Study on Correlation of Maedi-Visna Virus (MVV) with Ovine Subclinical Mastitis in Iran

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Abstract Maedi-Visna is an important slow viral disease of sheep leading to progressive pneumonia, encephalitis and mastitis. Udder is one of the organs affected by MVV. Despite the fact that in Iran Maedi-Visna is known since 2000, to the authors' knowledge correlation of subclinical mastitis and infection with MVV has not been assayed. In this study 50 milk samples from 10 flocks in East Azerbaijan Province of Iran were tested. None of the animals exhibited any clinical signs of the disease. Forty samples were collected from CMT positive ewes and ten were taken from CMT negative ewes. Milk samples were analyzed using PCR targeting gag sequence. Presence of provirus DNA was detected in one sample from CMT negative and seven samples from CMT positive ewes. These data demonstrate that 16.5 % of sheep with subclinical mastitis were infected to MVV. Thus this virus can be considered one of the main pathogenic agents of mastitis and can be dramatically transmitted to lambs by milk.

Keywords Maedi-Visna · Sheep · Milk · Mastitis · PCR

Introduction

Maedi-Visna (MV) is a disease of sheep caused by a group of viruses defined as small ruminant lentivirus (SRLV) that

belong to the genus lentivirus of the retroviridae family. This disease affects small ruminants causing chronic inflammatory lesions in the lungs, joints, mammary glands, and brain [1]. MV indurative mastitis is difficult to identify clinically, as the affected ewes usually would be older than 4 years and a degree of hardness in mammary glands is expected. In practice, the condition can only be diagnosed by histological examination. Milk production is significantly decreased and the somatic cell count (SCC) is increased, although there are no gross changes in the characters of milk. There is positive and significant correlation (0.53) between SCC and CMT [2]. Transmission occurs mainly by the respiratory route and also from dam to offspring via infected colostrum and milk [3, 4]. The results obtained from this study justify a large-scale experiment to investigate the relative contribution of milk from infected dams to the risk of infection in the offspring. We hypothesized that use of milk samples might be appropriate as an alternative to blood samples for molecular diagnosis of SRLV.

There are several serological techniques available for detection of specific antibodies against SRLV, the most commonly used being agar gel immune diffusion test (AGID) and, with higher sensitivity, enzyme-linked immunosorbent assay (ELISA) [5]. However, antibody detection methods may have limited sensitivity related to antigenic variation in SRLVs, to the existence of infected animals that do not develop a humoral immune response and to interfere with colostrum acquired antibodies in young animals. In addition, the presence of leukocytes in milk samples from cases of clinical mastitis and in milk samples with high SCC may also potentially inhibit growth of the agent [6, 7]. Due to the limitations of cultural methods, polymerase chain reaction (PCR) has been developed to identify various mastitis pathogens [8]. Recently, Extramiana et al. [9] have developed a single-step single-primer PCR for detection of

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sequences of the long terminal repeat (LTR) of MVV proviral DNA in blood, milk and tissues of naturally infected sheep. This PCR method might well be adapted to routine diagnosis of SRLV-infected animals due to its simple and easy-to-perform protocol.

SRLV infection can be detected by PCR in seronegative infected individuals [10]. Hence, application of PCR in along with serological tests potentially increases the detection sensitivity. Therefore, it was also investigated if PCR had additional diagnostic value when testing individual milk samples and bulk milk [11]. In the present study, we report genomic sequences analyses of SRLVs in a small geographic area of Iran for the first time. The aim of this study is to determine the presence/absence of MVV proviral-DNA using PCR method in ovine milk samples and to confirm its correlation with subclinical mastitis.

Materials and Methods

Animals

A total of 50 ewes, 2 to 4-year-old, belonging to 10 flocks were sampled in this study. The ewes were expected to be healthy, and free of clinical mastitis and any other palpable udder lesion. All of the ewes were in their mid-lactation stage and were milked by hand.

California Mastitis Test (CMT)

CMT was performed on all milk samples on the farms using the method described by Schalm et al. [12]. According to visible reactions the results were classified into 5 scores: (0) = negative, ($\pm$) = trace, (+1) = weak positive, (+2) = distinct positive, and (+3) = strong positive. Ewes that scored negative and trace were assumed healthy and the ewes with weak positive were assumed suffering subclinical mastitis.

Sample Collection

Udder halves were cleaned, disinfected with 70 % alcohol and dried with sterile cotton prior to sampling. The first three squirts of milk were discarded and approximately 5 mL of milk were taken in a sterile tube for bacteriological examinations, and 1 mL of milk was collected aseptically and transferred to the laboratory in a 1.5-mL microtube within 1–3 h in a 4–8 °C.

DNA Extraction from Milk Samples

DNA extraction was carried out as described by Meiri-Bendek et al. [8] with minor modifications. Samples were

centrifuged at 14,000 rpm for 4 min. The supernatant was discarded and the pellet was resuspended and washed 2–3 times with Tris–EDTA buffer until a clear solution was obtained. The pellet was washed once with PCR buffer and finally resuspended in 100 μ L of PCR buffer. Then, lysozyme (Merck, Germany) was added to each sample at the concentration of 2 mg/mL. Samples were incubated 15–20 min at room temperature. After that, proteinase K (Fermentas, USA) was added to individual sample at the concentration of 400 μ g/mL and samples were incubated at 56 °C for 1 h. DNA extraction was continued by a silica gel based kit (Aquaprep DNA extraction kit, Bionner, S. Korea) according to manufacturer instruction. Electrophoresis of each DNA sample on 2 % agarose gel in 1X TBE buffer was undertaken to check the integrity of the DNA. An aliquot of total DNA was produced from each sample and stored at –20 °C until required for analysis.

Polymerase Chain Reaction (PCR)

Detection of the gag gene of Maedi-Visna provirus was carried out with the forward 5' _GGGACGCCTGAAGTAG GTA-3' and reverse 5' _CAAAATCCTCGGACACAAG-3' primers, as described by Daltabuit-Test et al. [13], that specifically amplify a 748 bp fragment of the gag gene. For each sample, two 25 μ L PCR reactions containing 50 ng of DNA were carried out. The samples were considered positive when at least one of two replicates amplified the expected fragment size and all the controls gave the expected results. To analyze the PCR products, 10 μ L of each PCR product was electrophoresed on a 1.5 %

Fig. 1 PCR products gel electrophoresis. L: DNA ladder (100 bp), 1: negative control (fish sperm DNA), 2–4: positive samples, 5: negative sample, 6: positive control, 7/8: negative samples, 9: water blank

Fig. 2 Alignment of the sequence of Iran's isolate with nearest homologue sequence (Accession # HQ848062.1)

62	AAACTTCGGGGACGCCTGAAGTAAGTAAGAGAGACACCTACTGGGGAAAGTAGGGAAAA	121
284	AAACTTCGGGGACGCCTGAAGTAAGTAAGAGAGACACCTACTGGAGAGAGTAGGGGAAGG	343
122	GCACCCCTTCAAGGAAGAGAAAGTGTGCTTGGGGTCAGGAGGAGGATCCGCGACCCCTGGT	181
344	GCACCCCTTCAAGGAAGAGAAAGTGTGCTTGGGGTCAGGAGGAGGATCCGCGACCCCTAGT	403
182	CTTATTAAAGAGGGGTGCGGACGTCCTCCTGAGCCGAGGGAGCACAAGAAGCAACACTG	241
404	GAAAGAAAAAG–GGGGCGCGGACGTCCTTCTGGGCCGAGGGGACACAAAGGCAGCACCA	462
242	GTAAGAAAGCCGCTGTGGTGAATCTAGATAGAGACATGGCGAAGCAAGGCTCAAAGGAGA	301
463	GTAAGACAACCGCTGTGGTGAATCTAGATAAAGACATGGCGAAGCAAGGCTCAAAGGAGA	522
302	AAAAGGGATACCCGAGCTCAAAGAGGCTATAAAAGCAACATGTAAAAATAAGATTGGGC	361
523	AAAAGGGGTACCCGAGCTCAAAGAGGTAATTAAAGCAACTTGTAAAAATAAGATTGGGC	582
362	CAGGGAAGGAGACCTTGACAGAAGGGAATTGTCTATGGGCTTTAAAACTGTAGACTTCA	421
583	CCGGAAGGAGACCTTGACAGAAGGGAATTGTCTATGGGCATTAAAACTATAGACTTTA	642
422	TATTTGAAGACTTAAAGCAGAGCCATGGACACTAACAAAAA–GTACACAGTATGGGGGA	480
643	TATTTGAAGATATAAAGGCAGAACCTTGGACCATAACAAAAATGTATACAGTATGGGGAA	702
481	GATTAATAAAGTTAACTCCAGAGGAAACAAGCAAAAGGGAATTTGCTTCATTGCAAGCCA	540
703	GATTAGCGGCATTAAACGCCAGAGGAAACAAGCAAAAGGGAATTTGCTTCATTGCAAGCTA	762
541	CAATGGCATGTTTAATGTGTAGCCAAATGGGGATGAAGCCAGAGACAGTACAGGCAGCTA	600
763	CGATGGCATGTTTGATGTGTAGTCAAATGGGGATGAAGCCGAGAGAGTGCAGGCAGCTA	822
601	GGGGAATACTGAGTATGAAGGAAGGGCTACaagaaacagaagaagaaaaagagaaaaagG	660
823	GGGGAATAATAAGTATGAAAGGAGGGCTTCAAGAAAAACAAGAGGAGAAAGAAAGAAAA	882
661	TAGAACAGCTCTACCCAAACTTAGAGAGGCACAGA–GAAGCGT–ACCCTATAGTAAATT	717
883	TAGACAATTGTATCCTAATTTAGAGA–CACATAAGGAG–GTCTACCCCATAGTAAATT	939
718	TACAAGCAGGGGAAGAAGCTGGAAGGC	745
940	TACAAGCAGGGGAAGAAGTTGGAAGGC	967

agarose gel containing 0.5 µg/mL of ethidium bromide and visualized by ultraviolet light trans-illumination.

Sequencing

Positive PCR amplicons were sequenced from both ends. DNA sequencing was carried out using the dideoxy chain termination procedure (Chemistry V3.1, Applied Biosystems) and the 3730XL DNA analyzer (Applied Biosystems) by Bioneer sequencing service (Bioneer, Seoul, South Korea). The DNA sequence databases were searched using the nucleotide-nucleotide BLAST (BLASTn) at the National Center for Biotechnology Information, USA. Comparison of the different gag fragments with each other and with previously reported sequences were undertaken using align two sequence (bl2seq) (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>) at the National Center for Biotechnology Information, USA (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Results

Of fifty samples, eight samples were positive by PCR. Figure 1 shows the gel electrophoresis of PCR products. Among them seven samples belonged to CMT positive animals. These data demonstrate that 16.5 % of sheep with subclinical mastitis were infected to MVV. Ten percent of CMT negative sheep were MVV positive and they are likely at risk of mastitis. The PCR products were sequenced from both ends by gag 3 and 4. When the sequence results were subjected to BLAST search, the best score of 99 % homology was returned with Maedi-Visna virus (HQ848062, AY101611, HQ864608.1, L06906, M10608) in listed order. Figure 2 demonstrates the alignment of the sequence of Iran's Isolate with nearest homologue sequence (Accession # HQ848062.1). Figure 3 presents neighbour-joining phylogenetic tree based on 748 bp fragment of the gag gene.

Fig. 3 Neighbour-joining phylogenetic tree based on 748 bp fragment of the gag gene. NCBI registered isolates are shown by name except “Iran’s isolate”

Discussion

Maedi-Visna disease is caused by a non-oncogenic, exogenous retrovirus and manifests with a long incubation period of several months to years. The clinical manifestation of MV takes years to be detectable, and infected sheep are persistent sources of viral transmission. Therefore, economic losses due to this disease are considerable. These economic losses include marketing and export restrictions, decrease in weight of infected animals, decreased lamb birth weight and losses of milk production due to indurative chronic mastitis [3, 14]. To successfully utilize eradication programs, the infected animals should be accurately diagnosed and removed from their flocks. The virus is present in blood and milk samples. Transmission in a flock takes place predominantly between dam and lambs via infected mononuclear cells in colostrum and milk [15]. Thus, mammary glands are target of MVV, while little studies are carried out about MVV infection in this site. Most studies on MVV infection use blood samples. For investigation of relationship between mastitis and MVV infection, only milk samples were used. The use of milk samples rather than blood for molecular detection has many advantages: first, the use of milk provides similar information when compared with that obtained from blood. Second, the samples can be collected easily and stored directly by the owner of the flock, lowering the final cost of the test. Third,

detection of MVV in milk can be more reliable to prove the relationship between MVV infection and subclinical mastitis. The findings of this study showed that virus exists in milk and support the initial hypothesis of using milk samples as an alternative to blood samples for molecular diagnosis of SRLV. Different studies have used molecular or serological methods such as PCR, ELISA and AGID in order to survey the presence of MVV in milk samples [5, 6, 8, 9, 14, 15]. Unfortunately, serological methods aren’t able to detect infected animals prior to seroconversion, and the result of the tests cannot be interpreted during the first 6 months of life because of passively acquired antibodies in non-infected offspring [16].

Presence of SRLV antibodies in ovine milk was detected by Ploumi et al. [17], which indicated that MVV-seropositive sheep flocks are commonly associated with mastitis incidence. To our knowledge there is no study regarding the correlation between ovine mastitis and SRLV using molecular techniques in Iran. In the present study, the prevalence of Maedi-Visna virus infection was 16 % (Table 1).

Some authors [18] are of the opinion that ovine milk in which the SCC exceeds 500,000 Cells/mL is not acceptable as such milk samples appeared to be four times more frequently infected by pathogens as was compared to milk with lower SCC. On the basis of the present researches, it has been demonstrated that isolation of virus was associated with an increased CMT and reduced impedance in

Table 1 Negative and positive PCR results in comparison with CMT results of milk samples examinations

	CMT+	CMT–
PCR+	7	1
PCR–	33	9

sheep. The CMT score has positive correlation with SCC, infection status and number of bacteria isolated in small ruminant [2]. In the present study positive correlation were demonstrated between CMT and infection. This result is in agreement with Pekelder et al. [19] who indicated that MVV infection increases SCC in sheep.

It is reported that PCR has a sensitivity and specificity of 94.7 and 84.5 %, respectively, relative to the p28 ELISA [20]. Extramina et al. [9] evaluated the LTR-PCR technique for detection of the MV provirus DNA in blood, milk and tissue samples of infected sheep. They stated that LTR-PCR had 100 % specificity and 98 % sensitivity for tissue samples in comparison with the two serological methods, ELISA and AGID tests. Brinkhof et al. [10] reported that gag-PCR is more sensitive than LTR-PCR. They also revealed that the proportion of PBL samples from seropositive ewes that were LTR-PCR and gag-PCR positive were 44 and 64 % respectively. Therefore in the current study, SRLV infection was investigated by gag-PCR instead of LTR-PCR.

To conclude, the findings from this study extend our understanding of the role of MVV in subclinical mastitis and demonstrate the presence of MVV in milk samples of Iranian sheep flocks. The use of this assay may be most beneficial as a method of focusing on or justifying MVV-positive herds for development of control strategies and not as a definitive test to ensure MVV negative herd.

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