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Genetic Characterization of Encephalomyocarditis Virus Isolated from Aborted Swine Fetus in Korea

SONG, MIN-SUK[1], YOUNGHO JOO[2], EUN HO LEE[1], JIN-YOUNG SHIN[1], CHUL-JUNG KIM[2], KWANG-SOON SHIN[2], MOON-HEE SUNG[3], AND YOUNG KI CHOI[1]*

[1]*College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju 361-763, Korea*
[2]*College of Veterinary Medicine, Chungnam National University, DaeJeon 305-764, Korea*
[3]*Department of Bio and Nano Chemistry, College of Natural Sciences, Kookmin University, Seoul, Korea*

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Abstract An encephalomyocarditis virus (EMCV-CBNU) was isolated from an aborted swine fetus in October 2005. To investigate the genetic origin and virulence of the EMCV-CBNU strain, we determined the complete sequence of the virus and tested its virulence in mice. Genetic characterization revealed that the RNA genome was composed of 7,713 nucleotides with a single open reading frame (2,292 amino acids), coding 12 proteins. The EMCV-CBNU had the shortest poly(C) tract, consisting of 10 C's (C_{10}), compared with all the other EMCV strains reported in GenBank. Amino acid and phylogenetic analyses showed that EMCV-CBNU had the highest genetic identity with strain 2887A (99.7%), which was originally isolated from a fetus in a pig breeding farm that had a history of reproductive failure. Because rodents are the natural host of EMCV, we investigated the virulence of EMCV-CBNU in mice. Surprisingly, all mice inoculated with more than 1×10^2 $TCID_{50}$/0.1 ml of EMCV-CBNU showed symptoms of hind limb paralysis and eventually died during 3 and 8 days post-inoculation (DPI). Furthermore, when we inoculated the virus into pregnant mice, all dams and their fetuses died in 6 DPI. This is the first report on a full genomic analysis of swine EMCV in Korea, which exhibits high virulence in mice.

Key words: Swine, encephalomyocarditis virus, reproductive failure, mice

Encephalomyocarditis virus (EMCV) is a picornavirus that belongs to the Cardiovirus genus. This virus infects many animal species and shows a worldwide distribution. The picornavirus infects many animal species including pigs [17], rodents [29], cattle [31], elephants [30], and primates such as baboons, monkeys, chimpanzees, and humans [5, 10, 16, 20, 28]. The natural hosts of EMCV are rodents, including the water rat *Hydromys chrysogaster*. The virus is transmitted from rodents to humans, monkeys, horses, cattle, and swine. Recently, it has had a worldwide clinical significance in domestic pig herds with symptoms of acute fatal myocarditis, reproductive failure, or asymptomatic infection. Pathogenesis studies showed that EMCV in infected pigs is disseminated through blood to multiple organs, including brain, heart, lungs, kidneys, liver, spleen, pancreas, tonsils, skeletal muscle, and lymph nodes. However, EMCV persists only in the myocardial and central nervous system cells of the infected animal [4, 7, 16, 26].

Antibodies to EMCV have been detected in humans with no discernible illness, and EMCV has been associated with patients with encephalitis and meningitis [10, 20]. A porcine strain of EMCV has been shown to productively infect primary human cardiomyocytes [7, 15]. In addition, virulent EMCV strains have previously been isolated from non-human primates, and experimental infection of cynomolgus macaques with a porcine isolate of EMCV resulted in severe pathologic lesions, primarily in the heart and brain [21].

In Korea, EMCV was first isolated in 1992 from a porcine fetus associated with reproductive failure [27]. However, there were no available genetic information and additional reports in Korea regarding EMCV-related reproductive failure in porcine. Recently, the Korean swine industry has suffered from severe reproductive problems. During routine laboratory test for swine reproductive failure agents, we detected and isolated an EMCV from aborted swine fetuses and named the virus EMCV-CBNU.

In this study, we therefore investigated the virologic prevalence of EMCV from swine reproductive failure agents and determined the complete sequence of the EMCV-

*Corresponding author
Phone: 82-43-261-3384; Fax: 82-43-272-1603;
E-mail: choiki55@chungbuk.ac.kr

CBNU strain that caused reproductive failure in sows. The full genome of EMCV-CBNU was compared with the EMCV sequences previously published [3, 12, 18, 24, 25]. Furthermore, we tested the virulence of the EMCV-CBNU strain in mice and vertical transmission in pregnant mice.

MATERIALS AND METHODS

Clinical Specimens

In the virologic surveillance, diagnostic specimens were examined. Between November 2004 and December 2005, 56 specimens connected to swine reproductive failure problems were submitted to the Microbiology Laboratory of Chungbuk National University for etiological diagnosis of reproductive failure agents. The herds of origin (located mainly in Chungbuk and Chungnam provinces) were experiencing acute episodes of reproductive failures. Virologic analysis was performed to detect the most relevant pathogens for swine reproductive failure agents [porcine circovirus (PCV), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), and EMCV].

Cells and Viruses

MARC-145 cells and Baby hamster kidney cell line (BHK21) were maintained in Eagle's Minimal Essential Medium (EMEM) with Earle's salt solution supplemented with 8% fetal bovine serum (FBS), as previously described [2, 8]. The Korea EMCV-CBNU strain, isolated from a fetus at a pig breeding farm with a history of reproductive failure, was propagated on BHK21 cells in EMEM supplemented with 2% FBS. After approximately 48 h of incubation at 37°C, the viral suspension was clarified by centrifugation at 800 ×*g* for 10 min and stored at −80°C as the viral stock.

RNA Isolation

The EMCV-CBNU genomic RNA was extracted from the virus stock with TRIzol LS reagent, as recommended by the manufacturer (GIBCO/BRL, Gaitherburg, MD, U.S.A.). Briefly, 250 μl of the purified virus was mixed with 3 volumes of TRIzol reagent and extracted with 240 μl of chloroform. RNA containing the aqueous phase was precipitated with 600 μl of isopropanol. The RNA pellet was washed with 75% ethanol in DEPC-treated water, vacuum dried, and dissolved in 20 μl of DEPC-treated water. A 5-μl aliquot of the RNA was analyzed by electrophoresis on formaldehyde denaturing 0.8% agarose gel. The quantity of extracted RNA was estimated by optical density measurements.

cDNA Synthesis

cDNA was synthesized from 5 μg of extracted viral RNA using an oligo $(dT)_{12-18}$ primer and a Superscript II (Invitrogen, U.S.A.) commercial cDNA synthesis kit, according to the manufacturer's recommendation. RNA in a total volume of 20 μl was heated at 65°C for 10 min, and then chilled on ice. The denatured RNA and 1 μl of DTT solution were then added to the first-strand reaction mixture (Cloned Murine Reverse Transcriptase, RNAguard, RNase/DNase-free BSA, oligo $(dT)_{12-18}$ primer, dNTPs) and incubated at 42°C for 1 h.

Table 1. Oligonucleotide used for cDNA synthesis and PCR amplification.

Oligonucleotide	Sequence	Position	Polarity
EMCV First	TTGAAAGCCGGGGGTGGGAGATC	1-23	Sense
EMCV 3423R	TTTGTGCCAGCAAAGAACAG	3256-3275	Antisense
EMCV 1055F	TGACCCACCCAGAACCTACGG	1038-1058	Sense
EMCV 5131R	TGGAAGCATGGAAGAGGAGC	5094-5114	Antisense
EMCV 2895F	ACTTGCCCGAACTCAGTGAT	2773-2793	Sense
EMCV 6097R	TTCATTATCCCCGTGACTGGA	5925-5945	Antisense
EMCV 5725F	GCACCTTGGCAGTAAATAGA	5725-5744	Sense
EMCV 7095R	GACCACCGGTTATCAGAAAG	7059-7078	Antisense
EMCV 6964F	TGTTCCGCTTATTGGCTGAG	6947-6966	Sense
EMCV End	CCCGTCGACTTTTTTTTTTTTTTCTCT	7709-poly(T)	Antisense
EMCV 65F	CTACCCACTCCCCCTTTCAA	65-84	Sense
EMCV 3566F	GAGATCGAAGACCAAACAGG	3549-3568	Sense
EMCV 5483F	CGACAGATGAACAGCTTGAGG	5330-5350	Sense
EMCV 144R	TTGTTGTTTTGGGGTGGC	127-144	Antisense
EMCV 600R	ATCCCATACAATGGGGTACCT	578-598	Antisense
EMCV 1160R	GCCGGCAGTGTCTTGAGACAC	1162-1182	Antisense
EMCV 1238R	AAGACGGCCCACTGTTGACT	1202-1221	Antisense
EMCV 1280R	GCAGTGTCAGCACATGATGCC	1257-1277	Antisense
EMCV 2619R	TTTCAGCGTTTTCTACTCCCT	2584-2602	Antisense

DNA Sequencing and Phylogenetic Analysis

Amplification was performed using 5 μl of cDNA, 10 pmol each of primer (Table 1), 5 U of Taq polymerase (Takara, Japan), 3 μl of dNTPs, 2.5 mM $MgCl_2$, and 10× Taq buffer in a total of 50 μl. The mixture was heated for 2 min at 94°C, and amplification was carried out for 30 cycles for 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The mixture was then heated for 10 min at 72°C. PCR amplification was carried out on a GeneAmp 2400 thermocycler (Perkin-Elmer, U.S.A.). PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

PCR products were purified by using a QIAquick PCR purification kit (Qiagen, Germany). Nucleotide sequence analysis was carried out by direct sequencing of the PCR products with an automatic 3700 DNA sequencer. For comparison, the phylogenetic analysis included sequences from GenBank data. Neighbor-joining trees were constructed using the program Clustal X. Distance matrices were calculated according to sequence relatedness across the amino acid sequences of open reading frames. One-thousand bootstrap resamplings were performed with the Clustal X program and resulting trees were plotted using Tree View v1.5. The Mengo virus sequence was used to outgroup-root the trees.

In Vivo Growth Characteristics

A total of 56 4-week-old BALB/c mice were housed, 5 per each cage (215×320×140 mm). Viral replication and lethal dose (LD_{50}/ml) in the mice were measured after being intraperitoneally inoculated with virus-infected cell culture media containing 10^1 to 10^6 $TCID_{50}$/ml of virus. The body weight of each inoculated mouse was measured daily from 0 DPI to the end of the experiment. Twenty pregnant BALB/c mice were also housed (5 per each cage), and the virulence and vertical transmission from dam to fetus were tested. Fourteen pregnant mice were inoculated intraperitoneally with 0.1 ml of 2×10^2 $TCID_{50}$/head on 11 days of gestation (11DG). To investigate the distribution of virus in infected mice, we collected samples of brain, lung, liver, intestine, spleen, kidney, and skeletal muscle at 1, 2, 3, 5, and 7 DPI, and confirmed the viral RNA by RT-PCR. All tissues were collected with individual scissors to prevent cross contamination.

Nucleotide Sequence Accession Number

The sequence of EMCV-CBNU was submitted to GenBank and the assigned accession number is DQ517424.

RESULTS

Swine Reproductive Failure Agents

Out of 56 specimens, 15, 11, and 7 specimens were positive for PRRSV, PCV, and EMCV, respectively, by RT-PCR/or PCR. Although most of the PRRSV and EMCV were detected alone from specimens of reproductive failure, five PCVs were detected with combined infections of PRRSV or EMCV. These results suggest that EMCV is one of the etiological agents for reproductive failure in swine.

Molecular Analysis of EMCV-CBNU and Comparison with Other EMCV Strains

The complete nucleotide sequence of the EMCV-CBNU strain was determined by eight overlapping fragments that cover the entire genome, using individual primer sets (Table 1). The RNA genome is 7,713 nucleotides in length and contains a single open reading frame (2,292 amino acids), coding for 12 proteins. The 5' noncoding region (NCR) comprises 711 nucleotides and contains a poly(C) tract of 10 nucleotides. The 3' NCR comprises 126 nucleotides in addition to a 3' terminal poly(A) stretch of seven nucleotides.

The nucleotide sequence and predicted amino acid sequence were compared with the EMCV sequences previously published. The lengths of different genomic regions were similar to that of EMCV-2887A (Table 2). For nucleotide sequence comparisons, the sequence of the poly(C) tract was omitted. At the nucleotide level, the sequence identities of EMCV-CBNU were 99.3–97.7% and 84.2–81.7% with strains of group I (EMCV-2887A, -Rueckert, and -PV21) and group II (EMCV-30, -B, and -D), respectively (Table 3). Perhaps the most intriguing finding was that EMCV-CBNU had the shortest poly(C) tract consisting of 10 C's (C_{10}) without any U's (Table 2). The strain 2887A, another recent isolate from an aborted pig fetus, also had a relatively short poly(C) tract ($C_{10}UCUC_3UC_{10}$) compared with other EMCV strains [18].

Table 2. Comparison of genomic sequence length among different EMCV strains.

Strain	Total	Total without poly(C)	5'NCR total	5'NCR without poly(C)	Poly(C)	CR	3'NCR
CBNU	7713	7703	711	701	10 (C10)	6879	126
2887A	7730	7703	728	701	27 (C10TCTC3TC10)	6879	126
Rueckert	7835	7703	833	701	132 (C115TCTC3TC10)	6879	126
PV21	7861	7703	859	701	158 (C141TCTC3TC10)	6879	126
B	7825	7698	826	699	127(C127)	6879	123
D	7829	7699	828	698	130(C130)	6879	125
PV2	7820	7702	819	701	118(C118)	6879	125

Table 3. Nucleotide and amino acid identities of EMCV-CBNU between previously published EMCV sequences.

	2887A	Rueckert	PV21	B	D	30	Mengo
Total	99.3[a]	98.0	97.7	81.7	81.7	84.2	79.1
	99.7[b]	99.0	99.4	95.6	95.8	96.5	93.5
5'NCR	99.3	98.9	98.9	92.5	93.2	91.2	89.2
	-	-	-	-	-	-	-
L	99	99	98.5	81.1	81.1	99	80.1
	97	97	97	91	92.5	97	88.1
1A	100	99.5	99.5	79	79	80	74.8
	100	98.6	98.6	95.7	95.7	97.1	97.1
1B	99.9	99.3	99.5	80.1	79.9	95.1	95.1
	100	98.8	98.8	98.4	98.4	98.8	98.8
1C	100	100	99.9	81.1	81.1	83.7	78.2
	100	100	100	99.1	99.1	99.6	97.8
1D	99.5	99.2	99.5	80.7	81.1	84.8	79.1
	98.9	97.5	98.9	96.8	97.5	99.6	96.4
2A	100	99.2	99.6	73.9	74.3	75.4	63.5
	100	98.7	100	85.4	86	86	76.4
2B	100	99.8	99.5	92.2	92.2	93.9	89.5
	100	100	99.3	96.3	96.3	98.5	97.1
2C	99.8	99.7	99.7	81.4	81.4	83.3	79.3
	99.7	100	99.7	97.8	97.8	99.4	96.3
3A	99.2	98.5	99.2	77.3	77.3	79.5	69.7
	98.9	96.6	98.9	93.2	93.2	92	90.9
3B	100	100	98.3	70	70	80	56.7
	100	100	95	85	85	90	85
3C	99.7	99.5	99.5	78.4	78.4	80.8	72.5
	99.5	98.5	99.5	91.7	91.7	95.1	88.3
3D	100	99.9	99.9	84.3	84.3	85.9	82.4
	100	99.8	100	96.5	96.5	96.1	93.3
3'NCR	100	98.4	95.1	90	90.2	95.1	91.7
	-	-	-	-	-	-	-

[a]Identity of nucleotide sequences.
[b]Identity of amino acid sequences.

At the amino acid level, EMCV-CBNU shared sequence identities of 99.7–99.0% and 96.5–95.6% with strains of group I and group II, respectively. Among the noncoding regions of 5', EMCV-CBNU showed a nucleotide identity of 99.3% with -2887A and 98.9% with the -Rueckert and -PV21, but less than 93.2% with the other strains. The 1C protein was found to be the most conserved region in all EMCV strains (Table 3).

Mengo virus showed relatively low sequence identity with the EMCV-CBNU strain, sharing 79.1% in nucleotide and 93.5% in amino acid identities.

Phylogenetic Characterization

Phylogenetic comparison of the eight EMCV strains and Mengo virus revealed that EMCV-CBNU segregated with group I (EMCV-2887A, -R/45, and -PV21 strains), which shows high pathogenicity in mice [18] (Fig. 1). Mengo virus M/48 segregated far from the seven EMCV strains.

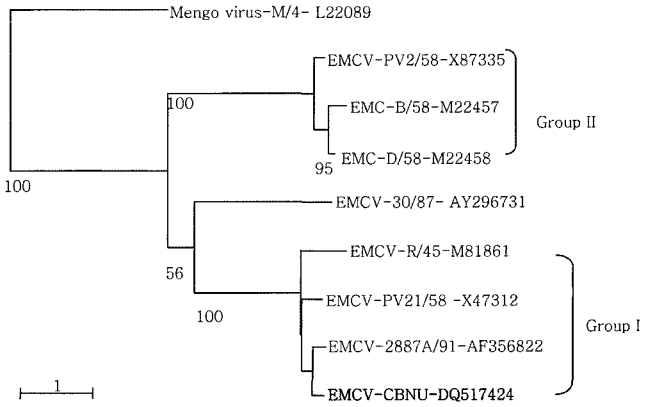

Fig. 1. Phylogenetic relationship between EMCV-CBNU and other EMCV strains, following the alignment of contiguous predicted amino acid sequences for all translated regions of the viruses.
The scale bar denotes the nucleotide substitutions per site. The bootstrap values are located at the nodes. GenBank accession numbers for the viruses are cited in the figure.

Virulence in Mice

The rodent is the natural host of EMCV. Therefore, many virulence studies of EMCV have been carried out with mice [1, 6, 11, 21–23]. To investigate the virulence and LD_{50} of EMCV-CBNU in BALB/c mice, 4 to 5-week-old female BALB/c mice were injected intraperitoneally with a 10-fold dilution of virus, which was passaged four times in BHK-21 cells. All mice inoculated with more than 1×10^2 $TCID_{50}$/0.1 ml of EMCV -CBNU died between 2 and 8 DPI (Fig. 2). The clinical signs in mice included hunched posture, ruffled fur, lethargy, anorexia, weight loss, and hind limb paralysis. Mice inoculated with 10^4 to 10^5 $TCID_{50}$/0.1 ml of EMCV CBNU developed disease at 2 to 4 DPI, whereas mice inoculated with 10^2 to 10^3 $TCID_{50}$/0.1 ml of virus developed disease between 5 and 7 DPI. Before becoming rapidly moribund, mice developed posterior limb paresis or complete paralysis.

All EMCV-CBNU-infected mice died within 3 to 8 DPI, except for one group that was infected with 10^1 $TCID_{50}$/0.1 ml of virus (Fig. 2). The LD_{50} of EMCV-CBNU was 2.5×10 $TCID_{50}$/head in mice. Following intraperitoneal inoculation, there was a widespread virus distribution, as demonstrated by the detection of EMCV RNA in the blood, spleen, kidneys, liver, heart, brain, and skeletal muscle at 2 DPI by RT-PCR. All specimens were positive for viral RNA, excluding the skeletal muscle (Fig. 3).

In pregnant mice, no dams showed any clinical signs and death until 2 DPI. After that, 6 out of 14 dams died by 5 DPI and the remaining dams died by 6 DPI with typical clinical signs. No fetuses died at 1 DPI, but deaths occurred to 45% of the fetuses at 3 DPI. Almost all fetuses were found to be dead at 5 DPI. In the control group, no deaths occurred to dams and fetuses. The virus titer of the dam's

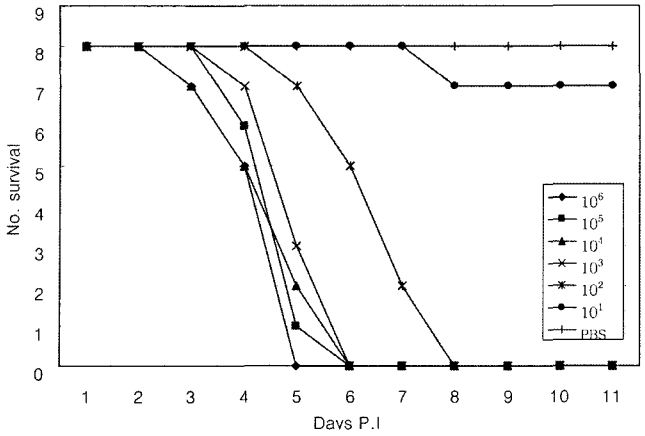

Fig. 2. Survival curve of mice infected with different amounts of EMCV-CBNU.
Mice were inoculated with 10^1 to 10^6 $TCID_{50}$/0.1 ml of EMCV-CBNU virus via the intraperitoneal route, and observed for 12 DPI. One group of mice was inoculated with PBS as a control. The deaths were recorded on the following day of observation.

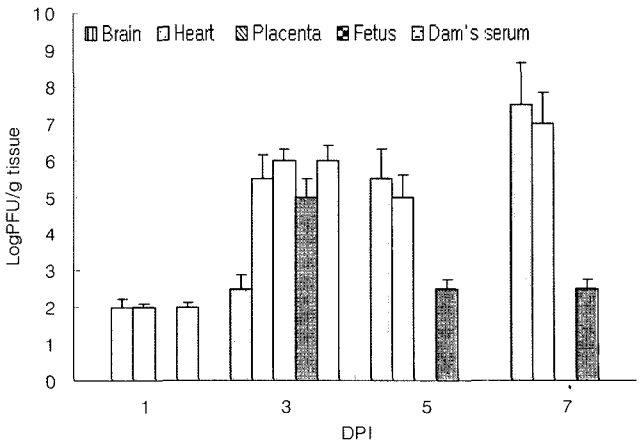

Fig. 4. The virus titers of the brain, heart, placenta, fetus, and dam serum after EMCV-CBNU virus infection in pregnant mice on 11 GD.
Data are represented as mean±standard deviation (SD).

serum was similar to that of the placenta at 1 DPI, and titers reached the highest at 3 DPI. The virus was no longer detected in the serum and placenta at 5 DPI, but the brain and heart tissues were positive until the end of the experiment (Fig. 4).

DISCUSSION

In this article, we describe for the first time the full genomic sequencing of a Korean EMCV strain (CBNU) from an aborted swine fetus. The length of the different genomic region is similar to that of the EMCV-2887A and -PV21 strains. Their major difference was only in the number of the C residues in the poly(C) tract.

Although the mechanisms involved in EMCV virulence and pathogenicity are not well known, the poly(C) tract

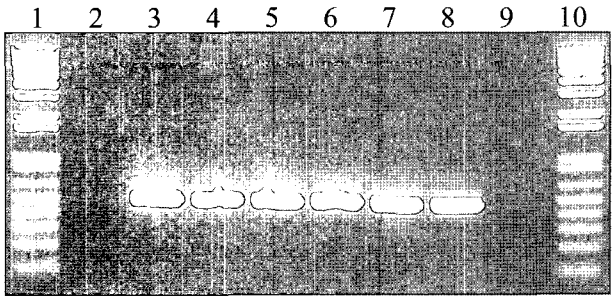

Fig. 3. Detection of EMCV from EMCV-CBNU-inoculated mice by RT-PCR. RT-PCR was conducted with the blood, spleen, kidneys, liver, heart, brain, and skeletal muscle tissues of mice at 2 DPI using the EMCV 2895F and EMCV 3423R primer set.
Lane 1, 1-kb ladder; lane 2, control mouse brain; lane 3, blood; lane 4, spleen; lane 5, kidney; lane 6, liver; lane 7, heart; lane 8, brain; lane 9, skeletal muscle; lane 10, 1-kb ladder.

may be a determinant in pathogenesis, virulence, and host ranges [21]. Studies to determine the role of the poly(C) tract in virus replication, virulence, and host range have been reported, with conflicting findings [21]. For instance, in earlier studies, truncation of the poly(C) tract of Mengo virus M/48 (from $C_{44}UC_{10}$ to C_8- or $C_{13}UC_{10}$) attenuated the viral pathogenicity in mice, resulting in lower virus titers in the brain and milder development of meningoencephalitis [15, 26]. On the other hand, LaRue *et al.* [21] demonstrated that a wild-type porcine EMCV containing a shorter poly(C) tract is more pathogenic in mice, pigs, and cynomolgus macaques. EMCV-CBNU has a poly(C) tract of only 10 C's, which is extremely short, compared with other EMCV strains. Although we did not compare the virulence with other EMCV strains, mice infected with 10^2 to 10^6 $TCID_{50}$/0.1 ml of EMCV-CBNU showed almost complete mortality in 8 DPI.

The pathogenicity of EMCV in various animal species was found to be strain dependent [19]. In swine, the virus can cause high mortality, which is attributable to myocarditis, encephalitis, and reproductive failure [9]. Sequence comparisons between different EMCV strains may allow speculation on the genomic regions involved in EMCV pathology and virulence. On the basis of sequence comparisons, EMCV strains could be separated into two groups. Group (I) consisted of strains EMCV-2887A, EMCV-R, EMCV-PV21, and EMCV-CBNU of this study, and group (II) consisted of strains EMCV-B, EMCV-D, and EMCV-PV2. Group (I) was characterized by its lethality and signs of hind leg paralysis in mice, whereas group (II) was essentially non-lethal in mice.

Amino acid sequence comparisons showed that the proteins 2A and 3B are less conserved between the strains of the two groups. The significance of this sequence divergence, with respect to the pathology and virulence of

the strains in mice, is unknown. Further experiments, including site-directed mutagenesis and production of chimeric viruses with cDNA clones, may elucidate the role of these proteins in affecting the virulence of EMCV-infected mice. The availability of an infectious cDNA clone of an EMCV strain causing reproductive failure in swine should be useful for further studies on the functions of viral gene products, and provide a basis for a molecular genetic approach not previously available for the analysis of EMCV-induced myocarditis and reproductive failure in swine. Recently, we are in a process to construct a cDNA clone of the EMCV-CBNU strain. This will allow us to study the molecular determinant of pathogenesis and virulence of EMCV in mice and swine.

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