Production and application of mouse monoclonal antibodies targeting linear epitopes in pB602L of African Swine Fever Virus

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Abstract

African swine fever (ASF) is an acute hemorrhagic disease of domestic pigs. The causative agent of ASF, ASF virus (ASFV), is a double-stranded DNA virus, the sole member in the family Asfarviridae. The non-structural protein pB602L of ASFV is a molecular chaperone of the major capsid protein p72 and plays a key role in the icosahedral capsid assembly. This protein has good antigenicity and is also a target for developing diagnostic tools for ASF. To understand the molecular basis for the antigenicity of pB602L, a procaryoticly expressed recombinant pB602L protein was produced and applied to immunize mice for producing monoclonal antibodies (mAbs). A total of eight mouse mAbs were obtained and their binding epitopes were screened by Western blot against overlapped polypeptides of pB602L. Three linear epitopes were identified and designated Epitope 1 (366ANRERYNY373), Epitope 2 (415GPDAPGLSI423), and Epitope 3 (498EMLNVPDD505). Based on their recognizing epitopes, the eight mAbs were placed to three groups: Group 1 (B2A1, B2F1 and B2D10), Group 2 (B2H10, B2B2, B2D8, and B2A3), and Group 3 (B2E12), accordingly. mAbs B2A1, B2H10 and B2E12 were applied to detect pB602L in ASFV infected porcine alveolar macrophages (PAM) and pig tissues by indirect florescence assay (IFA), immunohistochemical staining, and immunogold labeling for electron microscopy, respectively. The results showed that pB602L was well detected with all the three mAbs by immunohistochemical staining and immunoelectron microscopy; but only B2H10 was suitable for detecting the protein in ASFV infected PAM cells by IFA. Taken together, we developed eight anti-pB602L mouse mAbs recognizing three linear epitopes in the protein, which provide biological materials and molecular basis for the basic and applied researches on ASFV.

Production and application of mouse monoclonal antibodies targeting linear epitopes in pB602L of African Swine Fever Virus

mAbs against ASFV pB602L protein

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Summary

African swine fever (ASF) is an acute hemorrhagic disease of domestic pigs. The causative agent of ASF, ASF virus (ASFV), is a double-stranded DNA virus, the sole member in the family *Asfarviridae* . The non-structural protein pB602L of ASFV is a molecular chaperone of the major capsid protein p72 and plays a key role in the icosahedral capsid assembly. This protein has good antigenicity and is also a target for developing diagnostic tools for ASF. To understand the molecular basis for the antigenicity of pB602L, a procaryoticly expressed recombinant pB602L protein was produced and applied to immunize mice for producing monoclonal antibodies (mAbs). A total of eight mouse mAbs were obtained and their binding epitopes were screened by Western blot against overlapped polypeptides of pB602L. Three linear epitopes were identified and designated Epitope 1 (^{366}ANRERYNY^{373}), Epitope 2 (^{415}GPDAPGLSI^{423}), and Epitope 3 (^{498}EMLNVPDD^{505}). Based on their recognizing epitopes, the eight mAbs were placed to three groups: Group 1 (B2A1, B2F1 and B2D10), Group 2 (B2H10, B2B2, B2D8, and B2A3), and Group 3 (B2E12), accordingly. mAbs B2A1, B2H10 and B2E12 were applied to detect pB602L in ASFV infected porcine alveolar macrophages (PAM) and pig tissues by indirect florescence assay (IFA), immunohistochemical staining, and immunogold labeling for electron microscopy, respectively. The results showed that pB602L was well detected with all the three mAbs by immunohistochemical staining and immunoelectron microscopy; but only B2H10 was suitable for detecting the protein in ASFV infected PAM cells by IFA. Taken together, we developed eight anti-pB602L mouse mAbs recognizing three linear epitopes in the protein, which provide biological materials and molecular basis for the basic and applied researches on ASFV.

Keywords

African Swine Fever virus, pB602L, monoclonal antibody, Linear Epitope, Pig

Introduction

African swine fever (ASF) is an acute hemorrhagic disease of domestic pigs. This disease often results in devastating economic losses to the pig industries of suffered countries because of the high rates of morbidity and mortality, and it is therefore classified as a notifiable disease by the World Organization for Animal Health (OIE). An effective vaccine is still not available so far, and control of the disease relies mainly on rapid diagnosis and culling of infected pigs and close contacted with them (Rowlands et al., 2008).

ASF was first recognized in early 1900's in East Africa. Since then, it has spread to most sub-Saharan African countries(Boshoff et al., 2007). However, when the disease introduced in Georgia in the Caucasus in 2007 (Rowlands et al., 2008), it has spread subsequently toward the northern and eastern areas of Europe with many countries affected, such as the Russian Federation, Ukraine, and Poland (M. C. Gallardo et al., 2015; Goller et al., 2015; Smietanka et al., 2016). In 2018, the first ASF outbreak was reported in Liaoning Province in China (Ge et al., 2018; Zhou et al., 2018). Since then, the disease has occurred in almost every major pig production area of China (Tao et al., 2020). A recent surveillance showed that naturally attenuated ASFVs have been identified in several provinces of China, which makes the control situation of the disease even more complex in the future (Sun et al., 2021). The disease has also been reported in some other Asian countries recently (Mighell et al., 2021).

ASF virus (ASFV) is the only member of *Asfarviridae* family (Alonso et al., 2018). An ASFV virion has an overall icosahedral morphology with a diameter of 260-300 nm. It is composed of five layers including a viral core, a core shell, an inner lipid membrane, an icosahedral capsid, and an external envelope (Wang et al., 2019). The viral genome is a double-stranded DNA molecule of 170-190 kbp encoding more than 150 viral proteins. About 50 of the viral proteins are structural proteins that response for many important roles in the virus life cycle, such as viral particle assembly and the infection of a host cell; while the rest of them are non-structural proteins that are expressed during viral replication and function mainly as regulators of replication. Still, the functions of about 40 proteins of ASFV are unknown yet (Alejo et al., 2018).

The pB602L protein of ASFV is encoded by B602L gene, which contains a central variable region(CVR) and always severs as one of the targets for sub-genotype classification of ASFV isolates (Atuhaire et al., 2013; C. Gallardo et al., 2009; Mai et al., 2021; Owolodun et al., 2010). pB602L is one of the non-structural proteins and functions as a molecular chaperon the major structural protein p72, which formed aberrant "zipper-like"

structures instead of icosahedral virus particles in the absent of pB602L during the viral capsid assembly (Epifano et al., 2006; Liu et al., 2019). However, by which means that pB602L helps p72 to assemble correctly is still a mystery. Previous studies showed that pB602L has strong antigenicity and can be used to develop diagnostic tools for ASFV(Gutierrez-Castaneda et al., 2008). Given that live-attenuated ASFVs have been believed the most promising vaccination strategies against ASFV (Chen et al., 2020; Sang et al., 2020), pB602L is probability a suitable target for developing diagnostic tool for evaluating the humoral immune responses of these vaccines because of the antibodies against pB602Lproduced only after the expression of the protein in the host cells.

In this study, we expressed and purified a recombinant pB602L of ASFV strain HLJ/2018, which was then used as antigen to immunize mice for monoclonal antibody (mAb) development. A total of eight mAbs were obtained and they bound to three linear epitopes in pB602L. These results provide biological materials and molecular basis for the basic and applied researches on ASFV.

Materials and Methods

Ethics Statement

The animal experiment with mice was approved by the Animal Care and Use Committee of Harbin Veterinary Research Institute (ID: HVRI-IACUC-2019-348), and the experimental operation was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China.

Cells, serum samples, and experimental animals

The SP2/0 and Sf-21 cells used in this study were maintained in our laboratory. ASFV (Pig/HLJ/2018) infected porcine alveolar macrophages (PAM), tissues samples including the spleens, tonsils, and gastrohepatic lymph nodes from ASFV infected pigs, and anti-ASFV positive sera were obtained from the National High Containment Laboratory for Animal Diseases Control and Prevention, Harbin, China. The details of animal experiments were described previously (Zhao et al., 2019; Chen et al., 2020). Six-week-old female mice were purchased from Changsheng company Liaoning Province and kept in the experimental animal facility at Harbin Veterinary Research Institute.

Production of pB602L in Escherichia coli

The B602L gene of ASFV (Pig/HLJ/2018) was synthesized and cloned into pGEX-6p-1 vector by Genscript Biotech Corporation (Nanjing, Jiangsu, China). The recombinant plasmid pGEX-6p-1was transformed into *E. coli* BL21(DE3) (Qiagen, Hilden, Germany) for pB602L expression. The cells were cultured at 37 in LB medium containing 100 μg/ml ampicillin and transferred to 18 when the optical density at 600 nm (OD_{600}) reached 0.5˜0.6, then the protein expression was induced by 0.3 mM IPTG for 16 h. The cells were lysed using an ultrasonic crusher and then centrifuged for 40 min at 4 to remove the celluar debris. Glutathione Sepharose 4B (GE Healthcare, Uppsala, Sweden) was used to purify the GST-tagged pB602L protein, which was then treated with PreScission Protease to remove the GST taggs. The purified pB602L protein was abtained by passing through anion-exchange resin with Resource Q (GE Healthcare, Uppsala, Sweden), and identified by SDS-PAGE and Western-blot.

Recombinant baculovirus expressed pB602L protein

The B602L gene of ASFV (Pig/HLJ/2018) was cloned into pFastBac-HTA vector by Genscript Biotech Corporation (Nanjing, Jiangsu, China). The recombinant plasmid pFastBac-HTA-B602L was transformed into *E. coli* DH10 Bac (WEIDI, Shanghai, China) in LB plates, containing: 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal and 40 μg/mL IPTG, incubated at 37 ° C for 48 h. A white colony was piked and cultured in a liquid culture (containing 50 μg/mL kanamycin, 7 μg/mL gentamicin and 10 μg/mL tetracycline) at 37 for 24 h. Bacmid was purified with a DNA Isolation Kit (OMEGA bio-tek, USA) and transfected 25 mL insect cells (Sf-21) with a density of 2.5×10^6 cells/ml.

Rescued recombinant baculovirus was used to infected Sf-21 cells for expressing pB602L. IFA was used to identify protein expression.

Development of anti-pB602L monoclonal antibodies

Six-week-old female mice were immunized three times at a 14 d interval. Three days after the fourth enhanced immunization, the spleen cells of the mice were fused with SP2/0 cells. The cells were cultured in DMEM medium with HTA and 20% FBS. Ten days later, the cell culture supernatant was detected by indirect ELISA (iELISA), and the positive clones were selected and subcloned three times by a limited dilution method. The identified monoclonal cells were cultured in the medium containing 20% FBS and 1% Penicillin Streptomycin. Female mice aged 10 weeks were used to prepare ascites fluid of the monoclonal antibodies. The antibodies titer of ascites was detected by iELISA. SBA Clonotyping System-HRP (Southern Biotech, USA) was used to determine the subclasses of monoclonal antibodies. Western blot and IFA were used to determine the specificity of the monoclonal antibodies against the recombinant protein and virions.

Expression of pB602L polypeptide fragments

To map the epitope recognized by each of the mAbs, a progressive procedure was adopted to screen the mAbs by Western blot assay against overlapped polypeptides from the longest to the shortest. A total of 18 polypeptides were designed and screened, and their lengths and locations in pB602L are showed in Fig. 1A, B. Polypeptides S1-9 were expressed in*E. coli* BL21 (DE3) and their encoding gene fragments were amplified using the primers presented in Table 1. The plasmid pGEX-6P-B602L, which encoded the full-length pB602L protein, was used as a template to amplify these genes. The gene fragments encoding the polypeptides S10-18 were synthesized and cloned into pGEX-6p-1 vector by Genscript Biotech Corporation (Nanjing, Jiangsu, China). The production procedure of these polypeptides was the same with that of the above mentioned for producing the full-length pB602L.

SDS-PAGE and Western blot analysis

The protein sample prepared from empty vector transfected cells, uninduced cells, induced cells, purified pB602L or polypeptides was mixed with loading buffer and boiled for 10 min. The proteins in each sample were separated by 10% gel and stained with Coomassie Brilliant blue R-250 for SDS-PAGE. For Western blot analysis, the gel was blotted to PVDF transfer membrane with an electrophoresis apparatus. The membrane was blocked by 5% Difco Skim Milk for 2 h at room temperature and then incubated with each of the mAbs or a mouse monoclonal anti-GST antibody (Sigma-Aldrich, USA) as primary antibody at room temperature for 1 h. After four washes with Tris-Buffered Saline (TBS) add 0.5% Tween-20 (TBST), the membrane was incubated with IRDye 800CW goat anti-Mouse IgG (H + L) as secondary antibody (LICOR, USA) at room temperature for 1 h, followed by four washes with TBST. Western blot analysis was completed with an Odyssey CLX (LICOR, USA).

Indirect ELISA

The iELISA was performed according to the previous procedure (Gimenez-Lirola et al., 2016). In brief, purified recombinant pB602L protein was coated on 96-well ELISA plates(2.5 μg/ml, 100 μl/well) in Tris-HCl buffer (pH 8.5) and incubated overnight at 4. The coating solution was discarded and the plate was rinsed with phosphate buffered saline (PBS) (pH 7.2-7.4) add 0.5% Tween-20 (PBST) for three times. The hybridoma cell or monoclonal antibodies purified from ascites was added to the ELISA plate (100 μl/well) and incubated for 30 min at 37. After six washes with PBST for 1 min each, the rabbit anti-mouse IgG (whole molecule) (Sigma-Aldrich, USA) as secondary antibody was added at a dilution of 1:2000 and incubated for 30 min at 37, followed by six washes with PBST. Antibody binding was analyzed by adding TMB Substrate Solution (100 μl/well) (TianGen Biotech, China) for 20 min. The reaction was stopped by 2M H2SO4 (50 μl/cell), and the optical density value was measured at 450 nm using a microplate reader (ELx808, BioTek, U.S.A).

The antibody subtypes were identified using the SBA Clonotyping System-HRP Kit (SouthernBiotech, USA). The capture antibody was diluted to a concentration of 5-10 μg/mL in PBS and added to the ELISA plates

(100 μl/well) incubating at 4 overnight, then the HRP-labeled second antibodies (IgA-HRP, IgG1-HRP, IgG2a-HRP, IgG2b-HRP, IgG3-HRP, IgM-HRP, Kappa-HRP, and Lambda-HRP) were added (1:500 dilution in 5% Difco Skim Milk) and incubated for 1 h at 37. The other steps are the same as above.

Immunofluorescence assay (IFA)

The SF-21 cells, which infected with a baculovirus for expressing the pB602L protein, or ASFV infected PAM cells were fixed with 4% paraformaldehyde. After washing three times with PBS, the cells were blocked with 5% BSA at 37 for 1 h and washed three times with PBS. The cells were then inoculated with 1:1000 mAbs or a murine anti-pB602L serum for 1 h, followed by four washes with PBST. The Goat anti-Mouse IgG (H+L) (Invitrogen, USA) was added at a dilution of 1:2000 and incubated for 30 min at 37. The images were captured in an inverted fluorescence microscope (EVOS FL, Life, USA).

Immune electron microscopy

The ASFV (Pig/HLJ/2018) infected cells were collected and pelleted at 3000 rpm for 15 min, then fixed with 4% paraformaldehyde (Alfa Aesar, U.S.A) at 4 overnight. The supernatant was discarded and the cells were dehydrated with N, N-Dimethylformamidel (DMF) (SINOPHARM, China) at a concentration of 50%, 70%, 90%, and 100% step by step. The cells were then transferred into a mixture of DMF and LR White Resin (Electron Microscopy Sciences, USA) at a ratio of 2:1 and 1:2 at 4 for 30 min, respectively. Embedding was done in 100% LR White Resin at 4 overnight, then polymerized by ultraviolet radiation at -20 for 10 d. Ultrathin sections were prepared with a slicer (UC-6, Leica, Germany) and loaded on nickel grids. The grid was blocked by 3% BSA for 30 min at room temperature, inoculated with mAb (1:40) or 1% BSA for 40 min at room temperature. After three washes with PBS, the grid was incubated with a 10 nm gold-labeled goat anti-mouse IgG (SIGMA, USA) (1:100 diluted) for 40 min at room temperature. After washing with PBS three times, the grid was stained with 2% uranyl acetate dihydrate (TED PELLA, USA/Canada) for 10 min and dried grids were exanimated in an electron microscopy (Hitachi-7650, Japan) at an operation voltage of 80 KV.

Immunohistochemical analysis

For immunohistochemical analysis, tissue samples (including spleens, tonsils, and gastrohepatic lymph nodes) were collected from ASFV infected pigs and fixed in 10% formalin for 24 h at room temperature. Slides were prepared following a common procedure of dehydration, embedding, and section. The slides were dried in oven at a temperature of 56 for 15 min, incubated with 3% perhydrol liquid at room temperature and blocked with 5% Difco Skim Milk for 1h at room temperature, incubated with 1:100 of mAbs at 4 overnight. After five washes with TBST, the slides were incubated with a goat anti-mouse IgG (Invitrogen, USA) (1:100) for 30 min at 37. The slides were stained with ST Hematoxylin (Leica, Germany) and analyzed using a microscope (DFC550, Leica, Germany).

Epitope sequence analysis

A total of 69 full-length pB602L protein sequences of ASFVs were obtained from the GenBank database. All these sequences were aligned by ClustalW implemented in the MEGA 6.

Results

Preparation of recombinant pB602L

To prepare purified recombinant pB602L for mouse immunization, the B602L gene of ASFV (Pig/HLJ/2018) was cloned into pGEX-6p-1 vector and expressed as GST-tagged pB602L protein in *E. coli* BL21(DE3). The expression and purity of the recombinant pB602L protein were assessed by SDS-PAGE firstly. Specific bands corresponding to GST-pB602L and pB602L were observed (Fig. 2A). The expressed pB602L was further identified by Western blot assays using an anti-GST monoclonal antibody (Fig. 2B) and an anti-ASFV pig serum (Fig. 2C). Consensus results were obtained by SDS-PAGE and Western blot. These analyses also revealed 95% purity of the final purified recombinant protein (Fig. 2A, C), indicating the purified pB602L protein was suitable for mouse immunization.

Mouse anti-pB602L mAb production

To produce anti-pB602L monoclonal antibodies, mice were immunized with the purified recombinant pB602L protein. Following a commonly used protocol for producing mAbs in mouse (Petrovan et al., 2019), we finally produced eight clones of anti-pB602L mAbs and they were designated B2A1, B2F1, B2D10, B2H10, B2B2, B2D8, B2A3, and B2E12, respectively. These mAbs were identified as belonging to two subtypes of IgG1 and IgG2a (Table 2). All of them showed good reactivity with both prokaryoticly and eukaryoticly expressed recombinant pB602L protein indicated by the assays of Western blot, iELISA and IFA, respectively (Table 2).

Epitope mapping

To map the epitopes of the mAbs in pB602L, we screened the mAbs by Western blot against the pB602L polypeptide fragments as shown in Table 3 and Fig. 1B. As showed by above mAb characterizing assays, all mAbs recognized the largest polypeptide (2-531) (Fig. 1A). We then expressed three polypeptides of S1(2-256), S2(131-397), and S3(266-531). In contrast to S1 which was recognized by no one, S3 was recognized by all these mAbs. S2 was recognized by three mAbs B2A1, B2F1, and B2D10 (Fig. 1C), and they were therefore placed in Group 1. Based on these results, six polypeptides (S4-9) were expressed and screened. The results showed that the Group 1 mAbs recognized only S6 (333-397); S7(399-464) was recognized by four mAbs (B2H10, B2B2, B2D8, and B2A3) (Fig. 1C) and they were placed in Group 2; while S9 (465-531) was recognized by only one mAb (B2E12) (Fig. 1C) and it was placed in Group 3. To further shorten the recognized polypeptides by each group of the mAbs, we produced overlapped oligopeptides S10-18 and screening assays against them were conducted. By analyzing the overlaps of those polypeptides recognized by the mAbs in each group, three minimal recognized regions were finally determined and they were designated Epitope 1 (^{366}ANRERYNY^{373}), Epitope 2 (^{415}GPDAPGLSI^{423}), and Epitope 3(^{498}EMLNVPDD^{505}), and they were recognized by mAbs of Group 1 to 3, respectively (Table 3, Fig. 1B, C).

Given the B602L gene has a CVR and variations are always presented among different isolates, we then wondered whether the epitopes were conservative among different AFSV viruses. A sequence comparison was conducted based on 69 B602L genes of ASFVs downloaded from the GenBank database. The result showed that sequences of the three epitopes were completely conserved among these viruses (Table 4), suggesting that the mAbs developed in this study are capable of recognizing the pB602L proteins from all these AFSVs.

Preliminary applications of the mAbs

To explore the potential application of these mAbs, three mAbs B2A1, B2H10, and B2E12, which were the representative of each mAb group, were used in IFA, immunohistochemical staining, and immunoelectron microscopy for detecting pB602L in ASFV infected cells or tissues. During the mAb characterizing process, we proved that all these mAbs were performed well in detecting recombinant pB602L expressing in Sf-12 cells by IFA. But when they were used to stain ASFV infected PAM cell, strong florescence signals were detected only in the cells stained with B2H10 (Fig. 3). In the immunoelectron microscopy test, specific high electron density gold particles were observed in ASFV assembly factories of the cells which were labeled with B2A1 (Fig. 4A), B2H10 (Fig. 4B), and B2E12 (Fig. 4C), respectively, but not in the controls labeled with PBS instead of an antibody (Fig. 4D). Interestingly, we also found that few gold particles were localized on the surface of viral particles or at vesicular membranes. In the immunohistochemical test, very strong positive signs were observed in all the ASFV infected pig tissues including spleen, tosil, and gastrahepatic lymph node that were stained with each of the three mAbs, but not in the controls (Fig. 5), indicating all these mAbs can be used in immunohistochemical staining.

Discussion

ASF poses a great threat to the pig industry worldwide. Given there is no an effective vaccine available, the disease control relies mainly on rapid diagnosis, culling of infected pigs, and enhancing biosecurity measures on the threaten farms in those affected countries(Dixon et al., 2020). A better understanding of AFSV is

key to develop effective control measures. pB602L is an important non-structural protein of ASFV. To date, few studies focused this protein have showed that it plays an essential role during the viral particle assembly and can also be used to develop diagnosis tools(Epifano et al., 2006; C. Gallardo et al., 2009; Gutierrez-Castaneda et al., 2008). The most of the functions of this protein remains unknown. In the current study, we expressed a recombinant pB602L protein and it was then used to immunize mouse for screening mAbs. Eight mouse mAbs were produced and their recognized epitopes were mapped. To our knowledge, this is the first report on the mAbs and epitopes of pB602L and it provides new knowledge on the molecular basis of the antigenicity of this protein.

The mAbs against a viral protein are important biological materials for both basic and applied researches on the virus. Previous studies showed that the antibodies against pB602L were detectable as early as 10 d post infection in pig and ELISA assays developed based on recombinant pB602L proteins showed similar performances with that of the structural proteins p30 or p54, suggesting that pB602L has good antigenicity and is suitable for developing diagnostic tools(C. Gallardo et al., 2009; Gutierrez-Castaneda et al., 2008). However, the molecular basis for pB602L antigenicity remains largely unknown. Therefore, in this study, we developed firstly eight mouse anti-pB602L mAbs. All of them reacted well with recombinant pB602L. We then identified three epitopes ("^{366}ANRERYNY^{373}", "^{415}GPDAPGLSI^{423}" and "^{498}EMLNVPDD^{505}") recognized by these mAbs in pB602L. Multiple sequence alignment based on 69 pB602L amino acid sequences showed that all the three epitopes were completely conserved among these ASFVs, suggesting the antigenicity of pB602L probably comes from the conserved regions other than the CVR. This could partially explain, although the existence of CVR in B602L genes of different ASFV isolates (Mai et al., 2021), why a pB602L based ELISA assay worked well in detecting serum antibodies against different ASFV strains(C. Gallardo et al., 2009). Furthermore, it also indicates that a mAb targeting at any of these epitopes could possibly recognize the pB602L of all the currently identified ASFVs.

pB602L plays an essential role in a successful ASFV capsid assembly(Liu et al., 2019), which makes this protein one of the potential targets for anti-ASFV drug screening. However, the current knowledge on this protein is very limited, and much work needs to be done to understand its biological characteristics and the mechanism for functioning as a molecular chaperone of p72 protein. Visualization of the protein expression in ASFV infected cells is of great importance for such studies. To test the potentials of these mAbs for visualizing the pB602L protein in ASFV infected cells and tissues, three mAbs B2A1, B2H10, and B2E12 were used to detect pB602L in ASFV infected cells and tissues. The IFA assay revealed that B2H10, which recognized the Epitope 2, was good in detecting pB602L positive PAM cells, but which were failed by the other two mAbs. We repeated the IFA for three times and consensus results were obtained, indicating that only Group 2 mAbs are suitable for detecting pB602L in ASFV infected PAM by IFA. When these mAbs were employed to detect ASFV infected cells in different tissues that were collected from ASFV (Pig/HLJ/2018) infected pigs, all of them performed well with high specificity and efficacy, indicating that all of them are suitable for immunohistochemical staining. Immunoelectron micrographs provides very detailed information about the location of a target molecule in cellular departments and also commonly used for analyzing behaviors of a molecule. Therefore, we explored the potentials of these mAbs being used for gold-labeling immunoelectron microscopy. Since the high electron density particles were presented only in ASFV assembly factories, indicating that all the three mAbs detected pB602L specifically. Surprisingly, some gold particles presented on viral particles or the membrane of vesiculas. Given pB602L is a nonstructural protein of ASFV, therefore, these special distributions of the protein should be further studied in the future.

Conclusion

Taken together, the produced monoclonal antibodies in this study can be used as tools for developing ASFV detection tools and the identified epitopes in pB602L provide new knowledge for understanding the antigenicity of ASFVs.

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Conflict of Interest Statement

The authors declared that they have no conflict of interest.

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African swine fever (ASF) is widespread in Africa but is rarely introduced to other continents. In June 2007, ASF was confirmed in the Caucasus region of Georgia, and it has since spread to neighboring countries. DNA fragments amplified from the genome of the isolates from

domestic pigs in Georgia in 2007 were sequenced and compared with other ASF virus (ASFV) isolates to establish the genotype of the virus. Sequences were obtained from 4 genome regions, including part of the gene B646L that encodes the p72 capsid protein, the complete E183L and CP204L genes, which encode the p54 and p30 proteins and the variable region of the B602L gene. Analysis of these sequences indicated that the Georgia 2007 isolate is closely related to isolates belonging to genotype II, which is circulating in Mozambique, Madagascar, and Zambia. One possibility for the spread of disease to Georgia is that pigs were fed ASFV-contaminated pork brought in on ships and, subsequently, the disease was disseminated throughout the region.

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African swine fever is a devastating disease that can result in death in almost all infected pigs. The continuing spread of African swine fever from Africa to Europe and recently to the high-pig production countries of China and others in Southeast Asia threatens global pork production and food security. The African swine fever virus is an unusual complex DNA virus and is not related to other viruses. This has presented challenges for vaccine development, and currently none is available. The virus is extremely well adapted to replicate in its hosts in the sylvatic cycle in East and South Africa. Its spread to other regions, with different wildlife hosts, climatic conditions, and pig production systems, has revealed unexpected epidemiological scenarios and different challenges for control. Here we review the epidemiology of African swine fever in these different scenarios and methods used for control. We also discuss progress toward vaccine development and research priorities to better understand this complex disease and improve control.

Tables:

Table 1 Designed primers for amplifying linear palsmid pGEX-6P-1 and gene fragments encoding polypeptides (S1-S9)

Primers	Primer sequence	Target fragment length (bp)
pGEX-6P-1-F	GACTCGAGCGGCCGCAT	4965
pGEX-6P-1-R	TCCCAGGGGCCCCTG	
S1-F	CAGGGGCCCCTGGGAGCGGAGTTCAACATC	792
S1-R	ATGCGGCCGCTCGAGTCTTACGCACGCGGGTT	
S2-F	CAGGGGCCCCTGGGAAAAGAAGCGAAAACC	795
S2-R	ATGCGGCCGCTCGAGTCTTATTCTTTACCGATCTT	
S3-F	CAGGGGCCCCTGGGATTCAAGCCGATCCTG	798
S3-R	ATGCGGCCGCTCGAGTCTTACAGCTCCGCCTT	
S4-F	CAGGGGCCCCTGGGATTCAAGCCGATCCTG	201
S4-R	ATGCGGCCGCTCGAGTCTTACTGGTTGCTGCT	
S5-F	CAGGGGCCCCTGGGACTACGAGGAACTGCG	198
S5-R	ATGCGGCCGCTCGAGTCTTACTTGAAGCTGCCC	
S6-F	CAGGGGCCCCTGGGACAGAAAGTTGACGAG	195
S6-R	ATGCGGCCGCTCGAGTCTTATTCTTTACCGATCTT	
S7-F	CAGGGGCCCCTGGGAATCCTGCGTAACACCATC	198
S7-R	ATGCGGCCGCTCGAGTCTTAATCGTCGATGAA	
S8-F	CAGGGGCCCCTGGGACGGTATCAAGGGCCT	198
S8-R	ATGCGGCCGCTCGAGTCTTATTCCAGAGCGTTA	
S9-F	CAGGGGCCCCTGGGATGCGAGGAAAAGATC	201
S9-R	ATGCGGCCGCTCGAGTCTTACAGCTCCGCCTT	

Table 2 Summary of results for characterizing the mAbs

mAbs	Subtype	Activity	Activity	Activity
		ELISA+	IFA++	WB+
B2A1	IgG1	+	+	+
B2F1	IgG1	+	+	+
B2D10	IgG1	+	+	+
B2H10	IgG2a	+	+	+
B2B2	IgG2a	+	+	+
B2D8	IgG2a	+	+	+
B2A3	IgG2a	+	+	+
B2E12	IgG1	+	+	+

+: antigen expressed by E. coil

++: antigen expressed by baculovirus

Table 3 Polypeptides and their recognition by the mAbs

Polypeptide Fragment	Amino acid	Group 1 Group 2 Group 3	Group 1 Group 2 Group 3	Group 1 Group 2 Group 3
		B2A1	B2F1	B2D10
S1	2-256	-	-	-
S2	131-397	+	+	+
S3	266-531	+	+	+
S4	266-332	-	-	-
S5	300-365	-	-	-
S6	333-397	+	+	+
S7	399-464	-	-	-
S8	433-498	-	-	-
S9	465-531	-	-	-
S10	366-381	+	+	+
S11	374-389	-	-	-
S12	382-397	-	-	-
S13	398-414	-	-	-
S14	407-423	-	-	-
S15	415-432	-	-	-
S16	498-514	-	-	-
S17	506-522	-	-	-
S18	515-531	-	-	-

Table 4 Conservation analysis of the three epitops among 69 ASFVs

ASFV Strain (GenBank Accession)	Epitope 1								Epitope 2									Epitope 3								
	366	367	368	369	370	371	372	373	415	416	417	418	419	420	421	422	423	498	499	500	501	502	503	504	505
	A	N	R	E	R	Y	N	Y	G	P	D	A	P	G	L	S	I	E	M	L	N	V	P	D	D
ASFV Pig/HLJ/2018 (MK333180)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV BA71V (NC 001659)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV L60 (NC 044941)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV BA71 (NC 044942)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV NHV (NC 044943)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Ken05/Tk1 (NC 044945)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Ken06.Bus (NC 044946)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 26544/OG10 (NC 044947)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 47/Ss/2008 (NC 044955)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Benin 97/1 (NC 044956)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV OURT 88/3 (NC 044957)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV E75 (NC 044958)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Georgia 2007/1 (NC 044959)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Ken.rie1 (LR899131)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Germany 2020/1 (LR899193)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Kabardino-Balkaria 19/WB-964 (MT459800)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV NgheAn 2019 (MT180393)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Hanoi 2019 (MT166692)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Pol17 55892 C754 (MT847620)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Pol18 28298 O111 (MT847621)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Pol17 31177 O81 (MT847622)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Pol19 53050 C1959/19 (MT847623)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Georgia 2007/1 (FR682468)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV GZ201801 (MT496893)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV CN/2019/InnerMongolia-AES01 (MK940252)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 56/Ca/1978 (MN270969)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 57/Ca/1979 (MN270970)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 139/Nu/1981 (MN270971)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 140/Or/1985 (MN270972)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 85/Ca/1985 (MN270973)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 141/Nu/1990 (MN270974)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 142/Nu/1995 (MN270975)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 60/Nu/1997 (MN270976)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 26/Ss/2004 (MN270977)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 72407/Ss/2005 (MN270978)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 97/Ot/2012 (MN270979)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 22653/Ca/2014 (MN270980)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Zaire (MN630494)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV RSA W1 1999 (MN641876)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Wuhan 2019-1 (MN393476)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Wuhan 2019-2 (MN393477)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Liv13/33 (OmLF2) (MN913970)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV pig/China/CAS19-01/2019 (MN172368)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV HU 2018 (MN715134)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV LT14/1490 (MK628478)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Moldova 2017/1 (LR722599)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV CzechRepublic 2017/1 (LR722600)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Belgium/Etalle/wb/2018 (MK543947)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV wbBS01 (MK645909)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Belgium 2018/1 (LR536725)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV DB/LN/2018 (MK333181)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 2018/AnhuiXCGQ (MK128995)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Georgia 2008/1 (MH910495)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV R8 (MH025916)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV R7 (MH025917)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV R25 (MH025918)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV N10 (MH025919)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV R35 (MH025920)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Estonia 2014 (LS478113)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 26544/OG10 (KM102979)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV 47/Ss/2008 (KX354450)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV OURT 88/3 (AM712240)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV E75 (FN557520)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Ken05/Tk1 (KM111294)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Ken06.Bus (KM111295)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV L60 (KM262844)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV NHV (KM262845)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV BA71V (U18466)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ASFV Benin 97/1 (AM712239)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Fig. 1 Schematic map of the reactions between the polypeptides and the mAbs. (A) Full-length pB602L protein of ASFV Pig/HLJ/2018; (B) Ploypetide fragments (S1-S18) from different regions of pB602L expressed in E.coli and the position of three epitopes; (C) mAbs of the three epitopes.

Fig. 2 Expression and purification of pB602L. (A) SDS-PAGE analysis of pB602L protein expressed in *E. coli* . M: Protein molecular weight marker; Lane 1: Empty pGEX-6p-1 transfected cells; Lane 2: Non-induced cells; Lane 3: IPTG induced cells; Lane 4: Purified pB602L with GST tag removed. (B) pB602L identified by an anti-GST antibody. M: Protein molecular weight marker; Lane 1: Empty pGEX-6p-1 transfected cells; Lane 2: Non-induced cells; Lane 3: IPTG induced cells. (C) pB602L protein identified by an anti-ASFV serum. M: Protein molecular weight marker; Lane 1: Empty pGEX-6p-1 transfected cells; Lane 2: Non-induced cells; Lane 3: IPTG induced cells; Lane 4: Purified pB602L with GST tag removed.

Fig. 3 Immunofluorescent assay detecting pB602L in ASFV-Pig/HLJ/2018 infected PAM cells. Primary PAMs were plated on 24-well plates and infected with ASFV at an MOI of 0.2. At 24 h postinfection, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized in 0.1% (w/v) Triton-100 for 10 min at room temperature. B2E12, B2H10 and B2E12 are mAbs as primary antibodies. Cells stained with mouse anti-ASFV serum as positive controls.

Fig. 4. Localization of pB602L protein with monoclonal antibodies against different epitopes in PAM cells infected with ASFV (Pig/HLJ/2018). Primary PAMs were plated on 6-well plates and infected with ASFV at an MOI of 0.2. At 24 h postinfection, cells were fixed in 4% paraformaldehyde for 10 min at room temperature . pB602L protein was detected by mAbs B2A1(A), B2H10(B), or B2E12(C) and visualized with a gold-labeled goat-anti-mouse IgG in an electron microscopy. The control (D) was stained with PBS instead of a mAb. White arrows point to gold particles indicating the locations of pB602L in a ASFV assembly factory. Scale bars represent 100 nm.

Fig. 5. Immunohistochemical detection of ASFV pB602L protein in tissues of pigs. B2A1, B2H10, and B2E12 are monoclonal antibodies against different epitopes of pB602L protein. The spleens, tonsils, and gastrohepatic lymph nodes were collected from mock or ASFV (Pig/HLJ/2018) infected SPF pigs.