

Genetic characterization and isolation of porcine reproductive and respiratory syndrome virus*

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Summary

Porcine reproductive and respiratory syndrome virus (PRRSV) is an acute, febrile, highly contagious infectious disease characterized by reproductive dysfunction and respiratory failure. Its high morbidity and mortality have caused significant economic losses to the swine industry. With the continuous development of PRRSV, its genetic variation is also increasing, which causes great difficulties for the clinical prevention and control of infections caused by the virus. Monitoring the genetic variation of the virus and vaccine immunity at any time is the current research focus. In this study, patients suspected of having PRRSV were sampled, and the virus was successfully isolated. The genomic variation characteristics of the new isolate of HLJ03 were determined, especially the molecular characteristics of GP5 and NSP2, and the main focus was placed on the analysis of genetic variation. The results provide important information for further investigation of the molecular prevalence of porcine PRRSV and create a basis for the prevention and control of porcine PRRSV infections.

Keywords: porcine reproductive and respiratory syndrome virus, virus isolation, genetic analysis

Porcine reproductive and respiratory syndrome (PRRS) is an acute and highly contagious infectious disease caused by the porcine reproductive and respiratory syndrome virus (PRRSV) (17). A strain of the virus was first reported and isolated in China in 1996 (14, 19). Outbreaks and epidemics of PRRSV infection, leading to mass mortality, have posed a significant threat to the pig farming industry worldwide (2, 11, 20, 23). With the increasing prevalence and evolution of PRRSV, its clinical characteristics have changed from the original reproductive disorders, such as abortion, premature birth, and stillbirth in pregnant sows and respiratory diseases in piglets to highly pathogenic PRRS (HP-PRRS) characterized by rapid onset, rapid spread, high morbidity, and high mortality in all pig herds (6). PRRSV is constantly mutating in nature, resulting in the continuous emergence of new virus strains (8, 10, 12, 18). Genetic analysis of these strains has shown that the PRRSV genome can be divided into the European type (type 1, European type) and the North American type (type 2, North American type) and that the representative strains are Lelystad virus (LV) and VR-2332. The main circulating strains, including the highly pathogenic PRRSV in China, are the North American type (26).

The PRRSV has a positive-sense single-stranded RNA genome of approximately 15 kilobases (kb) (22), whose ORF1a and ORF1b encode non-structural proteins, including Nsp 1-12 proteins, and ORF2-7 encodes structural proteins, including GP2, GP3, GP4, GP5, M, E, and N proteins. Studies have shown that the hypervariable regions of the PRRSV genome are the NSP2 gene and the ORF5 gene. The NSP2 gene of the highly pathogenic PRRSV (HP-PRRSV) has 30 discontinuous amino acids deleted at positions 482 and 533-561 (12, 18). The main protective antigenic protein of PRRSV is the GP5 protein, whose variations at key sites are closely related to the cross-protection rate of vaccine strains (8). Studying the genetic variation of PRRSV virus strains is crucial for understanding and controlling PRRSV.

Piglets or weaners suspected of PRRSV were collected from the Heilongjiang province in China, and the virus was isolated by molecular experimental technology in this study. The identification results and animal regression test proved that PRRSV was successfully isolated and named HLJ03. To determine the genomic variation characteristics of the new isolates of HLJ03, especially the molecular characteristics of GP5 and NSP2, we focused on the genetic variation analysis. The data obtained by this research provide powerful support for clinical prevention and control of infections by this virus.

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Material and methods

All applicable international and national guidelines for the care and use of animals were followed. The animal experiments were approved by the Institutional Committee of Heilongjiang Bayi Agricultural University.

Samples, cells, and antibodies. The samples were collected from weaned piglets suspected of PRRS in a pig farm in Heilongjiang Province in 2021. The samples used for virus isolation were obtained from lung homogenates of pigs whose clinical signs suggested a PRRSV infection. They were dissolved in a medium (DMEM) containing 2.0 µg/mL trypsin (Sigma, USA). Marc-145 cells were obtained from the Xiamen Immocell Biotechnology company (Xiamen, China) and grown in DMEM (Gibco, USA) supplemented media with 10% fetal bovine serum (FBS). Rabbit anti-PRRSV N antibody was purchased from Bioss (China), and rabbit monoclonal anti-β-actin antibody was purchased from CST (BRD).

Virus isolation and titration. The confluent Marc-145 cells were plated in a 6-well plate and inoculated with virus-carrying tissue homogenate, which adsorbed at 37°C for 1 h. The Marc-145 cells maintenance solution containing 2% fetal bovine serum was added, cultured in an incubator with 5% CO₂ at 37°C, and the cell growth was observed every day. The cells were harvested and centrifuged after 4-5 days, and the supernatant was collected. The Marc-145 cells were inoculated with the supernatant and until a cytopathogenic effect (CPE) typical of infection with PRRSV occurred. When CPE reached about 80%, the virus collection operation was carried out, and the supernatant obtained was the isolated PRRSV. The cells were inoculated with PRRSV according to the above method for passage, purification, and analysis.

Immunofluorescence assay. The Marc-145 cells (cell confluence: 60-80%) were plated and infected with the HLJ03 strain in 24-well plates for 48 h. Rabbit anti-PRRSV N antibodies were used. The specific experimental steps are described in an article published by the author (3).

Reverse transcription-polymerase chain reaction, cloning, and sequencing. Viral RNA extraction from 200 µL of lung tissue homogenate samples was performed. The viral RNA was reverse transcribed into cDNA. The PRRSV Nsp2 gene was detected using the synthesized cDNA as a template as well as Nsp2 F and Nsp2 R as amplification primers (1). The PCR results showed a 2850 bp band as considered NSP2 gene amplification successful. To detect the GP5 gene, GP5 F and GP5 R were used. Then the regions of PRRSV were detected by specific primers (Tab. 1). All PCR products were ligated into the pGEM-T Easy vector system (Promega, USA) and sequenced.

Tab. 1. The summary of sequences used in this study

Primer names	Primer sequences (5'-3')
Nsp2 F	GAAGGGAATTGTGGTTGGCA
Nsp2 R	AGACCCAGAAAACACACCCA
PRRSV F	TCAGCTGTGCCAAATGCTGG
PRRSV R	AAATGGGGCTTCTCGGGTTTT
GP5 F	ATGTTGGGGAAGTGCTTGAC
GP5 R	CTAGAGACGACCCATTGTT

Electron microscopy. The Marc-145 cells were infected with HLJ03. The cells were processed, and the specific experimental steps are described in an article published by the author (15). Then, the particles of HLJ03 were observed by TEM.

Phylogenetic analysis. Nucleotide sequences from the PRRSV isolates (HLJ03) and 24 other PRRSV strains obtained from the GenBank database were used for phylogenetic analysis. The sequences were analyzed with the DNASTAR (DNASTAR, USA) and DNASIS software (Hitachi Solution, USA), respectively. The Mega X software was used for Phylogenetic analyses (14). The reliability of the inferred phylogenetic tree was measured by the bootstrap method with 1,000 replicates.

Pathogenicity of the HLJ03 strain. The group consisted of nine 11-week-old piglets free of maternally derived antibodies and unvaccinated for PRRSV, purchased from a pig farm. Six pigs were administered a 3 mL dose of 10⁵ TCID₅₀/mL HLJ03, and the remaining three pigs received 3 mL of DMEM. All pigs infected with HLJ03 were monitored for clinical signs, such as the body temperature change, anorexia or non-eating, conjunctivitis, cough, wheezing, and redness all over the body. The piglets were necropsied. The *in vivo* growth of HLJ03 was measured with a quantitative real-time RT-PCR (RT-qPCR) assay. The specific experimental steps are described in an article published by the author (15) and quantify the viral genome copy numbers in inguinal lymph node (LN) and tonsil samples of pigs using PRRSV F and PRRSV R primers (the sequences in Tab. 1).

Statistical analysis. Statistical analysis was done by Student's t-test using GraphPad Prism 5.

Results and discussion

Among the 30 lung tissue samples, 3 of them tested positive in RT-PCR. The virus was isolated using the lung tissues in Marc-145 cells, and named HLJ03. Clear CPEs, characterized by visible cell accumulation, rounding, falling off, and disintegration, were observed in the HLJ03 isolate after the fourth passage (Fig. 1B). The CPEs of PRRSV were usually observed in Marc-145 cells infected with each isolate at 30 h post-inoculation, which were collected for an indirect immunofluorescence assay. In the cytoplasm of cells infected with HLJ03, specific fluorescence was detected, whereas there was no specific green signal in the control cells (Fig. 1C, 1D). PRRSV particles from the HLJ03 strain were examined by EM. The diameter of the particles was 60 nm (Fig. 1E). The viral titers of HLJ03-P10, -P40, and -P70 peaked at 10^{5.0}, 10^{5.8}, and 10^{7.6} TCID₅₀/mL, respectively, at 72 h post-inoculation. The growth curves of the HLJ03 strain in Marc-145 cells were detected, as well as the viral titers of HLJ03-P10, -P40, and -P70 were detected at 12 h intervals after infection (Fig. 1F). The viral titers showed that the logarithmic phase of the HLJ03 strain occurred at 24 to 60 h post-infection, and the platform stage of the HLJ03 strain occurred at 72 h post-infection (Fig. 1G). The results illustrated that

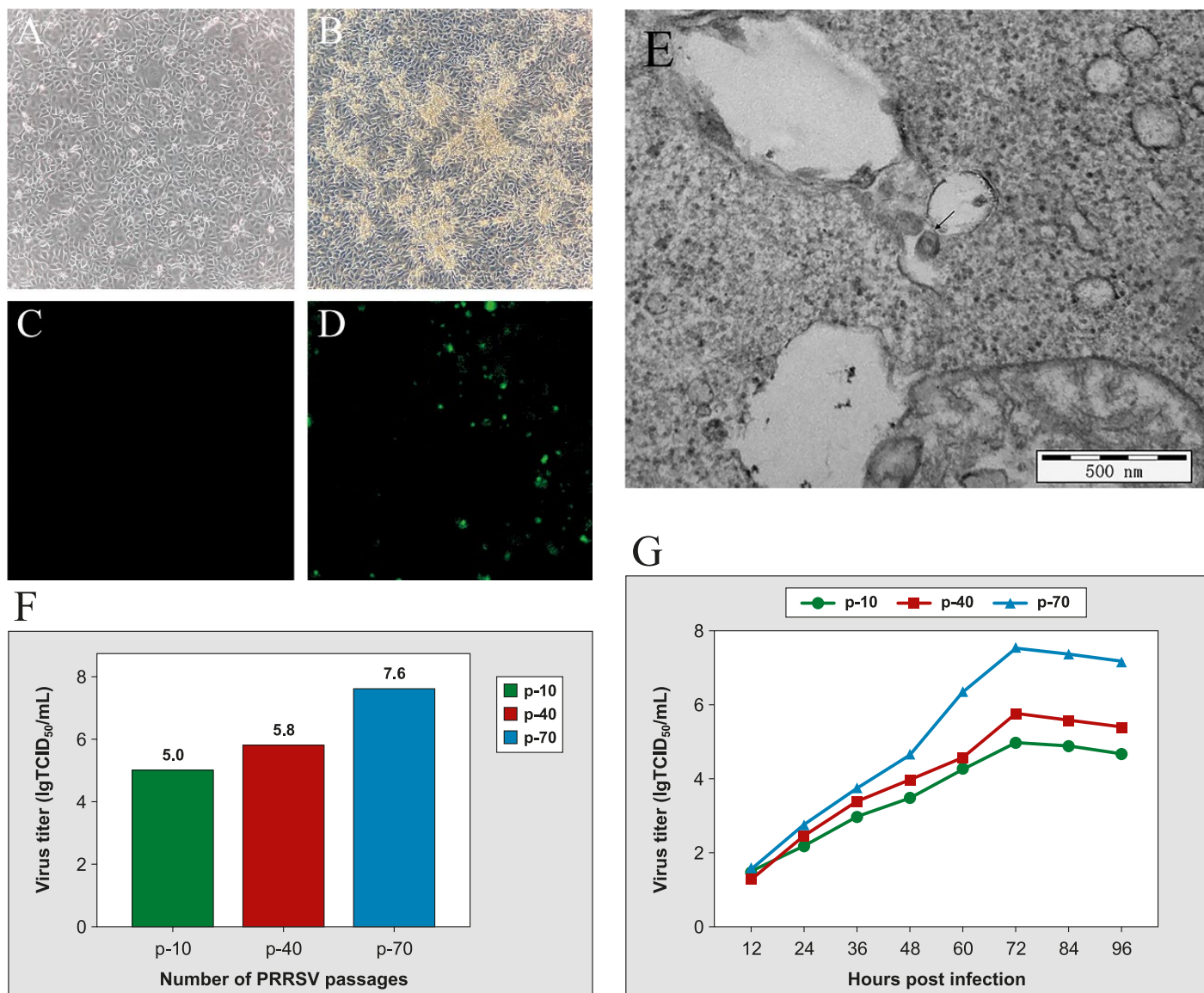


Fig. 1. A-D: Detection of the viruses by microscope and indirect immunofluorescence assay. After the Marc-145 cells were infected with HLJ03, rabbit-anti-PRRSV N was used as the first antibody and observed under the microscope. (A) Normal Marc-145 cells control. (B) Marc-145 cells infected with HLJ03. (C) Normal Marc-145 cells control. (D) Marc-145 cells infected with HLJ03. **E:** HLJ03 was determined by TEM. The Marc-145 cells were infected with HLJ03. The virions of HLJ03 are shown (scale bar 500 nm). The arrows indicate virions. **F:** The growth curves of HLJ03 in Marc-145 cells. Marc-145 cells were infected with HLJ03-P10, HLJ03-P40, and HLJ03-P70 viruses respectively, and test viral titers 72 h post-inoculation. **G:** Marc-145 cells were infected with HLJ03-P10, HLJ03-P40, and HLJ03-P70 respectively, and test the viral titers per 12 hours after infection.

the HLJ03 strain adapted to the cells, because the virus titer can increase to the 70th passage.

A 2850 bp sequence of the NSP2 gene from the PRRSV isolates was identified. To analyze epidemiological relationships, the nucleotide sequences from the PRRSV isolates were compared with those from 24 PRRSV strains retrieved from GenBank. Mega X was used to construct the phylogenetic tree of the NSP2 and GP5 gene sequences of 25 PRRSV (1 amplification sequence and 24 reference sequences). The results showed that the evolutionary tree was divided into five clusters, namely Lineage 1, Lineage 3, Lineage 5, Lineage 8, and PRRSV1. The NSP2 gene belonged to Lineage 8 of the PRRSV-2 type. The HLJ03 strains were most closely related to BB0907 (Fig. 2A). In

addition, the GP5 gene also belonged to Lineage 8 of the PRRSV-2 type and was closest to BB0907 (Fig. 2B).

Six piglets were inoculated with the HLJ03 strain, and the other three piglets were inoculated with DMEM as control. Then, the rectal temperature was recorded for 15 consecutive days. All piglets showed clinical signs associated with a PRRSV infection. The body temperature of piglets increased on the 1st day after the challenge and recovered slightly on days 2 and 3, and then the body temperature gradually increased. The temperature reached 40.0°C on day 5, accompanied by severe breathing difficulties, wheezing, redness of the skin, and cyanosis of the ears and limbs. On day 14, one piglet in the challenge group died. Piglets in

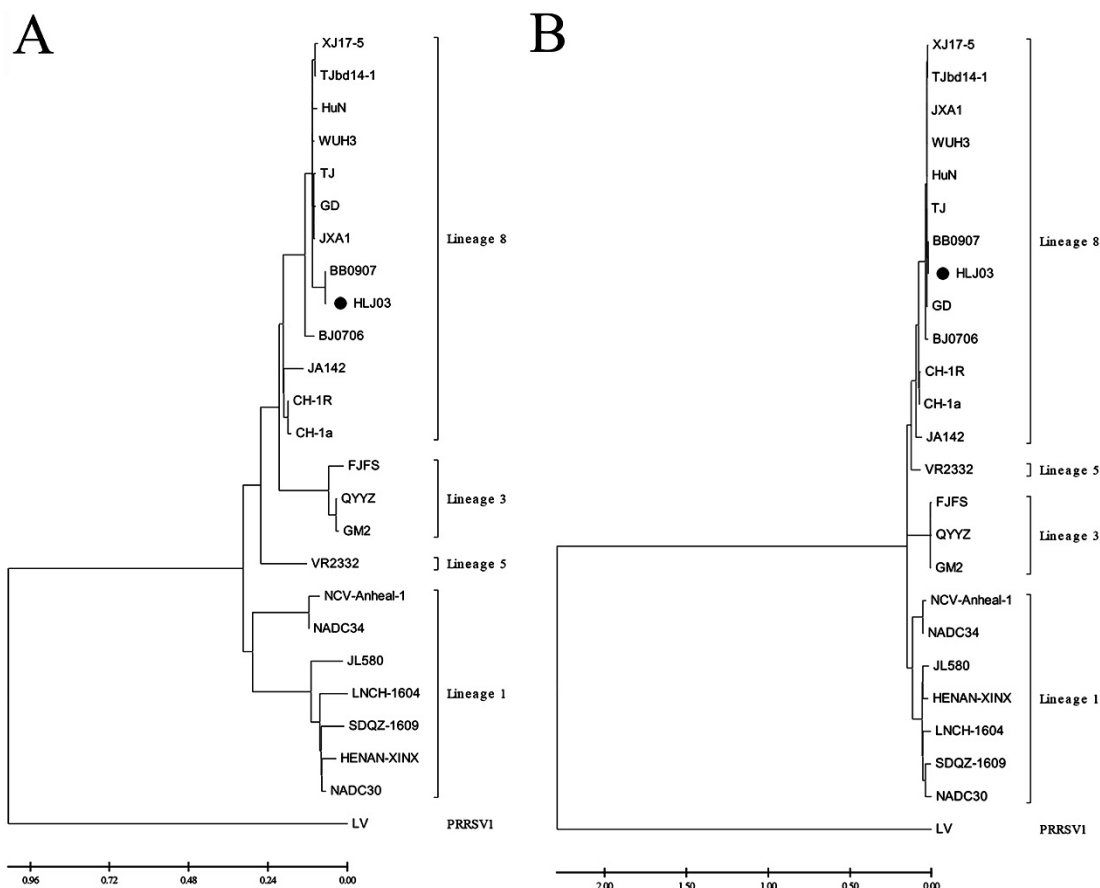


Fig. 2. For genetic evolution analysis, the PRRSV reference strain sequence was downloaded from the NCBI gene library, the gene sequence was obtained from HLJ03 sequencing, and Mega X was used to build the genetic evolution tree. (A) The genetic evolution tree for the Nsp2 gene. (B) The genetic evolution tree for the GP5 gene

the control group were all normal. We selected one pig from each of the challenge group and the control group, and recorded the changes in body temperature (Fig. 3A). The viral load was detected with the RT-PCR kit. Viral RNA was found in the inguinal lymph nodes and tonsils of pigs with HP-PRRSV, whereas no viral RNA was detected in the DMEM group (Fig. 3B, 3C).

Porcine Reproductive and Respiratory Syndrome is commonly known as PRRS. The representative virus strain Ch-1a was first reported and isolated in China in 1995. The disease has been widely present in pigs in China for more than 20 years. Researchers in China have developed a variety of vaccines to prevent PRRSV infections, but the disease still exists in pig herds in China. It is interesting that under the combined action of nature and immune pressure, PRRSV has been undergoing constant changes to adapt to the natural environment (13, 21). In 2006, a highly pathogenic strain of PRRSV appeared in Asia and then in North America, which posed a significant threat to pig farming worldwide (4). In the present study, the analysis of the molecular genetic evolution of the HLJ03 strain of PRRSV revealed that it had a high homology with the whole genome sequence of HP-PRRSV strains (such as the BB0907 strain) isolated after 2006. In terms of genetic distance between genes, wild-type PRRSV in China shows considerable genetic diversity,

which means that it is still very difficult to prevent and control PRRSV infections.

GP5 is the main structural protein of PRRSV and it is highly variable. Important structural features of GP5 are conserved among arteriviruses. The protein consists of an extracellular domain and is an important target for neutralizing antibodies (15). Studies have confirmed that R13 and R151 are virulence-related sites (16), and the HLJ03 strain or the HP-PRRSV HuN4 strain has mutated at these two sites, which indirectly proves that the virulence of the strain isolated in this study may be stronger.

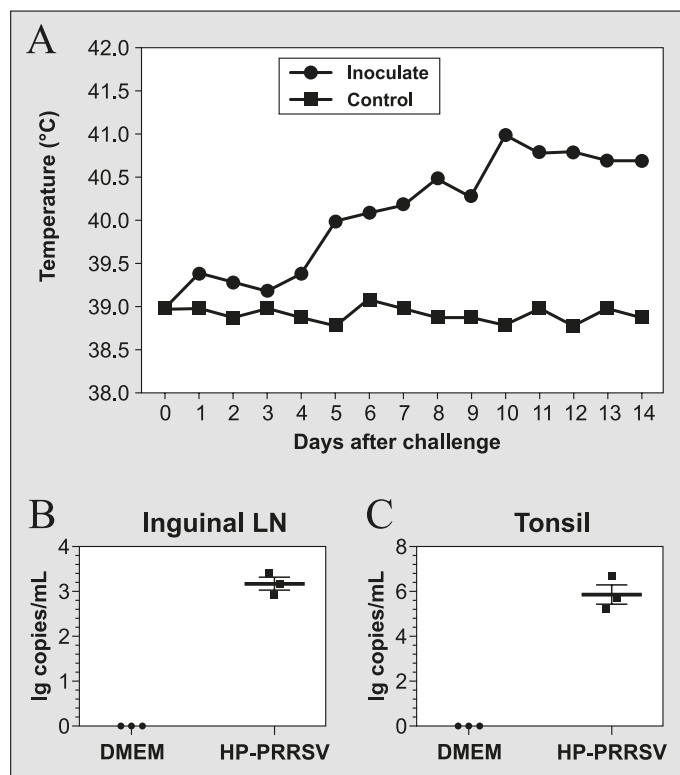


Fig. 3. A: Changes in the rectal temperature of pigs infected with HLJ03 and pigs infected with DMEM as control. The RNA levels of HLJ03 in inguinal lymph nodes (LN) (B) and tonsils (C) on day 14 p.i. determined by the RT-PCR kit

The NSP2 gene, as the most variable region in the whole genome of PRRSV, shows obvious diversity. Within the same genotype and between different genotypes, the NSP2 gene of PRRSV shows great variation and high resistance to deletion and insertion (7). In addition, the protease activity of NSP2 plays a crucial role in regulating host immunity and virus replication. Therefore, NSP2 is often used as an important marker for monitoring PRRSV variation (25). In this study, it was found that, compared with the classic strain CH-1A, NSP2 of the strain isolated in this study had a number of amino acid deletions. It also had deletions compared with the genome of the HP-PRRS virus from the outbreak in 2006 (5, 9, 24). Researchers have found that there are various deletion mechanisms in the hypervariable region of the protein, but it is still unknown which mechanism leads to deletions (jumping deletion) in the process of virus replication. In conclusion, the HLJ03 virus isolated in this study is a representative strain of HP-PRRSV, and its virulence and immunogenicity need to be further studied. However, the jumping mutation of the NSP2 protein of this virus strain provides molecular data for further research on the molecular evolution of PRRSV.

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