emission of a reporter dye, covalently attached to the 5' end of the probe, is quenched due to the physical proximity of a quencher dye at the 3' end. The 5' → 3' exonuclease activity of the DNA polymerase causes cleavage of the probe in the course of amplification, which leads to dislocation of both the reporter and the quencher. This results in an increase of the reporter dye's fluorescent emission, which is, at each time point measured, directly proportional to the amount of amplified target DNA.

A fluorogenic PCR specific for ovine herpesvirus 2 (OvHV-2)

DNA was developed by (Hussy et al., 2001) and compared to a previously established conventional seminested PCR. Testing of a total of 152 blood samples from both positive and negative animals revealed that the results of both assays corresponded to each other in 100% of the cases. The analytical sensitivity of PCR ranged between at least 10 copies and sometimes even 1 copy of target DNA per reaction mixture. In dilution series of the target DNAs, linear decreases of the signals were observed over 7 orders of magnitude. By this technique, it was possible for the first time to quantitatively characterize the course of OvHV-2 replication in naturally infected sheep. (Kim et al., 2003) used real time PCR to identify sheep experiencing an episode of intense OvHV-2 DNA shedding in their nasal secretions. Significantly, it has been discovered that a specific pattern of OvHv-2 shedding occurs in sheep (Hussy et al., 2001; Li et al., 2004).

A dually labeled real time PCR probe was designed and used to amplify the deer viral isolates by using real-time PCR. An absolute quantitation of DNA was carried out by generating a standard curve with serial dilutions of the plasmid-cloned target DNA and then comparing the signals obtained from samples with unknown amounts of DNA to the standard curve. The cycle number at which a positive signal can be discriminated from background values is termed the CT value and correlates with the amount of target templates in the initial reaction mixture. The CT values of standard plasmid dilutions allow calculation of the quantity of template molecules in a specific sample. The standard curve obtained showed that a dynamic range for detection of MCFV would be possible between 10 pg (2.2 x 103 copies) to 100 ng (2.2 x

107 copies). Therefore, our results indicated that the lower detection limit of the fluorogenic PCR was 2200 copies. The linearity persisted over the whole range of dilutions (at least 4 log units). (Hua et al., 1999) reported a sensitivity of 30 target copies for their assay and the linearity persisted over 7 log units. The copy number of MCFV per ml of blood samples ranges from 103 to 5 x 104 as reported by (Hussy et al., 2001). The first positive signal for OvHV-2 DNA was detected 6 weeks after introduction of the animal into the positive flock. At week 8, a maximum of 50,000 copies of OvHV-2 DNA per ml of blood was reached. The titer then dropped rapidly and undulated thereafter between 1,000 and 8,000 copies per ml until a second, smaller peak was observed between weeks 20 and 22 (11,000 copies). Afterward, until the end of the observation period at 39 weeks after introduction of the OvHV-2-negative sheep, the titer remained low, between 1,000 and 3,000 copies per ml. Compared with conventional PCR assays, real-time PCR assays are becoming invaluable in diagnostic labs not only for their ability to be run rapidly in a high throughput format yielding specific and sensitive quantitative information over a large dynamic range, but also for reducing potential cross contamination. This is because of the gel-free format endowed in real time PCR assays. More recently a real-time PCR specific for both AlHv-1 (Traul et al., 2005) and OvHv-2 (Hussy et al., 2001) were reported. The benefit of the real-time PCR assay compared to previous PCR assays has been the ability to rapidly ascertain specific patterns of viral shedding in the carrier host. We used the real time PCR technique to detect and quantitate the OvHv-2 DNA in animal samples and to establish a fast, simple, and

reliable fluorogenic PCR to quantitate deer MCFV DNA. The real-time PCR assay of deer viral isolates was optimized with respect to both annealing temperatures and magnesium ion concentration. The annealing temperatures was optimum at 60°C and the ideal magnesium ion concentration was 4 mM.

In this study CI-ELISA and real-time PCR assays were compared. Competitive inhibition ELISA (CI-ELISA) for detection of antibodies against MCF viruses was performed on plasma samples obtained from 433 bovine and 88 ovine samples. The CI-ELISA detects antibodies against a virus glycoprotein that is produced by OvHv-2 and other MCFVs such as AlHV-1 (Li et al., 2001b; Li et al., 1996; Li et al., 1994). A positive CI-ELISA result indicates previous infection but doesn't necessarily indicate current infection. Seroconversion from negative to positive status indicates recent infection or recrudescence of a latent infection. The limitation of the use of CI-ELISA is that it cannot differentiate between previous infection with viral clearance, acute infection, and continued latent infection. Another limitation is that the CI-ELISA cannot differentiate between exposure to OvHv-2 or other MCFVs (Powers et al., 2005).

Malignant catarrhal fever has been considered an uncommon disease in domestic cattle and bison in the USA, where it was defined as a fatal disease. Data from this study showed that only 27 (6.23%) out 433 cattle had positive CI-ELISA results. The 27 CI-ELISA positive cattle were analyzed for OvHV-2 DNA by using the ovine-PCR system. Only 5 cattle had positive CI-ELISA and PCR results, while 22 cattle had positive CI-ELISA but negative PCR results. This data from the current study showed that ovine herpesvirus type 2 DNA was not detectable

in blood samples of 22 out of the 27 cattle. Cattle with positive CI-ELISA results and negative PCR results are consistent with results of a study (O'Toole et al., 2002). They showed that 89% of bison with positive CI-ELISA results had negative OvHv-2 results as detected by a single PCR assay performed during entry into a feedlot. These cases may represent latent infection in which viral DNA is below the detectable limits of the PCR assay or clearance of the infection. Differentiation between clearance of acute infections, and persistent latent infections could not be made on the basis of data available from their study. Subclinical OvHv-2 infections can occur in cattle under natural exposure conditions. Most of the cattle infected with OvHv-2 develop subclinical infections rather than clinical MCF (Powers et al., 2005). These comparative results suggest that the biology of subclinical infections in cattle is different from that in sheep. Although SA-MCF has not been induced by inoculation of cattle with tissues from suspected carrier sheep, the disease has been reported most frequently when the cattle in close contact with sheep. So the presence of OvHv-2 in normal, healthy dairy cattle suggests the possibility of an additional reservoir for the virus (Collins et al., 2000) or this may indicate that some co-factors are needed for development of the disease. Continued investigation of the pathophysiology of OvHv-2, particularly in regards to cellular tropism, virus replication, and viral protein expression in cattle, is necessary for more complete understanding of the virus and its association with MCF in cattle.

Out of 88 sheep samples examined in this study by CI-ELISA and PCR, 79 (89.7%) had positive CI-ELISA results. Out of the 79 ELISA-positive samples, only 46 were also

positive by PCR. The proportion infected animals (58.2%) was lower than that reported by (Li et al., 1995b) who found that 143 out of 144 (99%) blood samples from adult sheep were positive for OvHV-2 DNA sequences. Ovine herpesvirus-2 (OvHv-2) can be detected in 100% of lambs by PCR testing of peripheral blood leukocytes and that lambs become infected before they are 6 months old(Li et al., 1995b). (Wani et al., 2006) reported PCR positives as high as 28 out of 33 (84.8 %) sheep samples. The failure to detect the 131 bp OvHv-2 DNA fragment in 33 CI-ELISA positive sheep samples agrees with the observations (Li et al., 1995a) who revealed that almost all the lambs became PCR-positive when they were between two and three months as samples may have a smaller amount of OvHV-2 DNA in their blood. It is probable that these cases may have been sampled at an early stage of the infection. The fact that the lambs did not become infected during the neonatal period could be related either to passively transferred maternal immunity or to the age-dependency of susceptibility at a cellular level (Li, et al 1998). But (Li et al., 2004) suggested that passively transferred immunity may not play an important role in the delay of infection of lambs with OvHV-2. They also suggested that the rate of infection in young lambs may depend on the intensity of their exposure to the virus in the environment.

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