Acta Veterinaria-Beograd 2023, 73 (1), 13-21
UDK: 578.8:577.213.3
615.371
DOI: 10.2478/acve-2023-0002

Research article

DESIGN OF A REPLICATIVE-COMPETENT MGF110 (1L-5-6L) DELETED AFRICAN SWINE FEVER VIRUS (GENOTYPE II)

Mariia V. NEFEDEVA[1]*, Alexander S. MALOGOLOVKIN[1,2,3], Ilya A. TITOV[1]

[1]Federal Research Center for Virology and Microbiology, Volginsky, Russia; [2]Sirius University of Science and Technology, Sochi, Russia; [3]Sechenov First Moscow State Medical Univesity, Moscow, Russia

(Received 09 November 2022, Accepted 24 February 2023)

Viral individual genes functions and their role in the interaction with the host cells remain the main area in the study of African swine fever virus (ASFV) biology. The extreme heterogeneity of the ASFV makes it difficult to develop vaccines against this pathogen. In this work, we generated the ASFV deletion mutant virus Volgograd/D(1L-5-6L) with the six genes deletion in multigenic family 110 (MGF110) (1L-5-6L) and studied its characteristics *in vitro*. The homologous recombination method was used for the deletion in ASFV parental strain Volgograd/14c. A series of six passages was carried out in the COS-1 cell culture using the limiting dilution method. The recombinant strain Volgograd/D(1L-5-6L) MGF110 was selected by the plaque formation method. Performed study of viral replication showed no changes in viral growth kinetics in comparison with the parental strain. The ASFV Volgograd/D(1L-5-6L) MGF110 is a great tool available to test the importance of MGF110 for virus virulence and vaccine development.

Keywords: African swine fever virus, multigenic family 110, recombinant virus, COS-1 cell culture.

INTRODUCTION

African swine fever (ASF) is a viral hemorrhagic disease with exceptionally high mortality in domestic pigs and wild boars [1]. Despite the measures taken, ASF continues to spread across borders, affecting many European countries, China and other Asian countries in 2018 [2]. In this regard, studies aimed at developing a vaccine against this dangerous disease have intensified. Researchers use various approaches to vaccine development, such as immunization with recombinant proteins [3-5], DNA vaccines [6], or vector vaccines [7,8]. However, so far, these approaches have not protected animals from infection. The only method that made it possible to obtain some positive results, expressed in the complete or partial protection of animals under experimental conditions from infection with a homologous virus strain, is the creation

*Corresponding author: e-mail: masha67111@mail.ru

of deletion mutants [9-12]. Two papers describing the creation of a vaccine candidate by this method have gained wide popularity. Chen et al., 2020 describes the creation and testing of an artificially attenuated strain with a deletion of seven genes based on the Chinese strain "HLJ/18" [13]. At the same time, Borca et al., 2020 deleted only one I177L gene, which according to them led to the attenuation of the original virus strain, "Georgia/2007" [14]. Vaccine trials on large livestock, carried out in a number of farms in Vietnam are pending and the results will demonstrate the potential and safety of deployed vaccine [15]. This fact may indicate the gap of scientific knowledge about the functions of certain genes in the ASFV genome, which is the reason for the difficulties in creating a universal and effective vaccine against this disease.

The ASFV high genetic diversity is determined by the right (5') and left (3') variable regions of the genome, represented by multigene families [16-18]. One of the representatives of multigene families is MGF110, which is located at the 5' terminal region of the ASFV genome. Despite active research in determining certain genes functionality, as well as identifying potential ASFV virulence genes, MGF110 is still insufficiently studied. At the same time, there is evidence of its possible role in virus attenuation. As an example, in a naturally attenuated ASFV strain isolated in 2014 in Estonia, the absence of some members of MGF110 was demonstrated [19,20].

Thus, in order to study the role of this multigene family in the processes of virus pathogenesis and attenuation, we aimed to develop a recombinant strain with a large deletion of MGF110 (1L-5-6L) genes.

MATERIALS AND METHODS

Virus and cells

The virulent ASFV Volgograd/14c strain (genotype II, serogroup 8) was obtained from the Federal Research Center for Virology and Microbiology, Vladimir region, Russia (FRCVM) strain collection. COS-1 cells were used to cultivate the recombinant virus and determine its characteristics (grown in DMEM/F-12 with 5% fetal bovine serum "Gibco", USA; 37ºC, 5% CO_2). Porcine blood derived macrophages were prepared by the method of cell separation in a Ficoll density gradient and grown in RPMI Medium 1640 (1x) with GlutaMAX^{TM}-I and 10% FBS ("Gibco", USA) and 30% of autologous porcine plasma, then incubated at 37 C° with 5% CO_2.

Experimental study

The ASFV Volgograd/14c strain DNA was used as a matrix for amplification. For deletion of six MGF110 (1L-5-6L) genes, specific oligonucleotide primers were designed. Recombination arms (744 bp left and 714 bp right) as well as the EGFP reporter gene under the control of the p72 promoter, was synthesized by amplification of overlapping fragments (Table 1).

Table 1. Oligonucleotides for 1L-5-6L MGF110 genes deletion

Title	Sequence 5'- 3'	Position on the ASFV genome Georgia_2007/1	Product length	GC, %	Tm, °C
LF_1_fwd	ggcgaattgggcccgacgtcgca tgtttcacttgtctcaagctcttc	6260	743	55.3	84.1
LF_1L_rev	ctccggcgacccgtgaaaatgatttta ttagacatgattgttg	7003		41.9	77.0
RF_6L_fwd	ctgtacaagtaatgctacggctggctgaacag	10108	713	50.0	73.8
RF_6L_rev	acgcgttgggagctctcccatatggataaa gagtagagcgttagctactactg	10821		49.1	70.6
GFP_1L_6L_fwd	aaatcattttcacgggtcgccggaggaaaagtc	6991-7003	956	48.5	73.9
GFP_1L_6L_rev	gccagccgtagcattacttgtacagctcgtccatgc	10108 – 10120		55.6	78.9

The oligonucleotides were designed using Oligo 6.0 program (http://oligo.net/downloads.html). LongAmpTaq 2X Master Mix kit («NEB», USA) was used to amplify PCR products.

The resulting PCR products were amplified by overlapping fragments and were used for cell transfection. The size of the deletion is 3103 bp.

For recombinant virus selection and screening, 96-well plates were infected with 10-fold dilutions of the cell lysate containing the Δ1L-5-6L-MGF110 recombinant virus. Plates were screened for 5-6 days. As a result, 6 passages were performed in the COS-1 cells. After 2 passages, the Δ1L-5-6L-MGF110 recombinant virus titer was 1.60 ± 0.10 lg $TCID_{50}$/ml, after 4 passages – 4.28 ± 0.15 lg $TCID_{50}$/ml. By the 6th passage, the recombinant virus titer reached 4.94 ± 0.13 lg $TCID_{50}$/ml. The performed sequencing analysis allows us to conclude that there are no nucleotide substitutions and confirms the presence of the specific EGFP marker gene with the p72 promoter.

Homologous recombination was carried out by COS-1 cells infecting (kidney of the African green monkey) in 6 well plates at a multiplicity of infection (MOI) equal to 0.3 with wild-type ASFV Volgograd/14c for 1 hour and subsequent cells transfection with 2.0 μg of PCR products. Single cells expressing eGFP were observed on the second to third days after infection-transfection under ZOE Fluorescent Cell Imager ("Bio-Rad Laboratories, Inc.").

Transfection of COS-1 cells was performed using Lipofectamine 3000 («ThermoFisher», USA) according to the manufacturer's instructions. For the Δ1L-5-6L-MGF110 virus selection, the limiting dilutions method and analysis of fluorescent plaques were used. Nucleotide sequencing was performed on the Genetic Analyzer 3130 automatic sequencer («Applied Biosystems», USA). The absence of the parental type in Δ1L-5-6L-MGF110 recombinant was confirmed by PCR followed by 1.5% electrophoresis.

In order to determine the ASFV Δ1L-5-6L-MGF110 titer, the porcine blood derived macrophages and COS-1 cells were used. Virus DNA was extracted using DNA-sorb-B

kit («InterLabService», Russia). Real-time quantitative PCR was performed in Real-time CFX96 Touch amplifier («Bio-Rad Laboratories», USA), with oligonucleotide primers complementary to the ASFV*B646L* gene [21].

To determine the effect of gene deletion on the virus replication kinetics, COS-1 cells were infected with the ASFV Δ1L-5-6L-MGF110 recombinant virus and the original Volgograd/14c with a multiplicity of infection of 0.1 and 1.0 $TCID_{50}$/cell. The experiment was carried out in 2 replicates. Samples aliquots were collected within 5 days.

RESULTS

Construction of ASFV Δ1L-5-6L-MGF110 deletion mutant

Presence of specific 1L-5-6L-MGF110 gene deletion was confirmed by PCR. After the amplification of DNA samples, isolated from the parental strain and the strain with deletion, specific amplicons with a length of 4561b.p. and 2394 b.p., respectively, were detected. This fact indicates the absence of the parental virus type in the Δ1L-5-6L-MGF110 recombinant (data not presented).

Infectious activity of Δ1L-5-6L-MGF110

Determination of the ASFV Δ1L-5-6L-MGF110 recombinant infectious activity was carried out in 96-well microplates with the porcine blood derived macrophages and COS-1 cells. The titration results showed that the ASFV Δ1L-5-6L-MGF110 virus infectious activity in the porcine blood derived macrophages is 7.25 ± 0.28 lg HAU_{50}/ml and 7.00 ± 0.21 lg $TCID_{50}$/ml in COS-1 cells. Thus, it can be concluded that the deletion of genes 1L-5-6L of the MGF110 multigene family does not affect viral replication *in vitro*.

Growth characteristics of constructed ASFV ΔMGF110 deletion mutant

According to the results, at 0.1 TCID50/cell multiplicity of infection, a growth curve was observed for both recombinant and original viruses, which reached the maximum copy number on 4-5 days after infection in COS-1 cell culture. At 1.0 TCID50/cell

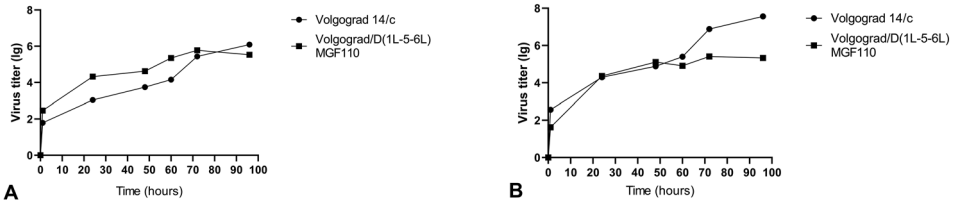

Figure 1. Quantification of the ASFV growth characteristics ASFV Δ1L-5-6L-MGF110 (square) and Volgograd/14c (circle) in COS-1 cell culture at a multiplicity of infection of 0.1 **(A)** and 1.0 **(B)** $TCID^{50}$/cell.

multiplicity of infection, a logistic growth curve is traced in infected cells and the increase of fluorescent foci within 5 days is also noted (Figure 1).

Thus, the ASF ΔMGF110 replication kinetics were determined at different multiplicities of infection in the COS-1 cell culture.

DISCUSSION

This work is devoted to the deletion of large MGF 110 fragment of the African swine fever virus and its implication on virus growth. Titers in primary and transplanted cell cultures, the absence of the original parental type of virus, and growth characteristics were determined. The results of the study will help understand of the MGF110 role in the ASFV pathogenesis.

Scientists have drawn attention to the multigenic family 110 of the African swine fever virus genome. A large amount of new data on the structural and functional organization of this multigenic family is presented in the work of Zhu et al., 2021. Thus, using the phylogenetic analysis method on 65 full length ASFV genomes, which are available in Genbank, it was possible to classify MGF 110 sequences in 8 groups (MGF-110A - MGF-110I). At the same time, MGF 100 and MGF 110 lack any similarity in nucleotide sequences with other multigene families present in the virus genome. Moreover, it has been predicted that members of MGF 110 may encode one or more transmembrane helices (6 groups of MGF110). They can also encode signal peptides. Most of the proteins encoded by MGF110 are located on the cell membrane or in the intercellular space [18]. There is evidence that proteins encoded by the 110 multigene family affect the structure and functions of the cell's endoplasmic reticulum [ER], involving it in the operation of viral factories and virion assembly. Also, due to a change in the endoplasmic reticulum with the participation of MGF110 proteins, regulation of the transfer of immunoregulatory or anticoagulant proteins to the surface of mononuclear phagocytic cells can be carried out. All these intended functions can influence the determination of the range of hosts and virulence [22]. However, despite all the data obtained, the functions performed by these proteins during the infectious cycle remain to be elucidated.

Many research, aimed at studying the functions of MGF 110 by creating deletion viruses. Thus, in the work of Ramirez-Medina et al., 2021 a deletion mutant that lacks the 1L gene, which is a member of MGF110, was studied [23]. It was found that this gene is not necessary for virus replication and does not affect its growth characteristics and attenuation. In another study on the deletion ASFVMGF110 5L-6L, similar results were obtained, indicating the absence of any effect from the deletion on virus replication and virulence. It was also showed, that such a deletion can serve as a DIVA marker for a potential vaccine, since a protein is expressed from the MGF110 5-6L genes, which can be detected by ELISA [24]. The results of our work do not contradict the data obtained by Ramirez-Medina et al., 2021. Moreover, the absence

of changes in the growth characteristics and replication of the virus *in vitro* that we observed indicate that not only 1L, but a much larger number of MGF110 members are not required for replication in cell culture. However, data on the effect of deletion of 1L-5-6L members on virulence remains to be elucidated in an *in vivo* experiment.

On the other hand, according to Li et al., 2021 deletion of the 9L gene in MGF 110 leads to significant changes in the reproduction and virulence of the virus [25]. As a result of the deletion, a decrease in virus replication was observed in the primary culture of porcine macrophages. In an *in vivo* experiment, low-dose infection of sensitive animals showed no clinical picture, low viremia, and a strong immune response [25]. This fact may indicate a significant role of the 9L gene in determining the ASFV properties. This gene may be a potential target in the development of vaccine candidates based on recombinant viruses. In our study, the 9L gene was not affected, but it seems a promising potential target for further research. Based on the obtained results, it can be concluded that there is no significant effect of the 1L-5-6L genes included in MGF 110 on virus replication and attenuation. However, to confirm the study results, it is necessary to conduct an experiment *in vivo*, which will allow to having final conclusions about the effect of the studied genes on the virus properties.

CONCLUSIONS

In this study, we successfully obtained and studied the growth characteristics of Volgograd/D(1L-5-6L) MGF110 recombinant virus with deletion 1L-5-6L members of multigenic family 110 from ASFV strain (genotype II, serogroup 8). Thus, it was demonstrated that the deletion of 1L-5-6L members of the multigene family 110 had no effect on virus replication in porcine macrophages and COS-1 cells. The results of this study may provide an opportunity to evaluate the role of MGF110 in immune evasion for the development of effective vaccines.

Acknowledgements

This work was supported by the Russian Science Foundation, project № 22-24-00552.

Authors' contributions

MVN continuous cell line management, cell transfection, determination of the virus growth characteristics, writing - original draft preparation. ASM conceptualization, data analysis, manuscript preparation. IAT study design, PCR experiments, construction of deletion mutant, data analysis, writing - review and editing. All authors have read and agreed to the published version of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Blome S, Franzke K, Beer M: African swine fever - A review of current knowledge. Virus Res 2020, 287:198099.
2. Sauter-Louis C, Conraths FJ, Probst C, Blohm U, Schulz K, Sehl J, Fischer M, Forth JH, Zani L, Depner K, Mettenleiter TC, Beer M, Blome S:African Swine Fever in Wild Boar in Europe-A Review. Viruses 2021, 13(9):1717.
3. Zsak L, Onisk DV, Afonso CL, Rock DL: Virulent African Swine Fever Virus Isolates Are Neutralized by Swine Immune Serum and by Monoclonal Antibodies Recognizing a 72-kDa Viral Protein. Virology 1993, 196:596-602.
4. Gómez-Puertas P, Rodríguez F, Oviedo JM, Ramiro-Ibáñez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM: Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. J Virol 1996, 70:5689-5694.
5. Gómez-Puertas P, Rodríguez F, Oviedo JM, Brun A, Alonso C, Escribano JM: The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective 636 immune response. Virology 1998, 243:461-471.
6. Argilaguet JM, Pérez-Martín E, Nofrarías M, Gallardo C, Accensi F, Lacasta A, Mora M, Ballester M, Galindo-Cardiel I, López-Soria S, Escribano JM, Reche PA, Rodríguez F: DNA Vaccination Partially Protects against African Swine Fever Virus Lethal Challenge in the Absence of Antibodies. PLOS ONE 2012, 7:e40942.
7. Jancovich JK, Chapman D, Hansen DT, Robida MD, Loskutov A, Craciunescu F, Borovkov A, Kibler K, Goatley L, King K, Netherton CL, Taylor G, Jacobs B, Sykes K, Dixon LK: Immunization of Pigs by DNA Prime and Recombinant Vaccinia Virus Boost To Identify and Rank African Swine Fever Virus Immunogenic and Protective Proteins. J Virol2018, 92:e02219-02217.
8. Lokhandwala S, Petrovan V, Popescu L, Sangewar N, Elijah C, Stoian A, Olcha M, Ennen L, Bray J, Bishop RP, Waghela SD, Sheahan M, Rowland RRR, Mwangi W: Adenovirus-vectored African Swine Fever Virus antigen cocktails are immunogenic but not protective against intranasal challenge with Georgia 2007/1 isolate. Vet Microbiol 2019, 235:10-20.
9. O'Donnell V, Holinka LG, Gladue DP, Sanford B, Krug PW, Lu X, Arzt J, Reese B, Carrillo C, Risatti GR, Borca MV: African Swine Fever Virus Georgia Isolate Harboring Deletions of MGF360 and MGF505 Genes Is Attenuated in Swine and Confers Protection against Challenge with Virulent Parental Virus. J Virol 2015, 89:6048-6056.
10. O'Donnell V, Holinka LG, Krug PW, Gladue DP, Carlson J, Sanford B, Alfano M, Kramer E, Lu Z, Arzt J, Reese B, Carrillo C, Risatti GR, Borca MV: African Swine Fever Virus Georgia 2007 with a Deletion of Virulence-Associated Gene 9GL (B119L), when Administered at Low Doses, Leads to Virus Attenuation in Swine and Induces an Effective Protection against Homologous Challenge. J Virol 2015, 89:8556-8566.
11. O'Donnell V, Risatti GR, Holinka LG, Krug PW, Carlson J, Velazquez-Salinas L, Azzinaro PA, Gladue DP, Borca MV. Simultaneous Deletion of the 9GL and UK Genes from the African Swine Fever Virus Georgia 2007 Isolate offers Increased Safety and Protection against Homologous Challenge. J Virol2017, 91:e01760-01716.
12. Li D, Liu Y, Qi X, Wen Y, Li P, Ma Z, Liu Y, Zheng H, Liu Z: African Swine Fever Virus MGF-110-9L-deficient Mutant Has Attenuated Virulence in Pigs. Virol Sin 2021, 36(2):187–195.

13. Chen W, Zhao D, He X, Liu R, Wang Z, Zhang X, Li F, Shan D, Chen H, Zhang J, Wang L, Wen Z, Wang X, Guan Y, Liu J, Bu Z: A seven-gene-deleted African swine fever virus is safe and effective as a live attenuated vaccine in pigs. Sci China Life Sci 2020,63(5):623-634.
14. Borca MV, Ramirez-Medina E, Silva E, Vuono E, Rai A, Pruitt S, Holinka LG, Velazquez-Salinas L, Zhu J, Gladue DP: Development of a Highly Effective African Swine Fever Virus Vaccine by Deletion of the I177L Gene Results in Sterile Immunity against the Current Epidemic Eurasia Strain. J Virol 2020, 94(7):e02017-19.
15. Khanh Vu: Reuters: Vietnam suspends African swine fever vaccine after pig deaths. [https://www.reuters.com/world/asia-pacific/vietnam-suspends-african-swine-fever-vaccine-after-pig-deaths-2022-08-24]
16. Yozawa T, Kutish GF, Afonso CL, Lu Z, Rock DL: Two novel multigene families, 530 and 300, in the terminal variable regions of African swine fever virus genome. Virology 1994, 202(2):997–1002.
17. Zsak L, Lu Z, Burrage TG, Neilan JG, Kutish GF, Moore DM, Rock DL: African swine fever virus multigene family 360 and 530 genes are novel macrophage host range determinants. J Virol 2001,75(7):3066–3076.
18. Zhu Z, Chen H, Liu L, Cao Y, Jiang T, Zou Y, Peng Y: Classification and characterization of multigene family proteins of African swine fever viruses. Brief Bioinform 2021, 22(4):bbaa380.
19. Zani L, Forth JH, Forth L, Nurmoja I, Leidenberger S, Henke J, Carlson J, Breidenstein C, Viltrop A, Höper D, Sauter-Louis C, Beer M, Blome S: Deletion at the 5'-end of Estonian ASFV strains associated with an attenuated phenotype. Sci Rep 2018,8(1):6510.
20. Nurmoja I, Petrov A, Breidenstein C, Zani L, Forth JH, Beer M, Kristian M, Viltrop A, Blome S: Biological characterization of African swine fever virus genotype II strains from north-eastern Estonia in European wild boar. Transbound Emerg Dis 2017, 64(6):2034–2041.
21. Fernández-Pinero J, Gallardo C, Elizalde M, Robles A, Gómez C, Bishop R, Heath L, Couacy-Hymann E, Fasina FO, Pelayo V, Soler A, Arias M: Molecular diagnosis of African Swine Fever by a new real-time PCR using universal probe library. Transbound Emerg Dis 2013, 60(1):48–58.
22. Netherton C, Rouiller I, Wileman T: The subcellular distribution of multigene family 110 proteins of African swine fever virus is determined by differences in C-terminal KDEL endoplasmic reticulum retention motifs. J Virol 2004, 78(7):3710–3721.
23. Ramirez-Medina E, Vuono E, Pruitt S, Rai A, Silva E, Espinoza N, Zhu J, Velazquez-Salinas L, Borca MV, Gladue DP: Development and In Vivo Evaluation of a MGF110-1L Deletion Mutant in African Swine Fever Strain Georgia. Viruses 2021, 13(2):286.
24. Ramirez-Medina E, Vuono E, Silva E, Rai A, Valladares A, Pruitt S, Espinoza N, Velazquez-Salinas L, Borca MV, Gladue DP: Evaluation of the Deletion of MGF110-5L-6L on Swine Virulence from the Pandemic Strain of African Swine Fever Virus and Use as a DIVA Marker in Vaccine Candidate ASFV-G-ΔI177L. J Virol 2022, 96(14): e0059722.
25. Li D, Liu Y, Qi X, Wen Y, Li P, Ma Z, Liu Y, Zheng H, Liu Z: African Swine Fever Virus MGF-110-9L-deficient Mutant Has Attenuated Virulence in Pigs. Virol Sin 2021,36(2):187–195.

DIZAJN REPLIKATIVNE KOMPONENTE SA MGF110 (1L-5-6L) DELECIJOM VIRUSA AFRIČKE KUGE SVINJA (GENOTIP II)

Mariia V. NEFEDEVA, Alexander S. MALOGOLOVKIN, Ilya A. TITOV

Funkcionisanje i uloga svakog virusnog gena u interakcijama sa ćelijama domaćina predstavljaju osnovno polje istraživanja biologije virusa, posebno uzročnika afričke kuge svinja (ASFV). Ekstremna heterogenost ASFV otežava razvoj vakcina protiv ovog uzročnika. U ovom radu, stvoren je mutant ASF Lolgograd/D(1L-5-6L) virus sa delecijom šest gena multigenetske familije 11 (MGF110) pri čemu je obavljeno ispitivanje njegovih karakteristika, in vitro. U cilju delecije parentalnog ASFV Volgograd/14c, korišćen je homologi rekombinantni metod. Virus je pasiran šest puta u COS-1 ćelijskoj liniji koristeći metod ograničenog razblaživanja. Selekcija rekombinantnog Volgograd/D(1L-5-6L) MGF110 virusa je obavljena metodom plakova. Rezultati obavljenih studija virusne replikacije ne pokazuju nikakve izmene u kinetici rasta, a u poređenju sa parentalnim sojem. Zaključeno je da ASFV Volgograd/D(1L-5-6L) MGF110 predstavlja alat za ispitivanje značaja MGF110 kako za virulenciju virusa tako i za razvoj vakcina.