FULL PAPER *Virology*

Isolation of Maedi/Visna Virus from a Sheep in Japan

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ABSTRACT. Maedi/visna (MV) is a lentiviral disease of sheep caused by the maedi/visna virus (MVV). Although MV is prevalent in many countries, it had not been reported in Japan. In 2011, however, three sheep in northern Japan were reported to be seropositive against the MVV antigen, indicating a persistent MVV infection. In the present study, we isolated MVV from one sheep to confirm MVV infection and conducted genomic classification of the virus. The co-culture of leukocytes from a seropositive sheep with fetal goat lung cells resulted in the formation of syncytial cells and the amplification of a long terminal repeat sequence of MVV by polymerase chain reaction. The isolate was confirmed as being MVV, rather than the caprine arthritis-encephalitis virus based on phylogenetic analysis of the *gag* gene sequence. Although the sheep was asymptomatic, nonpurulent meningitis and demyelination were found in the spinal cord. These were considered to be early lesions associated with pathogenic MVV infection. Therefore, the present study demonstrated that MVV is distributed in Japan.
KEY WORDS: maedi/visna, PCR, retrovirus, sheep, virus disease.

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Maedi/visna (MV) is a lentiviral disease caused by the maedi/visna virus (MVV) that mainly affects domestic sheep. Infected sheep develop fatal and progressive pneumonia and encephalomyelitis after a latent period of several months to years [7, 12, 13]. Because most MVV infected sheep produce a specific antibody that persists for life, MVV infection can be diagnosed serologically by the agar gel immunodiffusion (AGID) test and enzyme-linked immunosorbent assay [4]. MVV infection is recognized worldwide and leads to economic loss entailing not only death caused by fatal disease but also premature culling and the restriction of exports. Therefore, MVV infection has been considered a target for eradication, particularly in certain European countries [14]. In contrast, clinical MV has yet to be recognized in Japan, and no epidemiological studies of MVV infection had previously been reported. However, a recent serological survey demonstrated that three out of 267 sheep in the prefectures of Hokkaido and Iwate were seropositive against the MVV antigen, as based on AGID and enzyme-linked immunosorbent assay tests [5]. These sheep were asymptomatic, but the presence of an antibody indicated a persistent MVV infection. Therefore, in the present study, we tried to isolate MVV from one of the sheep on a farm in Iwate Prefecture, in order to confirm the infection and conduct genomic classification of the virus.

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MATERIALS AND METHODS

Virus isolation: Peripheral blood was obtained from a Cheviot sheep (estimated to be 13 years old) that had already been diagnosed as seropositive against the MVV antigen [5]. The blood was mixed with an equal volume of 0.83% ammonium chloride to lyse erythrocytes for the isolation of whole leukocytes. The isolated leukocytes were then co-cultured with the primary cultured cells of fetal lamb lung (FLL) (passaged 10 times) and fetal goat lung (FGL) (passaged 2 times) in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 unit/m*l* of penicillin, 100 μg/m*l* of streptomycin and 5 μg/m*l* of amphotericin B. The FLL and FGL cells were passaged approximately every five days. These cultured cells were subjected to Giemsa staining for the detection of multinucleated giant cells and to genomic DNA extraction for polymerase chain reaction (PCR), in order to analyze the integration and propagation of MVV.

PCR: PCR was performed to amplify a partial sequence of a long terminal repeat (LTR) and a complete sequence of the *gag* gene of MVV. The templates used for PCR were the genomic DNA extracted from the peripheral blood leukocytes of the seropositive sheep, those from the FLL or FGL cells co-cultured with infected sheep leukocytes or those from tissues taken at euthanasia of the sheep. The tissue samples

included the cerebrum, lung, spleen, turbinate and lymph nodes adjacent to the lung, intestine, spleen and spinal cord.

DNA was extracted using a Gentra Puregene Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCR was performed using a GoTaq Green Master Mix (Promega, Madison, WI, U.S.A.). Table 1 lists the primers used to amplify LTR and the *gag* gene, which were added at 0.5 μM. The primer pair used for LTR has been previously reported [1]. The amplification of LTR was performed as follows: initial denaturation at 94°C for two min, followed by 35 cycles at 94°C for 30 sec, at 58°C for 30 sec and then at 72°C for 30 sec. The *gag* gene was amplified as follows: initial denaturation at 94°C for two min, followed by 35 cycles at 94°C for 30 sec, at 52°C for 30 sec and then at 72°C for 1.5 min. The MVV positive control was kindly provided by Prof. Misao Onuma at Hokkaido University. Triplicate PCRs were performed for sequence determination to exclude PCR-generated errors. The amplified products were cloned into the pCR2.1-TOPO vector (Life Technologies, Gaithersburg, MD, U.S.A.). The LTR and *gag* gene sequences were analyzed using a BigDye Terminator v3.1 Cycle Sequencing Kit with an Applied Biosystems 3130 Genetic Analyzer (Life Technologies).

Phylogenetic analysis of the virus: Phylogenetic analysis was performed using Clustal W in MEGA5 software [21]. A phylogenetic tree based on *gag* gene nucleotide sequences was generated using the Neighbor-Joining method with a *p*-distance model. The analysis included MVV strain 1514 as the reference strain (M60610) [20], 85/34 (AY101611) [10], P1OLV (AF479638) [2], SA-OMVV (M31646) [16] and EV1 (S51392) [18]. The caprine arthritis-encephalitis virus (CAEV) strains included in the study were Cork as the reference strain (M33677) [17], Ov496 (FJ195346) [6], Gansu (AY900630) and No. 40, which was isolated from a goat in Japan [8]. The *gag* gene of equine infectious anemia virus (EIAV) (AF247394) was used as the outgroup.

AGID: The serum sample taken from the sheep used in the present study was analyzed by the AGID test to confirm the animal's seropositivity against the MVV antigen. The reference MVV antigen and antiserum were provided from National Institute of Animal Health (Tsukuba, Japan). In addition, the antigen of the present isolate was prepared to confirm its antigenicity against the reference MVV antisera. Briefly, virus-infected FGL cells were cultured in tissue culture flasks and passaged every five days. The culture fluids collected from each passage were pooled and concentrated 100 times using ammonium sulfate, as previously described for the preparation of bovine leukemia virus antigen for the AGID test [9].

Histopathology and immunostaining: The sheep used in the present study was humanely sacrificed for pathological analysis. Tissue samples were fixed in 10% formalin and embedded in paraffin wax. Sections (4 μm) were stained with hematoxylin and eosin (HE), along with Klüver-Barrera staining for histological examination. Immunostaining was performed using the antibodies for MVV (1:100, VMRD Inc., Pullman, WA, U.S.A.), CD3 (1:50, Dako, Glostrup, Denmark) and CD20 (1:2,000, Thermo, Waltham, MA, U.S.A.).

Table 1. Sequence of the primers used for LTR and *gag* gene amplification

Name	Sequence (5'→3')
LTR 2s	CAGAAATCATAGTCAGGATGACAC
LTR 2a	CCACGTTGGGCGCCAGCTGCGAGA
Gag-F	AACTTCGGGGACGCCTGAAG
Gag-R[a,b)]	WTCCCATTTTTCYCCTTCTA

a) W: A+T. b) Y: C+T.

RESULTS

Virus isolation from a seropositive sheep: Although the presence of MVV in peripheral blood leukocytes and tissues from the MVV-seropositive sheep was investigated by genomic PCR, the amplification of LTR was not observed. However, the FGL cells cultivated with the leukocytes formed numerous multinucleated giant cells after the fourth passage (Fig. 1). In contrast, FLL cells did not show any syncytial cells. The MVV LTR fragment was amplified by genomic PCR from the FGL cells forming multinucleated giant cells (Fig. 2), and a 253-bp long sequence without 48 bp of primer sequences was determined (AB821356). The ampricon of the positive control was 346-bp long (data not

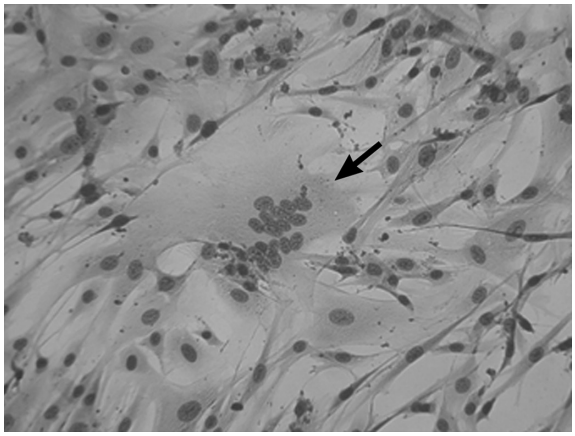

Fig. 1. Representative syncytium (arrow) formed in FGL cells. The FGL cells co-cultured with leukocytes from a MVV-seropositive sheep were passaged four times and stained with Giemsa.

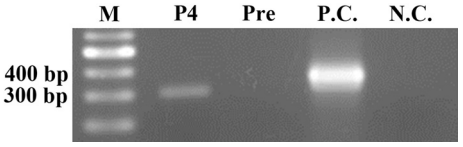

Fig. 2. Amplification of LTR fragments by genomic PCR. DNA from FGL cells passaged four times (P4) produced a band representing a 301 bp fragment, whereas DNA from seropositive sheep leukocytes before co-culture (Pre) did not undergo amplification. The positive control (P.C.) sample was 346-bp long. Autoclaved double-distilled water was used for negative control (N.C.). M: DNA marker.

A

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Isolate      1  ATGGCGAAGC AAGGCTCAAA GGAGAAAAAG GGATACCCCG AGCTCAAGGA AGTAATAAAA   60
MVV-1514     1  .......... .......... .......... .......... .......... ......T...   60
CAEV-Co      1  .......G.. ...T...CGG ..G.....GA .AT..T..T. .....G.AA. .TGT..C..G   60

Isolate     61  GCAGCATGTA AAGTAAGGAT AGGGGCCGGG AAGGAGACCT TGACAGAAGG GAACTGTCTA  120
MVV-1514    61  ...A.T.... ..A.....G. ....C..... .......... .......... ...T......  120
CAEV-Co     61  CAT.....C. .GA...AAG. TC.ACT.A.A GG....CA.. .......... A..T...T..  120

Isolate    121  TGGGCATTAA AAACTGTAGA CTTTATATTT GAAGATATAA AAACAGAGCC GTGGACACTA  180
MVV-1514   121  .......... .....A.... .......... ..G...T... .......... ......GA.T  180
CAEV-Co    121  ...TGCC.T. ....AT.... T.AC..G... ..G..CCAT. ..GAG..A.. T......AA.  180

Isolate    181  ACAAAAATGT ATACAGTATG GGACAGACTA AAACAGTTGA CTCCAGAAGA GACAAGTAAG  240
MVV-1514   181  .......... .......... ...T...T.. ...GG.C.A. ....G..G.. A.....C..A  240
CAEV-Co    181  GT....T.TA GG...A.... .C.G.AGG.G ..GA.TC.A. ....T..G.. ..GT.AC..A  240

Isolate    241  AGAGAATTTG CTTCTTTGCA AGCTACACTA GCTTGCCTAA TGTGTAGTCA AATGGGCATG  300
MVV-1514   241  ........C. .C..C..... ......GT.G ......A... .......... ..........  300
CAEV-Co    241  .A...C...A TG........ G..C...T.. ..GG.T.... .....T.C.. ......G...  300

Isolate    301  AAGCCTGAGA CAGTGCAGGC AGCACAGGGA ATAATAAAAA TGAAACAGGG ACTACACGAG  360
MVV-1514   301  ..A..C.... .......... ....A..... .......GT. .....G.A.. ......A..A  360
CAEV-Co    301  .GA..... ..T....A.A T...AT..CT .C.G...TC. .....G.T.. GT...TG..A  360

Isolate    361  AATAAGGAGC AA---GAGAA GAAGGTAGAA CAACTCTACC CA-------- ----------  399
MVV-1514   361  .........G CCAAG.G.G. .........G .......... .CAACTTAGA GAAACATAGG  420
CAEV-Co    361  C.AG....AA .GAAG..AG. C..AAG.... A.GGAAG.GA GT-------- ----------  402

Isolate    400  ---------- --ATTGTGAA CTTGCAAGCA GGGGGAAGAA GTTGGAAGGC AGTAGATTCA  447
MVV-1514   421  GAAGTTTACC CT........ T......... ..A..G.... .......... G.....G...  480
CAEV-Co    403  ---GTCTTCC CA..A..AGT GCAAGC.... ..A..G.... .C.....A.. .........T  459

Isolate    448  ATAGTCTTCC AGCAGCTACA AACAGTAGCA ATGCAGCATG GACTTGTGTC AGAGGACTTT  507
MVV-1514   481  G......... ....A..G.. ......G... .......... .......... C.....T...  540
CAEV-Co    460  G..A.G.... ....A..G.. .......... .......... .C..C..... T.........  519

Isolate    508  GAGAGACAAA TGGCGTATTA TGCCACCACA TGGACAAGTA AGGATATATT AGAAGTATTA  567
MVV-1514   541  .....G...T ....A..... ...T..T..C .....T.... .A........ .........G  600
CAEV-Co    520  ..A..G..GT ....A..... ...T..T..C .......... .A..C...C. .........G  579

Isolate    568  GCCATGATGC CTGGGAACAG AGCACAGAAA GAGCTAATAC AAGGGAAATT AAATGAAGAA  627
MVV-1514   601  .TT..... .......T.. .........G ..AT..... ....A..... ..........  660
CAEV-Co    580  .......... ....A..T.. ...T..A..G ...T....T. .......... ..........  639

Isolate    628  GCAGAAAGAT GGGTGAGGCA AAATCCGCCG GGACCAAAT- -----GTTCT CACGGTGGAT  681
MVV-1514   661  ........G. ....A..A.. ......A..C ..G..G...- -----..C.. ..........  714
CAEV-Co    640  ........G. ..AGA...A. T.....A..A CCT...GCAG GAGGA.GAT. A..A......  699

Isolate    682  CAGATAATGG GCGTAGGACA AACAAATCAG CAGGCATCTC AGGCCAACAT GGATCAAGCA  741
MVV-1514   715  ..A..... .A........ ...C...... ..A..... .A.....T.. ......G...  774
CAEV-Co    700  ..A..T.... .G........ .........A GCA...G.A. .A..T..... ......G...  759

Isolate    742  AGACAAATAT GTTTGCAATG GGTAATAAAT GCACTAAGGT CAGTGAGACA TATGTCACAT  801
MVV-1514   775  .....G.... .CC....G.. ........CA ..GT....A. .......G.. ..........  834
CAEV-Co    760  ..G..... .CC..... .......... ...T....AG ....A..... ....G.G..C  819

Isolate    802  AGACCAGGGA ATCCTATGTT GGTAAAGCAA AAGAACAATG AAAGCTATGA GGATTTTATA  861
MVV-1514   835  ........A. .C..... A..G.....G .....T.C.. .G..T..... A..C..C...  894
CAEV-Co    820  ..G..... ....A...C. A......... ..A.CG.... .GCCA..... A......GC.  879

Isolate    862  GCAAGGTTGT TGGAAGCAAT TGATGCAGAA CCAGTTACGG AGCCTATAAA AACATATTTG  921
MVV-1514   895  ..TC.CC.AC .A..G..T.. ......G... .....A.T.. .C........ .........A  954
CAEV-Co    880  .....AC..C .A........ A........G ........AC .......... .GAT...C.A  939

Isolate    922  AAAATAACTC TGTCATATAC AAATGCTAGC ACAGATTATC AAAAGCAAAT GGACAGAATA  981
MVV-1514   955  ...G....GT .......... .......... .....C.G.. .......G.. ...T..G.C. 1014
CAEV-Co    940  ..GC....A. .A..T..... ......ATCA G......G.. .G........ ...T....C.  999

Isolate    982  TTGGGAACTC GAGTACAACA GGCAACAGTG GAAGAAAAAA TGCAAGCATG TCGGGATGTG 1041
MVV-1514  1015  ........GA .G..T..... A.....G..A ........G. .......... ...A...... 1074
CAEV-Co   1000  C.A...CAAA .......... A..T.GT..A .......... .......... .A.A...... 1059

Isolate   1042  GGATCAGAAG GGTTTAAGAT GCAGCTATTA GCACAAGCTT TAAAGCCAGA AAGAAACATA 1101
MVV-1514  1075  .....C.... .A........ ...AT..... .......... .G.GA..GC. .G.G..GGC. 1134
CAEV-Co   1060  .......... ....C..A.. ...AT.G... ........A. ...G.....G ..A.GGA.A. 1119

Isolate   1102  GGAAATCGAG GAGGAGGACA AAAGTGTTAT AATTGTGGAA AACCGGGACA TTTGGCAAGA 1161
MVV-1514  1135  ...C.CAA.. .G.T.AAT.. .......... .......... ....A..... .C.C...... 1194
CAEV-Co   1120  ..G---AAT. ..CAGCC... ..G......C ..C....... .......... .CAA.....G 1176

Isolate   1162  CAATGTAGGC AAGGCATAAT ATGCCATCAT TGTGGAAAAA AGGGACATAT ACAGAGAGAT 1221
MVV-1514  1195  ..G.....A. ....A..... .......... .......... GA........ G..A.AG..C 1254
CAEV-Co   1177  ........A. ....A..C.. ...T..CA.C ........G. GA........ G..A.A...A 1236

Isolate   1222  TGCAGAAAAA AGAAAAGT-- -GAGATAAAA CAGCAGGGAA ACAGCAGGAG GGGGCCACGT 1278
MVV-1514  1255  ...C.GC.G. .....----- ---------- .......... ...A...... .......... 1299
CAEV-Co   1237  ......GG.. ...G.GACAT AAG.GG.... .......... ..G.G..... ....AT.... 1296

Isolate   1279  GTGGTGCCGT CCGCACCCCC TATGTTGTAA                                    1308
MVV-1514  1300  .......... ....G..... ..........                                    1329
CAEV-Co   1297  .......... ....T..T.. ....GAA...                                    1326
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Fig. 3. Multiple alignment of the nucleotide (A, this page) or amino acid (B, next page) sequences of *gag* genes. (A) MVV-1514 and CAEV-Cork (CAEV-Co) were used for nucleotide comparison as the MVV and CAEV reference strains, respectively. (B) Nine viruses including our isolate were compared in their amino acid sequences. Nucleotides or amino acids identical to the present isolate are shown as dots. Gaps are indicated by dashes.

B

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Isolate       1  MAKQGSKEKK GYPELKEVIK AACKVRIGAG K-ETLTEGNC LWALKTVDFI FEDIKTEPWT   59
MVV-1514      1  .......... .......... .T..I.V.P. .-........ ......I... ...L......   59
MVV-85/34     1  .......... .........R .T..IKV... .-........ .......... ..........   59
MVV-P1OLV     1  .......... .......... .T..IKV.P. .-........ ......I... .....A....   59
MVV-SA-OMVV   1  ..T....... .......... TT..IKV.P. .-........ ......L... ..........   59
MVV-EV1       1  ......R... .........R KT.RI.V.PR EG.P...... .......... ...L.G....   60
CAEV-Co       1  ..R.V.GG.R D....EKC.. H...IKVRLR G-.H...... ..C...L.YM ...H.E....   59
CAEV-Ov496    1  ..R.V.GG.R D....EKC.. Q...IKVRVR G-.H...... ..C...L.YM ..EH.E....   59
CAEV-Gansu    1  ..R.V.GG.R D....EKC.. Q...IKVRLR G-.H...... ..C...L.YM ...H.K....   59

Isolate      60  LTKMYTVWDR LKQLTPEETS KREFASLQAT LACLMCSQMG MKPETVQAAQ GIIKMKQGLH   119
MVV-1514     60  I......... ..G....... .......... ...I...... .........K ...S..E..Q   119
MVV-85/34    60  .......... .R........ .......... M......... .........R ...S..E..Q   119
MVV-P1OLV    60  I.......G. .AA....... .......... M......... .........R ...S..G..Q   119
MVV-SA-OMVV  60  ........EK ...V...... .......... ...I...... .R.......R ...S..E...   119
MVV-EV1      61  I......... .......... .......... M.......L. .........R ..MH..E..Q   120
CAEV-Co      60  KV.FR.I.QK V.N.....SN .KD.M..... ..G...C... .R...L.D.M ATVI..D..L   119
CAEV-Ov496   60  KV.FN.I.QK V.L...A.SN .KD.M..... ..G...CN.. .....L.D.M ATVT..D..L   119
CAEV-Gansu   60  KG.FRNI.QK V.N.....SN .KD.M..... ..G...C... .....L.D.M ATVT..D.VL   119

Isolate     120  ENKEQ-EKKV EQLYP----- -----IVNLQ AGGRSWKAVD SIVFQQLQTV AMQHGLVSED   168
MVV-1514    120  ....AKGE.. .....NLEKH REVYP..... .........E .V........ ..........   179
MVV-85/34   120  ....--.... .....NLEKH KEVFP..... .........E .V......N. ..........   177
MVV-P1OLV   120  .KQ.EK...I .....NLETH KEVYP..... .........E .V........ .....I....   179
MVV-SA-OMVV 120  .KQ.DK.... .....NLEKH REVYP..... .........E .VT....... ..........   179
MVV-EV1     121  ....EK.... .....NLEKH REVYP..... .......... .V........ ..........   180
CAEV-Co     120  .QE.KK.D.R .KEES----- --VFP..VQA .......... .VM....... ..........   172
CAEV-Ov496  120  .QE.KR.E.K .KEES----- --VFP..VQA .......... .VM....... ..........   172
CAEV-Gansu  120  .QE.KKGEEK .KEES----- --VFP..VQA ....N..... .VM....... ..........   172

Isolate     169  FERQMAYYAT TWTSKDILEV LAMMPGNRAQ KELIQGKLNE EAERWVRQNP PGPN--VLTV   226
MVV-1514    180  ....L..... .......... .V........ .......... .......... ....--....   237
MVV-85/34   178  .......... .......... .......... .......... .......... ....--....   235
MVV-P1OLV   180  ....L..... .......... .......... .......... .......... -...--A...   236
MVV-SA-OMVV 180  ....L..... .......... .......... .......... .......... ....--....   237
MVV-EV1     181  ....L..... .......... .......... .......... .......... ....--....   238
CAEV-Co     173  ....L..... .......... .......... .......... .....R.N.. .P.AGGG...   232
CAEV-Ov496  173  ....L..... .......... .......... .......... .....R.N.. .PQAGGG...   232
CAEV-Gansu  173  ....L..... .......... .......... .......... .....R.N.. .P.VGGG...   232

Isolate     227  DQIMGVGQTN QQASQANMDQ ARQICLQWVI NALRSVRHMS HRPGNPMLVK QKNNESYEDF   286
MVV-1514    238  .......... .......... .......... T......... .......... ...T......   297
MVV-85/34   236  .......... .......... .......... T......... .......... ...T......   295
MVV-P1OLV   237  .......... .......... .......... T.......L. .......... ...T......   296
MVV-SA-OMVV 238  .......... .......... ...L...... T......... ........I. ...S......   297
MVV-EV1     239  .......... .......... R.EL...... T...A..... .......... ...T......   298
CAEV-Co     233  .......... .A.A...... .......... ....A....A .......... ..T..P....   292
CAEV-Ov496  233  .......... .A.A...... .......... S...A....A .......... ..S..P....   292
CAEV-Gansu  233  .....A.... .A.A...... .......... I...A....A .........R ..A.....E.   292

Isolate     287  IARLLEAIDA EPVTEPIKTY LKITLSYTNA STDYQKQMDR ILGTRVQQAT VEEKMQACRD   346
MVV-1514    298  .......... ...MD..... ..V....... ...C...... T......... ..........   357
MVV-85/34   296  .......... ....D..... ..V....... .A.C...... V......... ..........   355
MVV-P1OLV   297  V......... ....D..... ..V....... ...C...... V..A...... ..........   356
MVV-SA-OMVV 298  .........T ....D..... ..V...F... ...C...... V........S ..........   357
MVV-EV1     299  .......... ....D..... F.SDSA.... ...C...... V..N.....S ..........   358
CAEV-Co     293  A......... ....Q...D. ..L....... .A.C...... T..Q.....S ..........   352
CAEV-Ov496  293  A......... ....Q...E. ..L....... .S.C...... V..Q.....S ..........   352
CAEV-Gansu  293  A.K....... ....Q...D. ..L....... .S.C...... V..Q.....S ..........   352

Isolate     347  VGSEGFKMQL LAQALKPERN IGNRGGGQKC YNCGKPGHLA RQCRQGIICH HCGKKGHIQR   406
MVV-1514    358  .......... .....R.QGK A.HK.VN... .......... .......... ....R..M.K   417
MVV-85/34   356  .......... .....R..K. P....P.... .......... .......... ....R..M.K   415
MVV-P1OLV   357  .......... .....R...K ARPQ.EK... .......... .......... ....R..M..   416
MVV-SA-OMVV 358  .......... .....R.P.K E.KQ.V-... .Y........ .......... ....R..M.K   416
MVV-EV1     359  .......... .....R.P.K G..V.SS... .....TRTSC KTMQARDNLP SVW.R..VRK   418
CAEV-Co     353  .......... .....R.GKG K.-N.QP.R. ........Q. .......... N...R..M.K   411
CAEV-Ov496  353  .......... .....R...K K.-I.PA.R. ........R. .......... N...R..M.K   411
CAEV-Gansu  353  ...D...... .....R.GKG KR-P.QS.R. ........Q. .......... N...R..M.K   411

Isolate     407  DCRKKKS-EI KQQGNSRRGP RVVPSAPPML *                                  435
MVV-1514    418  ...Q..---- -....N.... .......... *                                  442
MVV-85/34   416  ...Q..NKDM .......... .......... *                                  445
MVV-P1OLV   417  ...Q.R.GDQ .....I.... .......... *                                  446
MVV-SA-OMVV 417  ...Q..GNPT S......... .......... *                                  446
MVV-EV1     419  ...Q.NKDN. QL...N.... .......... *                                  448
CAEV-Co     412  E..G.RDIRG .....G...I .........E *                                  441
CAEV-Ov496  412  ...G..TG-- M.S..G...L .........E *                                  439
CAEV-Gansu  412  ...G..EM-G R....G...I .........E *                                  440
```

(Fig. 3. Continued.)

shown). The shorter LTR length in the present isolate mainly resulted from the lack of a 36 bp sequence in the U3 and a 11 bp sequence in the R region, in addition to sparse single nucleotide insertions and deletions.

Gag gene sequence of the isolated virus: The complete *gag* gene sequence of the isolated virus was found to be 1,308-bp long and encoded 435 amino acid residues (AB818536). When compared with the MVV and CAEV reference strains, the nucleotide sequence showed 82.8 and 72.0% homology with MVV strain 1514 and the CAEV strain Cork, respectively (Fig. 3A). The homology of the gag protein N-terminal region was higher to MVV strains than that to CAEV strains (Fig. 3B). However, a 21 bp sequence between nt 403 and nt 423 of MVV-1514 was lacking in the isolate. The seven corresponding amino acid residues were also found in the other four MVV strains, but not in all three CAEV strains. The homology with the reported partial *gag* gene of CAEV-No. 40 was 84.5% (Fig. 4).

Phylogenetic analysis of the virus: Phylogenetic analysis based on the *gag* gene sequence was performed to confirm that the isolated virus was MVV. The *gag* sequence obtained was compared with those of viruses that have already been reported as MVVs or CAEVs. The isolated virus was classified as MVV in the phylogenetic tree containing MVV and CAEV clusters (Fig. 5). In addition, the virus was similar to MVV strain 85/34, which was isolated in North America. In contrast, CAEV-No. 40 isolated in Japan was classified in the CAEV cluster, although its available *gag* gene sequence was as short as 129 bp.

Confirmation of MVV infection in the seropositive sheep: The sheep used for virus isolation in the present study was reconfirmed as being seropositive against the MVV antigen by the AGID test (Fig. 6A). In addition, the antigen prepared from the present isolate formed a clear precipitation line and connected with the line produced between the positive reference serum and the reference antigen (Fig. 6B). The prepared antigen, which was the culture fluid concentrated to about 1/100 of the original volume, formed a precipitation line even when the antigen was diluted to eight fold.

Pathological examination: The sheep had not shown any clinical manifestations. Gross examination revealed pulmonary atelectasis in a restricted region of the right cranial lobe, but prominent lesions were not observed in the cerebrum, cerebellum and spinal cord. However, mononuclear cell infiltration was identified in many areas of meninges between the cervical and lumbar cords (Fig. 7A and 7B). The infiltrates included CD3 or CD20 positive lymphocytes (Fig. 7C and 7D). The white matter of these segments also sparsely showed demyelination (Fig. 7E), vacuolation and the swelling of astrocytes. The MVV antigen was not detected by immunohistochemistry (data not shown).

DISCUSSION

MVV is a slow virus, and the latent period of MV is generally several months to years. Because infection with lentiviruses persists for life, serological surveillance is important to prevent the spread of disease from asymptomatic carrier animals. The three sheep reported in Japan as being seropositive against the MVV antigen were highly likely to be infected persistently with MVV. Therefore, the present study aimed to isolate MVV from one of those sheep

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Isolate     481   CAGCATGGAC TTGTGTCAGA GGACTTTGAG AGACAAATGG CGTATTATGC CACCACATGG    541
CAEV-No.40    1   ........C. .C........ .........A ..G..GC.A. .A........ T..T..C...     60
Isolate     542   ACAAGTAAGG ATATATTAGA AGTATTAGCC ATGATGCCTG GGAACAGAGC ACAGAAAGAG    602
CAEV-Jpn     61   .....C..A. ....T..... ......G... .......... ....T..... T..A......    120
Isolate     603   CTAATACAA                                                          611
CAEV-No.40  121   T....T...                                                          129
```

Fig. 4. Alignment of the partial *gag* gene sequences from the isolated MVV (Isolate) and CAEV-No. 40 isolated from a goat in Japan.

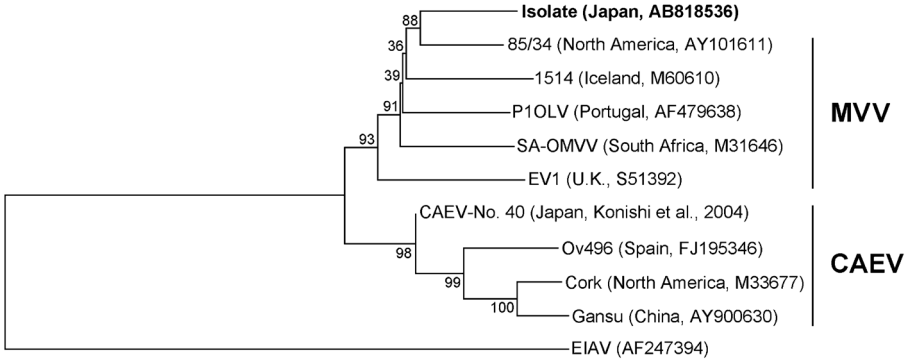

Fig. 5. Phylogenetic analysis of the isolated virus (Isolate) and other MVV and CAEV strains. The phylogenetic tree was generated using the entire *gag* gene sequences of previously reported MVVs and CAEVs, except for CAEV-No. 40, the available sequence of which was 129-bp long.

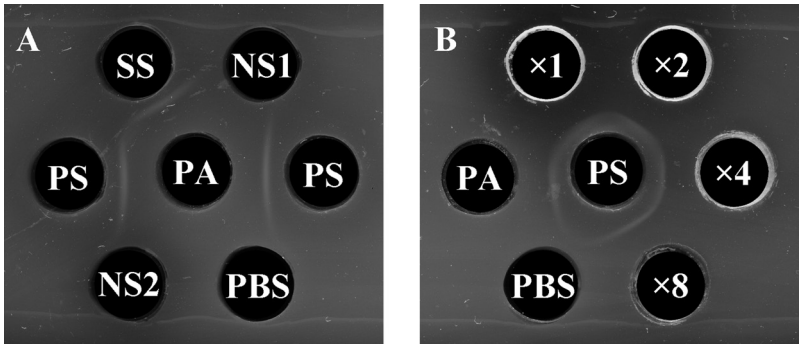

Fig. 6. A) Confirmation of MVV-seropositivity of the sheep analyzed in the present study by the AGID test. SS: Serum of the MVV-seropositive sheep. NS: Negative serum (NS1 and NS2). PS: Positive reference antiserum. PA: Positive reference antigen. Phosphate buffered saline (PBS) was used for a negative control reaction. B) Antigenicity of the isolated MVV. The prepared antigen was used without dilution (×1) or was diluted two (×2), four (×4) or eight (×8) times with PBS. The negative control reaction was carried out using PBS. PS: Positive reference antiserum. PA: Positive reference antigen.

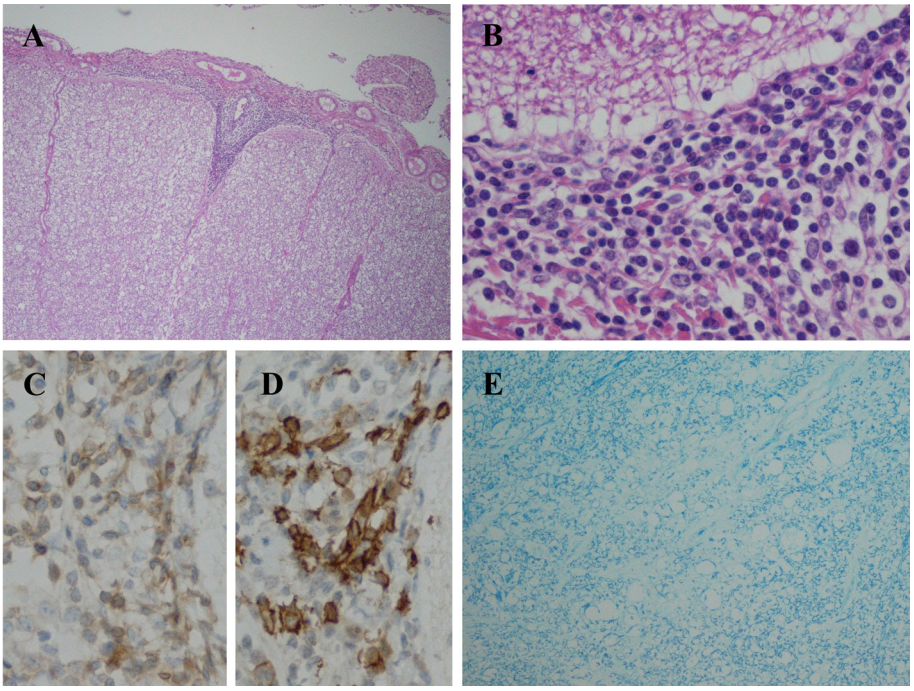

Fig. 7. Spinal cord lesions in the MVV-seropositive sheep. (A) Infiltration of mononuclear cells into meninges of the thoracic spinal cord region (HE staining). Higher magnification is shown in (B). The infiltrates include CD3 positive T cells (C) and CD20 positive B cells (D). (E) Demyelinating lesion in white matter (Klüver-Barrera staining).

and characterize the virus based on comparisons with other MVV and CAEV strains.

Although MVV and CAEV are now classified as small ruminant lentiviruses (SRLVs), both were considered to be distinct viral species restricted to their respective hosts. Therefore, the viruses isolated from sheep and goats were referred to as MVV and CAEV, respectively [11]. However, MVV in the present study was isolated from a co-culture of peripheral blood leukocytes taken from a seropositive sheep with primary cultured goat cells, but not with sheep cells, probably due to the effect of the low passage times of FGL cells as compared with the high passage times of FLL cells.

It has become evident that MVV and CAEV can cross the species barrier [19]. An epidemic of CAEV in goats oc-

curred in 2002 on a farm in Nagano Prefecuture, where a CAEV strain was isolated and characterized [8]. Thus, one possible source of the isolate's origin was CAEV that may have spread from goats on the farm to sheep probably related to the sheep used in the present study. However, the *gag* gene and amino acid sequence of the isolate were closer to that of MVV than to that of the CAEV reference strain and apparently distinct from that of CAEV reported in Japan, although the analyzed partial *gag* gene sequence of which was as short as 129 bp. Moreover, our phylogenetic analysis revealed that the present isolate belonged to the MVV cluster. These data indicated that the present isolated virus was MVV, but not CAEV. It was also demonstrated that the present MVV (named MVV-Iwate) was similar to MVV strain 85/34 isolated in North America. This suggests that the present isolate might be related to the North American strain. Another point of view is that the present isolate might be a recombinant of MVV and CAEV, which could be attributed to the lack of a 21 bp nucleotide sequence in the isolate and in all three CAEV strains analyzed. The recombination of MVV and CAEV reportedly occurred due to their coinfection or superinfection, resulting in the generation of replication-competent chimerical viruses [15]. However, the sheep used in the present study were not considered simultaneously infected with MVV and CAEV, as the several *gag* gene clones amplified had a uniform sequence despite PCR using primers that were designed in highly conserved regions among several MVV and CAEV strains. The ancestral virus might have originated from a recombination of MVV and CAEV.

Although the sheep used in the present study had not shown any clinical signs, degenerative and nonpurulent inflammatory lesions were identified in the histological examination of the spinal cord. Benavides *et al.* classified spinal cord lesions associated with MV into three patterns: the *vascular* pattern characterized by perivascular cuffs with minimal lesions in the adjacent neuroparenchyma; the *malacic* pattern (the most common type) characterized by severe white matter destruction and small numbers of macrophages; and the *infiltrative* pattern characterized by a severe infiltrate of histiocytes in the parenchyma [3]. However, the present spinal lesions did not match any of these patterns. Because Benavides's classification is based on the spinal lesions of 12 sheep with clinically recognized neurologic signs, it was considered that the present findings were early lesions that formed during latent MVV infection. The negative result of MVV immunostaining might be attributed to limited viral antigens in the tissues or the reactivity of the primary antibody.

The MVV infection route to the sheep used in the present study remains unknown due to a lack of available information about this animal. The quarantine of imported sheep and goats against SRLVs that entails serological tests had not been performed in Japan before the outbreak of CAEV. Therefore, it was suggested that MVV-infected sheep had been imported prior to the SRLV quarantine and thus became a source of infection. The present study reconfirmed the importance of serological surveys to identify sheep with subclinical but persistent MVV infection. Further surveillance is required to elucidate the current distribution of MVV in Japan.

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