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Rovac is the possible ancestor of the Russian lapinized vaccines LK-VNIVViM and CS strains but not the Chinese strain (C-strain) vaccine against classical swine fever

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ABSTRACT

Classical swine fever (CSF), or hog cholera, is a highly contagious disease that emerged in the first half of the nineteenth century. To fight against the disease and protect pigs, different vaccines were developed, including early generation of lapinized Rovac strain and the later development of the "Chinese" strain (C-strain). However, details of the development of these vaccines are lost in history. In order to investigate the phylogenetic relationship between the Rovac and other lapinized vaccines, this study determined the genome sequence of the Rovac, which comprised 12,304 nucleotides, notably with the 3′untranslated region (3′UTR) containing a 13-nucleotide insertion. The near-complete genome of Russian vaccine strain LK-VNIVViM was determined by next-generation sequencing on Illumina MiSeq platform. Whole genome phylogenetic analysis revealed a closer relationship of the Rovac strain with the Russian LK-VNIVViM, CS strain and its derivative RUCSFPLUM (genotype 1.2), rather than with the C-strain (genotype 1.1). In addition, it demonstrated an ancestry role of the LK-VNIVViM in relation to the CS strain and RUCSFPLUM. The study suggested that the Rovac vaccine is the possible ancestor of the Russian vaccine strains but not the C-strain vaccine.

1. Introduction

Classical swine fever virus (CSFV) is a single-stranded, positive-sense RNA virus belonging to the genus of *Pestivirus* of the family *Flaviviridae* [1]. The viral genome consists of a 5′untranslated region (5′UTR), N-terminal protease (N^{pro}), capsid (C) protein, envelope (E) proteins E^{rns}, E1, E2, p7, nonstructural (NS) proteins NS3, NS4A, NS4B, NS5A, NS5B and 3′UTR [2]. This pestivirus can infect naturally both domestic pigs and wild boar and cause a highly contagious disease, classical swine fever (CSF), which was identified and confirmed in 1904 [3]. Although there is no record showing the exact date when CSF emerged, it was believed that the disease was first noted in Ohio, USA, in 1833 [4], and other reports suggest presence of CSF in Europe in the first part of the 19th century [5]. A molecular evolutionary study reveals that the virus might have emerged in 1825 [6]. The disease was then named hog cholera, suggestive of a highly fatal infectious disease that might have progressed quickly in an acute or peracute form upon infection and caused massive death. Chronic and atypical forms were also likely present.

To fight against CSF, different vaccines have been developed and applied [7]. While initial trials with simultaneous injection with highly virulent viruses and positive sera, so called "seroinfection" failed [8], crystal violet inactivated vaccine was unable to provide long-lasting protection of pigs in China [9]. Successful adaptation of

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rinderpest virus to rabbits [10] has promoted a similar approach to attenuate the CSFV. Koprowski et al. described in 1946 the propagation of a virulent CSFV strain in rabbits, which had been used by the Lederle laboratories in St. Joseph, Missouri, USA for the production of vaccine and immune serum [11]. The method and the "attenuated" hog cholera vaccine were later patented on August 1950 (United States Patent Office, number 2,518,978). Similarly, Baker reported passage of CSFV strain A in rabbits [12]. However, both the vaccine strains proved to be too virulent to pigs [13]. Further vaccine developments have achieved great success with live attenuated vaccines such as the Taiwanese Lapinized Philippines Coronel (LPC) that is based on more than 900 passages of the Rovac strain in rabbits [14], the Chinese Hog Cholera Lapinized virus (HCLV) [15] and its derivative Riems strain, the Russian CS strain [16], the Indian HCLV [17], the Japanese guinea pig exaltation-negative (GPE^-) (in interference with the growth of Newcastle disease virus in swine testicle cell culture) [18], and the low-temperature-adapted French Thiverval virus [19].

The HCLV strain was first named "Chinese" strain or C-strain in 1963 [20]. Although there is no specification of its parental strain in the initial report [15], Shimen strain is indicated as the parental strain of the HCLV [21,22]. However, this has been proved to be incorrect by molecular phylogenetic analysis of complete genome sequences [23]. Therefore, the exact origin of this vaccine strain remains unknown. According to Prof. Mészáros, Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest, Hungary, Rovac vaccine was mentioned as the likely parental virus of the HCLV strain during his brief discussion with a group of Chinese veterinary scientists. Prof. Mészáros received the HCLV vaccine of the 350th rabbit passage and brought them from China back to Hungary in 1958. One of the authors of this article, Dr. Liu had visited Prof. Mészáros twice and got to know stories behind his visit to China. Therefore, the objective of this study was to infer the phylogenetic relationship between the Rovac strain and other lapinized vaccine strains by molecular approaches in order to elucidate the mystery of the C-strain origin.

2. Materials and methods

2.1. Rovac vaccine virus, PCR amplification and Sanger sequencing

The Rovac vaccine virus was obtained from The National Veterinary Services Laboratories (NVSL), Ames, Iowa, and maintained at National Veterinary Research Institute (NVRI) in Pulawy, Poland since 1994. The virus was primarily propagated in PK-15 cells and the last three passages were performed using SK-6 cells. The infection of cells was confirmed by a peroxidase-linked assay (PLA) based on C16 monoclonal antibody (Community Reference Laboratory for CSF, Hannover, Germany). The cell cultures were frozen, thawed and centrifuged. The supernatant was sent to National Veterinary Institute, Uppsala, Sweden for this study.

RNA was extracted from supernatant with TRIzol reagent and cDNA synthesis was primed by hexamers in a 20-μl volume using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. *Pfu* High-fidelity DNA polymerase (Agilent Technologies, Inc. Santa Clara, CA) was used for PCR amplification with primers listed in Table S1. PCR products were separated on 1–2% agarose gel, and bands with correct size were sliced and purified using Wizard® SV Gel and PCR Clean up system (Promega, Madison, USA). ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) was used for sequencing of PCR products and capillary electrophoresis was performed in ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed with multiple programs of the Lasergene SeqMan (DNAStar, Inc., Madison, WI).

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2.2. Determination of the 5′ and 3′ ends by RNA ligase-mediated RACE

The 5′ and 3′ends of the viral genome were determined by RACE method using a GeneRacer Kit (Invitrogen, Carlsbad, USA), as previously described [24]. Briefly, the GeneRacer RNA Oligo, and RNA Oligo 2 (Table S1) were ligated to the 5′end and 3′end of the viral genome, respectively. The 5′ and 3′end sequences were amplified with specific primers (Table S1) by a one-step RT-PCR kit (Qiagen, Hilden, Germany). The products were gel-purified, cloned into pCR4-TOPO (Invitrogen, Carlsbad, USA) and four clones were sequenced.

2.3. Next-generation sequencing of CSFV LK-VNIVViM

LK-VNIVViM was one of the CSFV vaccines produced in the former Union of Soviet Socialist Republics (USSR) and described previously [25]. TRIzol reagent was used to lyse the virus and total RNA was purified from the aqueous part using RNeasy kit (Qiagen, Hilden, Germany). The procedures for tag labeling and random amplification were described previously [26]. Library preparation was done using Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) and next generation sequencing was performed on MiSeq desktop sequencer, according to manufacturers' instructions. Data were analyzed by CLC Genomics Workbench (CLC Bio, Aarhus, Denmark).

2.4. Phylogenetic analysis

Bayesian inference analysis was performed using the software MrBayes 3.1 [27], as previously described [6,24]. The Bayesian analysis used the evolutionary model General Time Reversible (GTR), with substitution rate heterogeneity and proportion of invariable sites (GTR + I + G). The Markov chain Monte Carlo (MCMC) search was run with four chains for 3 million generations or until the convergence of the chains was reached, sampling the Markov chain every 1000 generations. The first 25% trees were discarded as "burn-in".

3. Results and discussion

In order to reveal the phylogenetic relationship between the Rovac strain and other lapinized vaccine strains especially C-strain vaccine, the complete genome of the Rovac was determined in this study. The viral genome was 12,304 nucleotides long, consisting of 5′UTR (368 nt), N^{pro} (504 nt), C protein (297 nt), E^{rns} (681 nt), E1 (585 nt), E2 (1119 nt), p7 (210 nt), NS2-3 (3420 nt), NS4A (192 nt), NS4B (1041 nt), NS5A (1491 nt), NS5B (2154 nt) and 3′UTR (242 nt). The 5′UTR, which was sequenced from 4 individual clones, lacked the first five nucleotides (GUAUA) compared to that of the CSFV reference strain Eystrup (GenBank access no. NC_002657) and the vaccine strain HCLV (AF091507). As this region is involved in the 5′UTR stem-loop structural motif, any deletion might affect virus replication and translation. Therefore, further studies are needed in order to verify this deletion and to investigate its possible roles via reverse genetics. By contrast, the Rovac genome 3′UTR contained a 13-nt insertion (UUUAUUUAUUUAG), while it was a 12-nt insertion (CUUUUUUCUUUU) in the HCLV strain (AF091507). This 13-nt insertion is also present in the 3′UTR of another vaccine strain, Porcivac [25]. A previous study has demonstrated an important role of

the 12-nt insertion in 3′UTR of the HCLV strain in the attenuation of CSFV [28], whereas the 13-nt insertion is believed not to be a marker for virulence [25]. Therefore, the possible role of this 13-nt insertion in attenuation of virulence needs more rigorous evaluation. The overall similarity between the Rovac and the Chinese HCLV strains was 93.6%. The Rovac genome sequences has been deposited in GenBank with the accession number KJ873238.

From the Illumina NGS data, 1.6 million reads were mapped to the CSFV reference genome (NC_002657), and a consensus sequence of 12,130 nt was obtained for the Russian LK-VNIVViM strain. The near-complete genome contained a 379-nt 5′UTR, an ORF of 11,697 nt that could be divided into the same 11 gene regions as those of the Rovac strain and the reference strain, and a 54-nt fragment of 3′UTR where the 3′end ambiguous nucleotides were removed. It was unable to know if this genome contains a similar insertion at the 3′UTR but there was such an insertion according to a previous study [25]. The LK-VNIVViM had a sequence similarity of 99.1% to Rovac (KJ873238), 95.3% to the reference strain Eystrup (NC_002657) and 93.4% to HCLV (AF091507). This genome sequence has also been deposited in GenBank with the accession number KM522833.

The phylogenetic relationship between the Rovac strain and other lapinized vaccines including the Russian LK-VNIVViM strain was determined by Bayesian analysis of 44 complete genome sequences of CSFV (Fig. 1). The overall tree topology was consistent to that reported previously [23]. The Rovac strain was placed unexpectedly in the basal position to three attenuated vaccine strains, namely the Russian LK-VNIVViM, the CS strain and its derivative, RUCSFPLUM. The LK-VNIVViM occupied a similar basal position to the CS and RUCSFPLUM. The relationships were strongly supported by the maximal posterior probability value of 1.0. On the other hand, the lapinized vaccines strains, such as the Taiwanese LPC, the Indian HCLV (thought to be derived from CSF "Weybridge") and the Chinese HCLV, formed a distant cluster with strong support. The whole-genome phylogeny showed that the Rovac strain was more closely related to the Russian vaccine strains LK-VNIVViM, CS and its derivative RUCSFPLUM (genotype 1.2) than to the C-strain clade (genotype 1.1), suggesting that Rovac is the ancestor strain of the Russian vaccine strains.

According to Lin and Lee [14], the Taiwanese LPC strain originated from a lapinized virus that had already undergone about 250 passages in rabbits by the Lederle laboratories, from which the Philippines government had purchased large doses of Rovac vaccine. The lapinized virus that was introduced to Taiwan in 1952 was believed directly from the Lederle laboratories [29]. Therefore, the monophyletic lapinized vaccine strains (LPC, HCLV, C-strain) would have shared the same ancestor, Rovac strain, and this would fit well to pieces of information gathered by Prof. Mészáros during his visit to China in 1958. However, the close relationship between the Rovac and Russian vaccine strains clearly diminished this possibility. It is likely that the Rovac strain, determined in this study, is different from the virus that was used to develop LPC vaccine. Therefore, the ancestor of the C-strain might be another, yet to be determined strain.

Zhou [9,30] reviewed the history of C-strain development in China and stated that former USSR scientists visited China in 1957 and 1958 and took the C-strain vaccine of the 360th passage in rabbits back to USSR. Prof. Mészáros also sent the C-strain vaccine from Hungary to USSR scientists upon their request. The analysis presented in this paper showed that Russian scientists had developed their own vaccine strains, which are likely based on the Rovac strain as suggested by the whole genome phylogeny. Indeed, LK-K (genotype 1.1) and LK-VNIVViM (genotype 1.2) are two different CSF vaccines that have been used in the National Institute of Veterinary Virology and Microbiology, Pokrov, Russia, and only LK-VNIVViM, from which the CS strain was derived, has been in use for

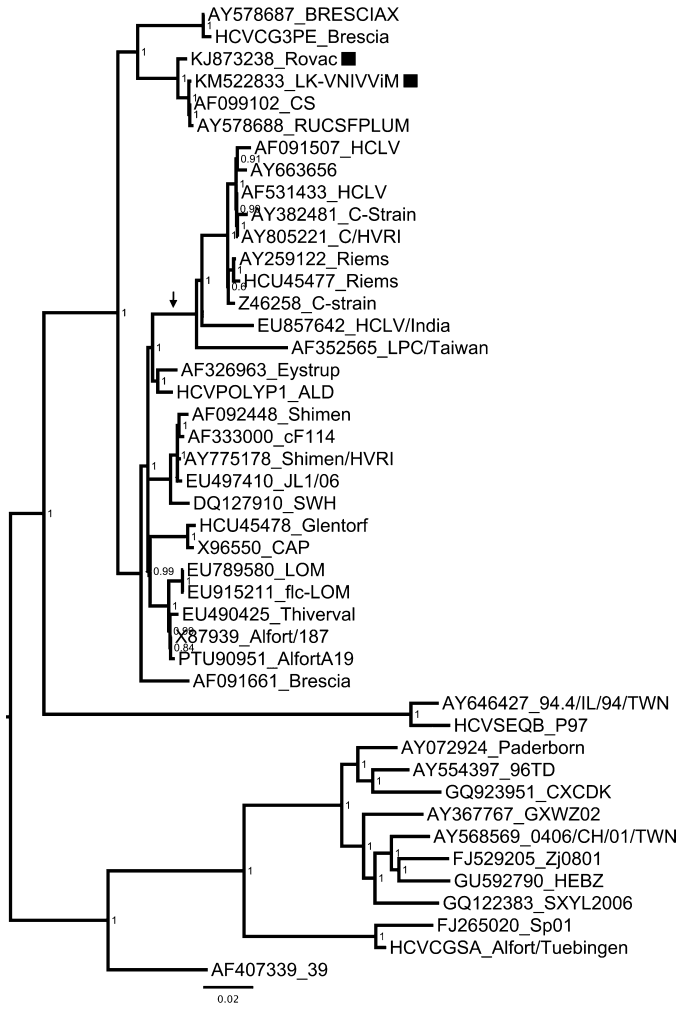

Fig. 1. A closer phylogenetic relationship between the Rovac strain and three Russian vaccines, rather than with the C-strain in the consensus tree of CSFV whole-genome phylogeny. The Rovac and LK-VNIVViM are marked with a "■" symbol. The sequences are labeled with accession number followed by strain name (if any). The lapinized vaccine clade is indicated with an arrow symbol (↓). Numbers indicate the posterior probability on the nodes. Bar indicates changes per nucleotide site.

vaccine production for more than 30 years (Malogolovkin, personal communication). This fact corroborated well with our phylogeny, which demonstrated an ancestry position of the LK-VNIVViM in relation to the CS strain and its derivative RUCSFPLUM strain. Due to the lack of access to, or the genomic sequence, in GenBank, of the LK-K strain and the K vaccine that is also produced in Russia, it is not possible to perform molecular phylogenetic analysis to determine their relationship with the C-strain or the LPC strain.

In summary, the genome sequences of the vaccine strains Rovac and LK-VNIVViM were determined. Molecular phylogenetic analyses revealed an unexpected closer relationship of the Rovac vaccine with the Russian vaccines LK-VNIVViM, and CS strain and its derivative RUCSFPLUM, rather than with the Chinese C-strain, suggesting that the Rovac strain is the likely ancestor of the Russian lapinized vaccine strains against classical swine fever. Further investigation is therefore still needed to elucidate the origin of the C-strain.

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References

[1] Pletnev A, Gould E, Heinz FX, Meyers G, Thiel HJ, Bukh J, et al. Flaviviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus taxonomy. 9th ed. Oxford: Academic Press; 2011. p. 1003–20.
[2] Rümenapf T, Thiel HJ. Molecular Biology of Pestiviruses. In: Mettenleiter TC, Sobrino F, editors. Animal viruses: molecular biology. Norwich: Caister Academic Press; 2008. p. 39–96.
[3] Dorset M, Bolton BM, McBryde CN. The etiology of hog cholera. USDA, Bur Anim Ind. 21st Ann Rep; 1904. p. 138.
[4] USDA. Hog cholera: its history, nature and treatment as determined by the inquires and investigations of the Bureau of Animal industry. Washington, DC: Government Printing Office; 1889.
[5] Beynon AG. Swine fever in Great Britain. Bull Off Int Epiz 1962;57:1461–87.
[6] Liu L, Xia H, Wahlberg N, Belák S, Baule C. Phylogeny, classification and evolutionary insights into pestiviruses. Virology 2009;385:351–7.
[7] Van Oirschot J. Vaccinology of classical swine fever: from lab to field. Vet Microbiol 2003;96:367–84.
[8] McNutt SH. Swine diseases. In: Brandly CA, Jungherrr EL, editors. Advances in veterinary science 1. New York: Academic Press Inc.; 1953. p. 283–328.
[9] Zhou T. Classical swine fever virus and progresses in its control (Part I). Chin J Vet Sci Technol 1980;4:23–33 [in Chinese].
[10] Baker JA, Rinderpest VIII. Rinderpest infection in rabbits. Am J Vet Res 1946;7:179–82.
[11] Koprowski H, James TR, Cox HR. Propagation of hog cholera virus in rabbits. Proc Soc Exp Biol Med 1946;63:178–83.
[12] Baker JA. Serial passage of hog cholera virus in rabbits. Proc Soc Exp Biol Med 1946;63:183–7.
[13] Szent-Iványi T. Classical swine fever: new control and eradication methods. Rev Sci Tech Off Int Epiz 1984;3:465–86.
[14] Lin TTC, Lee RCT. An overall report on the development of a highly safe and potent lapinized hog cholera virus strain for hog cholera control in Taiwan. Natl Sci Coun Spec Publ 1981;5:1–44.
[15] Research Group of CSF Vaccine. Studies on the avirulent Lapinized Hog Cholera virus. Acta Vet Zootech Sin 1979;10:1–34 [in Chinese].
[16] Zaberezhny AD, Grebennikova TV, Kurinnov VV, Tsybanov SG, Vishnyakov IF, Biketov SF, et al. Differentiation between vaccine strain and field isolates of classical swine fever virus using polymerase chain reaction and restriction test. Dtsch Tierarztl Wochenschr 1999;106:394–7.
[17] Gupta PK, Saini M, Dahiya SS, Patel CL, Sonwane AA, Rai DV, et al. Molecular characterization of lapinized classical Swine Fever vaccine strain by full-length genome sequencing and analysis. Anim Biotechnol 2011;22:111–7.
[18] Sasahara J, Kumagai T, Shimizu Y, Furuuchi S. Field experiments of hog cholera live vaccine prepared in guinea-pig kidney cell culture. Natl Inst Anim Health Q 1969;9:83–91.
[19] Aynaud JM, Lejolly JC, Bibard C, Galicher C. Studies of the properties of cold induced classical swine fever virus mutants. Application to vaccination. Bull Off Int Epizoot 1971;75:654–9 [in French].
[20] Bognár K, Mészáros J. Experiences with a Chinese strain of lapinized swine fever virus. Acta Vet Hungaricae 1963;13:69–74.
[21] Wu HX, Wang JF, Zhang CY, Fu LZ, Pan ZS, Wang N, et al. Attenuated lapinized Chinese strain of classical swine fever virus: complete nucleotide sequence and character of 3′-noncoding region. Virus Genes 2001;23:69–76.
[22] Dong X, Chen Y. Marker vaccine strategies and candidate CSFV marker vaccines. Vaccine 2007;25:205–30.
[23] Xia H, Wahlberg N, Qiu HJ, Widén F, Belák S, Liu L. Lack of phylogenetic evidence that the Shimen strain is the parental strain of the lapinized Chinese strain (C-strain) vaccine against classical swine fever. Arch Virol 2011;156:1041–4.
[24] Liu L, Kampa J, Belák S, Baule C. Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen. Vet Microbiol 2009;138:62–8.
[25] Björklund HV, Stadejek T, Vilcek S, Belák S. Molecular characterization of the 3′ noncoding region of classical swine fever virus vaccine strains. Virus Genes 1998;16:307–12.
[26] Granberg F, Vicente-Rubiano M, Rubio-Guerri C, Karlsson OE, Kukielka D, Belák S, et al. Metagenomic detection of viral pathogens in Spanish honeybees: co-infection by Aphid Lethal Paralysis, Israel Acute Paralysis and Lake Sinai Viruses. PLOS ONE 2013;8(2):e57459.
[27] Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003;19:1572–4.
[28] Wang Y, Wang Q, Lu X, Zhang C, Fan X, Pan Z, et al. 12-nt insertion in 3′ untranslated region leads to attenuation of classic swine fever virus and protects host against lethal challenge. Virology 2008;374:390–8.
[29] Lee RCT. Lapinized hog cholera vaccine in Taiwan. Sci Agric (Taiwan) 1954;2(11):4–14 [in Chinese].
[30] Zhou T. Classical swine fever virus and progresses in its control (Part II). Chin J Vet Sci Technol 1980;5:30–9 [in Chinese].