Virus Research 269 (2019) 197632

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Development and characterization of monoclonal antibodies against p30 protein of African swine fever virus

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ARTICLE INFO

Keywords:
Monoclonal antibodies
African swine fever virus
p30 protein
Diagnostic assays

ABSTRACT

Among the structural proteins that compose the virion of African swine fever virus (ASFV), p30 is one of the most immunogenic proteins and is produced during early stage of ASFV infection. These two characteristics make p30 a good target for diagnostic assays to detect ASFV infection. In this study, we describe a panel of newly generated p30-specific monoclonal antibodies (mAbs). The reactivity of these mAbs was confirmed by immunoprecipitation and Western blot analysis in Vero cells infected with alphavirus replicon particles that express p30 (RP-p30). Furthermore, this panel of mAbs recognized ASFV strains BA71 V (Genotype I) and Georgia/2007 (Genotype II) in immunofluorescence assays on virus-infected Vero cells and swine macrophages, respectively. These mAbs also detected p30 expression by immunohistochemistry in tissue samples from ASFV-infected pigs. Epitope mapping revealed that a selected mAb from the panel recognized a linear epitope within the 32-amino acid region, 61–93. In contrast, two of the mAbs recognize the C-terminal region of the protein, which is highly hydrophilic, enriched in glutamic acid residues, and predicted to contain an intrinsically disordered protein region (IDPR). This panel of mAbs and mAb-based diagnostic assays potentially represent valuable tools for ASFV detection, surveillance and disease control.

1. Introduction

African swine fever virus (ASFV) is a causal agent of lethal hemorrhagic fever in domestic pigs characterized by high mortality and morbidity. The virus is transmitted through contact with infected animals and through contaminated feed or fomites. ASFV is also transmitted through the soft ticks of genus *Ornithodoros* (Sánchez-Vizcaíno and Neira, 2012). ASFV originated in Africa, where warthogs and bushpigs are reservoirs of the virus (Montgomery, 1921; Oura et al., 1998). The virus initially spread from West Africa to Europe in the mid-20th century. While ASFV was eradicated from most of Europe during the mid-1990s, the virus remains endemic on the island of Sardinia, Italy (Martinez-Lopez et al., 2015). Since 2007, ASFV again spread out of Africa to the Caucasus and subsequently to Eastern Europe, resulting in outbreaks in the Russian Federation and in neighboring countries, including Belarus, Ukraine, Lithuania, Estonia, Poland, Latvia, Czech Republic, Romania and Hungary. Recently, ASFV outbreaks have occurred in major swine-producing countries in Europe, China and Mongolia. In China, up to 1 million pigs were culled, following the confirmation of the first case in Shenyang province on August 3, 2018 (Ge et al., 2018). Therefore, ASFV poses the most significant threat to the global swine industry.

ASFV is a large double-stranded DNA virus belonging to the family *Asfarviridae, genus Asfivirus* (Dixon et al., 2013). The virus is enveloped, possessing inner and outer lipid membranes surrounding an icosahedral viral capsid (Alejo et al., 2018). The virion is composed of more than 50 polypeptides (Alejo et al., 2018; Esteves et al., 1986). The viral phosphoprotein p30, encoded by the CP204L gene, is located in the inner membrane of the viral envelope. The protein is produced before the initiation of viral DNA synthesis and protein expression persists until the end of the viral life cycle (Afonso et al., 1992; Prados et al., 1993).

P30 protein is immunogenic and stimulates the highest level of viral antibody response during ASFV infection (Giménez-Lirola et al., 2016). Antibodies against p30 are detected as early as 8 days post-infection in pigs (Giménez-Lirola et al., 2016; Gomez-Puertas et al., 1998, 1996). Moreover, a recombinant fusion protein of p30 and the ASFV structural

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https://doi.org/10.1016/j.virusres.2019.05.010
Received 25 March 2019; Received in revised form 22 May 2019; Accepted 22 May 2019
Available online 23 May 2019
0168-1702/

protein p54 induces neutralizing antibodies (Barderas et al., 2001). These properties make p30 an important antigen for serological detection of infection and for incorporation into vaccines (Barderas et al., 2000; Oviedo et al., 1997).

Since no vaccine or other treatments for ASF are available, highly sensitive and specific diagnostic tests are needed for early virus detection, control of viruses in infected herds, and for surveillance to demonstrate freedom from disease. For this study, we have developed and characterized a panel of monoclonal antibodies (mAbs) against p30, which can be applied for the detection of ASFV-specific antigens and antibodies.

2. Materials and methods

2.1. Production of recombinant p30 in Escherichia coli

The ASFV p30 (631 bp) gene sequence from strain BA71 V (GenBank Accession # U18466.1) was used for the preparation of p30 recombinant protein fragments. The corresponding nucleotide sequences were codon optimized for expression in *Escherichia coli (E. coli)* and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). His-tagged full-length or truncated p30 constructs were cloned into the pHUE vector and recombinant proteins were expressed in *E. coli* as described previously (Catanzariti et al., 2004; Heimerman et al., 2018). Recombinant proteins were purified with PrepEase His-Tagged Protein Purification Kit (USB) following the manufacturer's instructions. Specificity of the recombinant protein was verified by western blotting using an anti-His-Tag mAb (Clone J099B12; Biolegend, San Diego, CA).

2.2. Expression of p30 fragments in eukaryotic expression system

ASFV p30 was codon-optimized for expression in mammalian cells and the sequence was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). To map p30 epitopes recognized by monoclonal antibodies, p30 gene fragments (Fig. 5C) were cloned into the pEGFP-C3 vector (Clontech Laboratories, Mountain View, CA). These p30 fragments were designed based on *in silico* B-cell linear epitope predictions [Fig. 5C; (Larsen et al., 2006)].

2.3. Monoclonal antibody production

Monoclonal antibodies against p30 were produced as previously described (Fang et al., 2006; Li et al., 2012). Briefly, 6–8 weeks old BALB/C mice were immunized with 50–100 µg/mouse of purified p30 protein mixed with an equal volume of incomplete Freund's adjuvant. Mice were immunized intraperitoneally three times with two weeks between each immunization. The mice were euthanized three days after the final immunization, after which splenocytes were collected and fused with NS1 myeloma cells. After fusion, cells were cultured in 24-well plates in HAT selection media (Cat# 21060-017, Gibco, Life Technologies, Thermo Fisher Scientific Inc., Pittsburgh, PA). Culture supernatants were initially screened for p30-specific antibodies by immunofluorescence assay (IFA) on Vero cells infected with a defective alphavirus replicon particle that expresses p30 from strain BA71 V (RP-30). Hybridoma clones that produced p30-specific antibodies were subcloned into single cell clones (monoclones). These were further screened by IFA on RP-30-infected Vero cells. In addition, p30 mAb-positive hybridoma clones were confirmed by IFA using ASFV-infected Vero and swine macrophages (see below).

2.4. Immunofluorescence assay (IFA)

IFA tests on RP-30-infected cells were carried out using the modified method as we described previously (Murgia et al., 2019). Vero cells at 70-80% confluence in 96-well plates were infected with RP-30 (1.89×10^9 RP/ml) at 1:400 dilution. At 24 h post infection (hpi), cells were fixed with ice-cold methanol for 30 min at −20 °C. Cell monolayers were incubated with undiluted hybridoma culture supernatants (100 µl/well) overnight at 4 °C. Alexa-Fluor-488 goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) diluted 1:400 in PBS was used as a secondary antibody for detection. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA). Plates were monitored using an EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA).

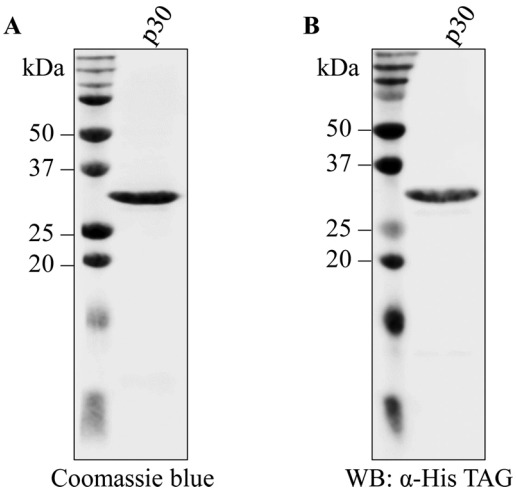

Fig. 1. ASFV p30 antigen production. (**A**) SDS-PAGE of His-tagged p30 recombinant protein preparation, followed by Coomassie blue staining. (**B**) Western blot analysis using anti-His-tag mAb. In both panels A and B, the left lane shows the molecular weight marker, while the right lane shows p30 protein.

IFA tests on ASFV-infected cells were conducted on Vero cells infected with ASFV strain BA71 V (200 $TCID_{50}$/ml) and on porcine alveolar macrophages infected with ASFV strain Georgia/07 (500 $TCID_{50}$/ml). At 48 hpi, cell monolayers were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized by incubation with 0.1% Triton X-100 for 5 min and incubated with anti-p30 mAb followed by incubation with Alexa-Fluor-488 conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA). Nuclei were stained with DAPI and the plates were examined using the EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA).

For epitope mapping, IFA test was performed using Vero cells transfected with pEGFP-C3 constructs (Fig. 5D) containing p30 gene fragments. At 24 h post transfection (hpt), cell monolayers were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After permeabilization with 0.1% Triton X-100 for 5 min, cells were incubated with anti-p30 mAb followed by incubation with Alexa-Fluor-594 conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA). Nuclear staining with DAPI was performed. The plate was checked under EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA).

2.5. Western blot analysis

RP-30-infected Vero cells were harvested at 36 hpi with Pierce IP Lysis Buffer (Thermo Fisher Scientific, Waltham, MA) containing Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris was removed by centrifugation at 15,000*g* for 15 min at 4 °C. Cell lysates were mixed with Laemmli sample buffer (4X) and boiled at 95 °C for 5 min. Following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto a nitrocellulose membrane. The membrane was blocked overnight with 5% skim milk at 4 °C and incubated with anti-p30 mAb at room temperature for 1 h. After washing with 1xPBS (containing 0.05%

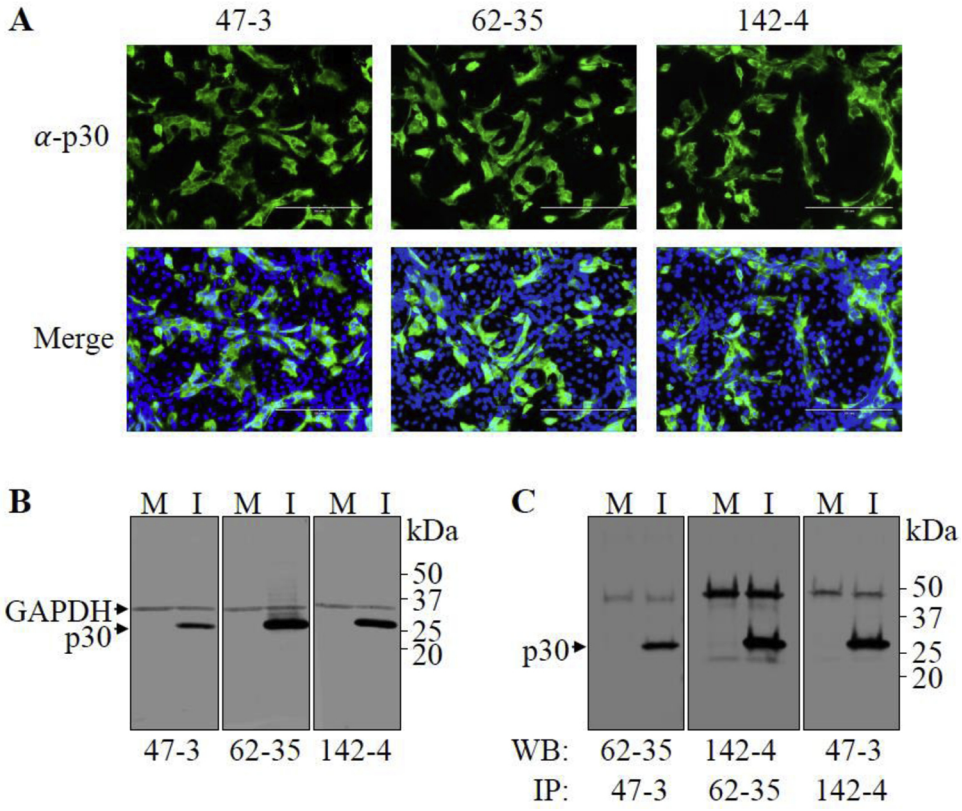

Fig. 2. Detection of p30 expression in Vero cells infected with recombinant alphavirus replicon particles expressing p30 (RP30). (**A**) Immunofluorescent detection of p30 expression. RP30 infected cells were fixed at 36 h post infection. Cells were incubated with p30-specific mAbs listed on the top of each panel and stained with Alexa-Fluor-488 conjugated goat anti-mouse IgG (green). Cells were counterstained with DAPI (blue). (**B**) Western blot detection of p30 expression. RP30 infected cells were harvested at 36 h post infection, and western blot analysis was performed using the p30-specific mAbs indicated below each panel. (**C**) Immunoprecipitation analysis of RP-30 infected cell lysate with p30-specific mAbs. For each panel, two mAbs were used. IP: mAb used to precipitate the proteins from cell lysate; WB: mAb used to detect the p30 protein in the membrane after immunoprecipitation. In both panels 2B and 2C, the size of the protein is labeled on the right side of the panel. M: Mock-infected cell lysate; I: RP30-infected cell lysate.

Table 1
Summary of anti-p30 mAbs generated in this study.

mAb	Isotype	IFA	IHC	IP/WB	ELISA	Epitope region
47-3	IgG1 lambda	+	+	+	+	aa 61-93
62-35	IgG1 lambda	+	+/-	+	+	aa 120-204
142-4	IgG1 lambda	+	+/-	+	+	aa 120-204

TWEEN 20; PBST), the membrane was further incubated with IRDye® 800CW Goat anti-Mouse IgG (H + L) secondary antibody (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature. After washing with PBST, the blots were imaged using a digital image system (Odyssey infrared imaging system; LI-COR Biosciences, Lincoln, NE). Expression of the housekeeping gene GAPDH was detected as a loading control.

2.6. Immunoprecipitation assay

Immunoprecipitation assays were performed on lysates of Vero cells infected with RP30. At 36 hpi, cell lysates were harvested with IP lysis/wash buffer supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris was removed by centrifugation at 12,000*g* for 15 min at 4 °C. Cell lysates were immunoprecipitated with anti-p30 mAb using the Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Expression of p30 in RP-30-infected cells was detected by western blot analysis with a second mAb against p30.

2.7. Immunohistochemistry on ASFV-infected tissues

Tissues from pigs infected with ASFV strain Georgia 2007 were fixed in formalin, embedded in paraffin, and sectioned at 4 µm onto positively charged slides. Slides were stained using a Leica Bond-Max autostainer with the Polymer Refine Red Detection kit (Cat# DS9390, Leica Biosystems Inc., IL), and retrieved with Proteinase K for 10 min at room temperature. Anti-p30 monoclonal antibodies (47-3, 62-35, and 142-4), diluted 1:100 in Bond Primary Antibody Diluent (Leica Biosystems, Tris-buffered saline), were added to the slides and incubated for 15 min at room temperature. Polymerization was performed with Polymer-AP α-Rabbit (Leica Biosystems Inc.) for 25 min at room temperature. The reaction was visualized using Fast Red chromogen and slides were counterstained with hematoxylin. Slides were imaged at 10 X magnification using a Nikon Eclipse 501 microscope (Nikon Corporation, Japan). The slides were also counterstained with hematoxylin and eosin (HE).

2.8. Indirect ELISA

Purified recombinant p30 protein constructs (full-length or fragments) were coated on flat bottom polystyrene plates (4 ug/ml; 100ul/well) in carbonated coating buffer (pH 9.6). Each plate was incubated for 1 h at 37 °C followed by three washes with PBST (1xPBS containing 0.05% Tween 20). The plate was then blocked for 1 h at 37 °C with 10% goat serum in PBS (PBS-GS). After washing with PBST, 100 µl of anti-p30 mAb was added to each well and incubated for 1 h at 37 °C, followed by 1 h incubation at 37 °C with HRP-conjugated goat anti-mouse IgG (ICN Biomedical) diluted at 1:2000 in PBS-GS. The HRP activity was measured using an ABTS 1-Component Microwell Peroxidase Substrate kit (KPL). The reaction was stopped by adding 1% SDS and the absorbance was measured at 405–650 nm. An unrelated monoclonal antibody was used as negative background control.

3. Results

3.1. Generation of monoclonal antibodies against ASFV p30

To produce p30 antigen for mouse immunization, a synthetic gene corresponding to the CP204 L gene sequence of ASFV strain BA71 V was cloned and expressed as a His-tagged recombinant protein in *E. coli*. On SDS-PAGE gel, the purified His-tagged p30 migrated at the apparent

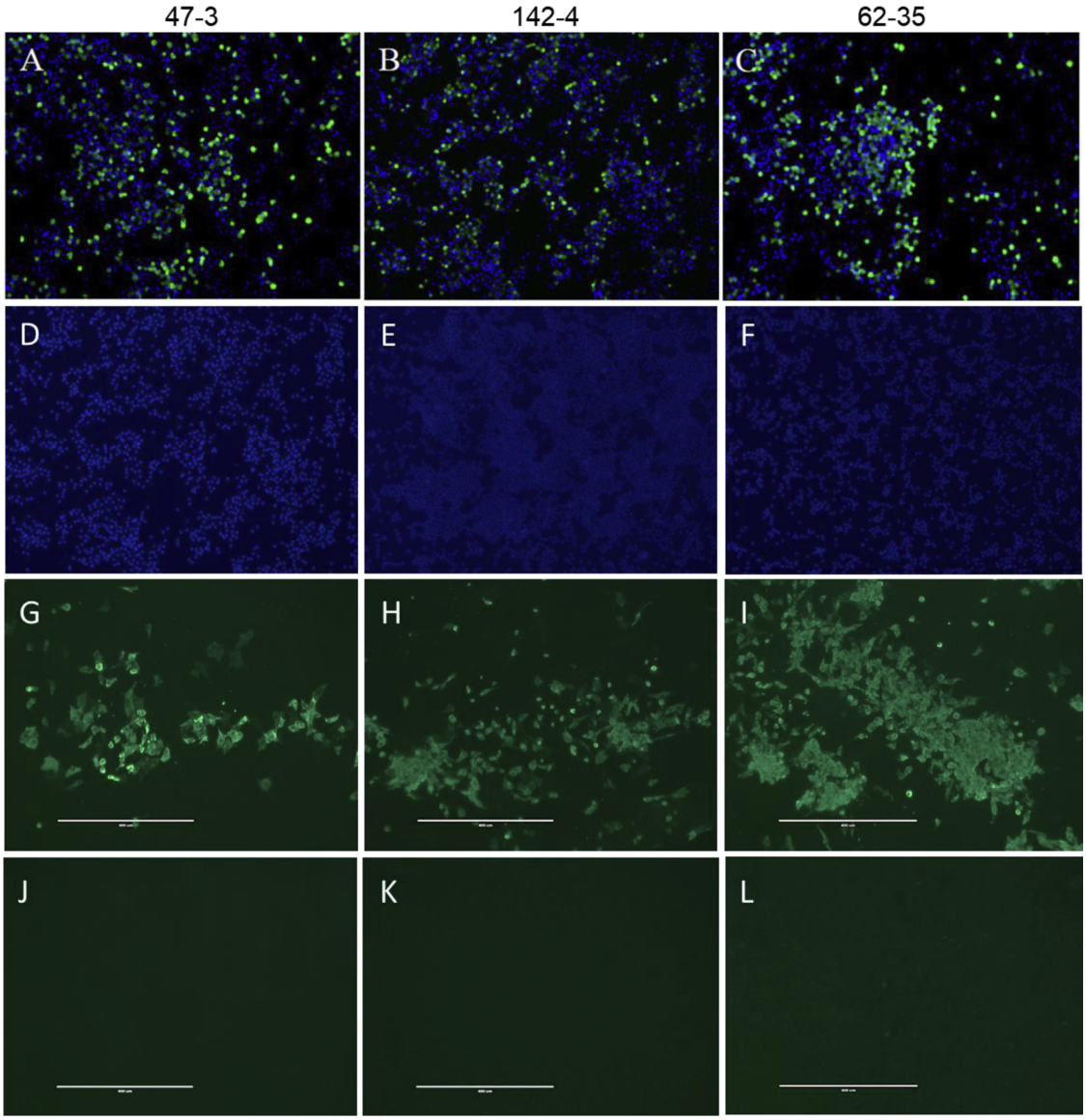

Fig. 3. Immunofluorescent assay detection of ASFV in virus-infected cells. (**A-C**) porcine alveolar macrophages (PAMs) were infected with ASFV strain Georgia/2007. (**D-F**) Mock-infected PAMs. Cells were fixed and incubated with an anti-p30 mAb as indicated and stained with Alexa-Fluor-488 conjugated goat anti-mouse IgG (green). Cell nuclei were counterstained with DAPI (blue). (**G-I**) Vero cells were infected with ASFV strain BA71 V. (**J-L**) Mock-infected Vero cells. Cells were fixed and stained with an anti-p30 mAb as the primary antibody and Alexa-Fluor-488 conjugated goat anti-mouse IgG as the secondary antibody.

molecular mass around 30 kDa, which is larger than its predicted size of 23.3 kDa (Fig. 1A). This observation is consistent with the finding of a previous study (Prados et al., 1993). The identity of the protein was confirmed by Western blot analysis with anti-His-Tag antibody (Fig. 1B).

To generate anti-p30 mAbs, mice were immunized with recombinant p30 protein. After fusion process, supernatants from the resulting hybridoma cells were screened by IFA using Vero cells infected with RP-30 (Fig. 2A). Initial screening yielded three primary hybridoma clones, which were subcloned to obtain 31 monoclones. One mAb from each primary clone, mAb #47-3, #62-35, and #142-4, was selected for further characterization. Isotype analysis showed that each of the three mAbs consisted of IgG1 heavy chains combined with lambda light chains (Table 1).

This panel of mAbs was further tested by western blot analysis and immunoprecipitation using cell lysates from RP-30-infected Vero cells. In western blots, all three mAbs specifically detected a protein band above the 25 kDa protein marker, which is consistent with results generated from a previous study of p30 protein (Prados et al., 1993). As expected, this band was not detected in mock-infected cells (Fig. 2B). The mAbs also detected p30 protein in immunoprecipitated proteins from RP-30 infected Vero cell lysates (Fig. 2C).

3.2. Reactivity of anti-p30 monoclonal antibodies in ASFV-infected cells and tissues

We further confirmed the reactivity of this panel of mAbs in ASFV-infected cells. IFA was performed on porcine alveolar macrophages (PAMs) infected with ASFV strain Georgia/2007. As shown in Fig. 3, all three mAbs, 47-3, 62-35, and 142-4, recognized ASFV-infected PAMs (Fig. 3A–C). We further confirmed this result in Vero cells infected with the BA71 V strain (Fig. 3G–I). All three mAbs strongly labeled BA71V-infected Vero cells, while no fluorescent signal was detected for uninfected cells (Fig. 3D–F; J–L). Together, the results show that these mAbs recognize ASFV in Georgia/2007 and BA71V- infected cells.

Next, we tested the anti-p30 mAbs on thin sections of paraffin-embedded tissues from pigs experimentally infected with ASFV Georgia/2007 (Popescu et al., 2017). Representative IHC results are shown in

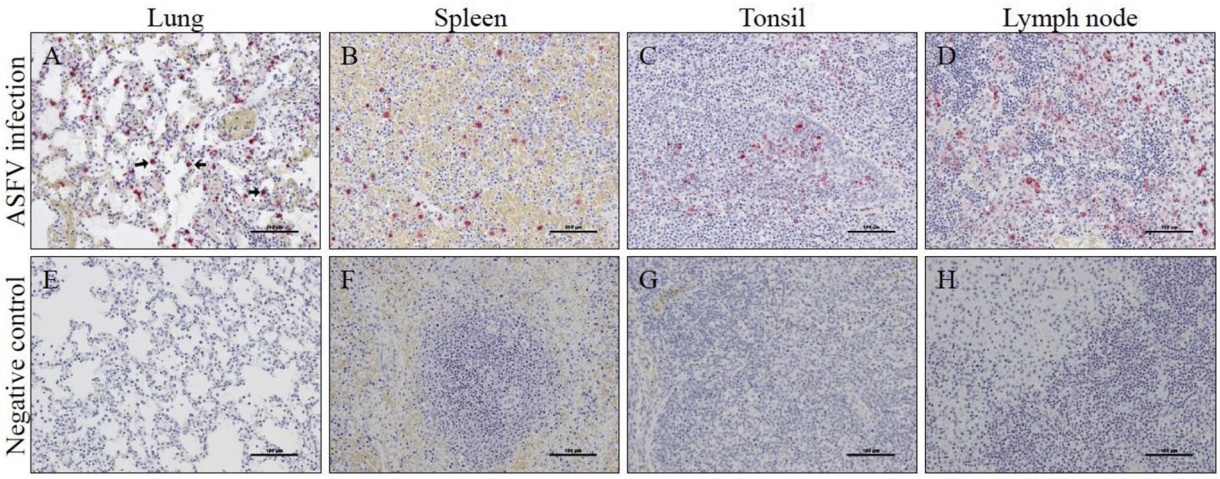

Fig. 4. Immunohistochemical analysis of tissue samples from ASFV-infected pig. (**A-D**) Tissue samples from pigs infected with ASFV strain Georgia/2007. (**E-H**) Tissue samples from negative control pig. Immunohistochemistry (IHC) was performed using thin section of paraffin-embedded tissues, including lung (**A, E**), spleen (**B, F**), tonsil (**C, G**) and lymph node (**D, H**). Tissue sections were incubated with primary mAb 47-3 and polymerization was performed with Polymer-AP α-mouse. Colors were developed using Fast Red chromogen and slides were counterstained with hematoxylin. Bar = 100 μm.

Fig. 4. The mAb 47-3 showed strong reactivity for the detection of ASFV in lung, tonsil, spleen and lymph nodes from infected animals, while tissues from mock-infected pigs were negative. The other mAbs, 62-35 and 142-4, showed relatively weak staining in the same tissues from ASFV-infected pigs. Since BA71 V does not infect pigs, reactivity against BA71 V was not tested in paraffin-embedded tissues.

3.3. Epitope mapping of anti-p30 mAbs

To map the epitope of each mAb, we used the antibodies for western blotting to investigate their abilities to react with four large p30 fragments expressed in *E. coli* (Fig. 5A), which included the full-length 204 amino acids (aa) of p30 protein, and three overlapping fragments, p30(1-100 aa), p30(101-204 aa) and p30(50-150 aa). All three antibodies recognized the full-length recombinant protein (Fig. 5B). The mAb 47-3, recognized p30(1-100 aa) and p30(50-150 aa); whereas mAbs, 62-35 and 142-4, recognized p30(101-204 aa). To refine epitope mapping of mAb 47-3, we divided the N-terminal polypeptide into p30(24-60 aa), p30(40-80 aa) and p30(61-100 aa). Mab 47-3 only recognized p30(61-100 aa).

Within the C-terminal end, four overlapping polypeptides, p30(91-130 aa), p30(111-160 aa), p30(143-182 aa), and p30(161-204 aa) were constructed. Interestingly, mAbs 62-35 and 142-4 recognized none of these fragments in Western blot. The experiment was repeated using polypeptides immobilized on ELISA plates. The results were consistent with data from the Western blots (data not shown). Next, we tested the possibility that the mAbs may recognize larger conformational epitopes or native epitopes possessing a post-translational modification. As shown in Fig. 5C, we designed p30 fragments from the BA17 V based on *in silico* predicted B-cell linear epitopes obtained using BepiPred (Larsen et al., 2006). Each truncated p30 peptide was constructed as an EGFP-tagged fusion protein and expressed in Vero cells (Fig. 5D). IFA results showed that mAb 47-3 recognized an epitope located within p30 residues 61-93 of the protein, which is consistent with the results obtained in the Western blots. The mAbs 62-35 and 142-4 only recognized the relatively large p30(120-204 aa) fragment (see Fig. 5D–E). The IFA result suggests that p30(120-204 aa) region contains a conformational epitope, but it is also recognized by mAbs when present in a denatured form in Western blot. To reconcile these data, we analyzed the p30 protein sequence using the GeneSilico MetaDisorder server, which is used to predict protein regions possessing intrinsic disorder. As shown in Fig. 6, the computer program predicted a region covering the peptide sequence between aa 91-143 as disordered or flexible, with only modest secondary structure. This region is highly enriched in glutamic acid residues, serines and other polar or charged residues characteristic of loops and disordered regions. However, the region contains only 3 aliphatic residues over a 45 residue stretch (92-136 aa), suggesting that it may be labile and highly surface-exposed overall.

To determine whether these epitope regions are conserved among different genotypes of ASFV, we analyzed p30 sequences from 19 genotypes (Fig. 7). The amino acid alignment identified a total of 5 aa differences in the mAb 47-3 epitope region. One single aa difference, His_{67} to Arg_{67} between Georgia/07 and BA71 V, did not impair the binding of the mAb to its targeting epitope. It is likely that other ASFV strains with an Arg at position 67 (belonging to genotypes III, IV, V, VI, VII, XIX, XX, XXI) are recognized by mAb 47-3. On the other hand, mAbs 62-35 and 142-4 recognized a larger region located between aa 120-204. A number of strain-dependent sequence differences are scattered throughout this region. ASFV strains in genotype III, IV, VII, XIX, XX and XXI has the same change in aa 139 (E to V) as that in Georgia/07; this residue change did not affect mAb recognition of this region. Whether the other aa changes in genotypes III-XXI affect the binding of the mAbs needs to be further analyzed.

4. Discussion

Monoclonal antibodies (mAb) are key reagents for diagnostic detection of viral infection. In this study, we produced a panel of mAbs against the p30 protein of ASFV. We selected three mAbs (47-3, 62-35, 142-4) for further characterization and assay development. These mAbs recognized the p30 protein in IFA, ELISA, Western blot and immunoprecipitation assays. We determined that the mAbs recognize two distinct ASFV strains that we tested in infected cells, including viruses of genotype I (BA71 V) and genotype II (Georgia/2007). IHC assays on Georgia/ 2007-infected tissues demonstrated that mAb 47-3 is a suitable reagent for the detection of ASFV antigen in formalin-fixed tissues. The mAb detected virus in all tested tissues (lung, tonsil, spleen, lymph nodes) at 4 days post infection, remarkably even before the appearance of lesions. This is consistent with a previous study, which showed that p30 antigen can be detected in tonsils at 5 days post-infection (Dixon et al., 2017).

Our preliminary epitope mapping determined that mAb 47-3 recognizes an epitope located between aa 61 to 93 of p30. This region is partially conserved with only a single amino acid difference among at least 10 genotypes, suggesting that the mAb 47-3 should be able to detect at least 10 genotypes of ASFV. Interestingly, the region that

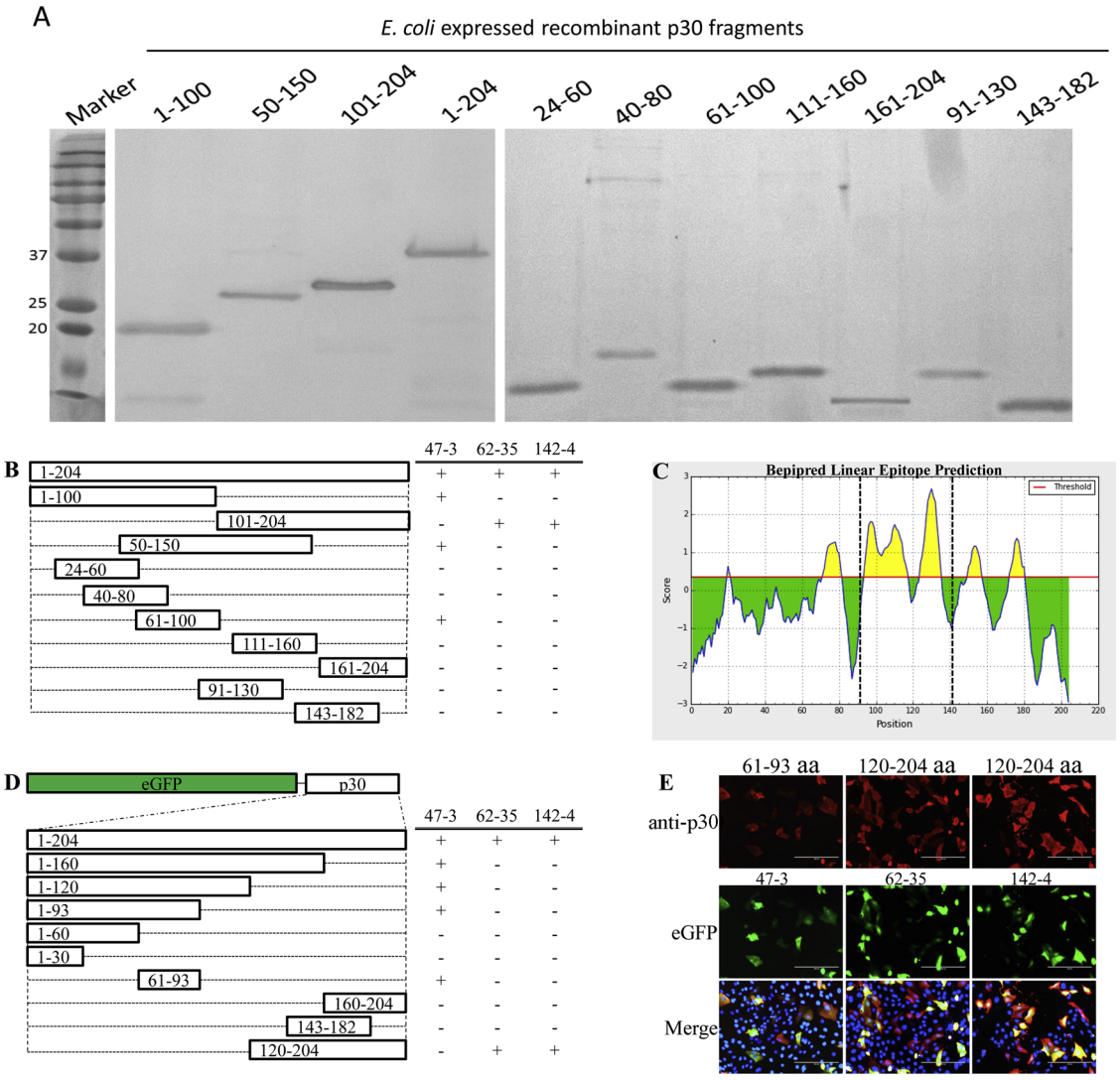

Fig. 5. Epitope mapping of p30 monoclonal antibodies. (A) SDS-PAGE detection of purified p30 fragments expressed in E. coli. **(B)** A schematic diagram of *E. coli* expressed constructs used as antigens in western blot analysis for epitope mapping. **(C)** Antigenic regions of p30 were predicted by *in silico* analysis of Bepipred Linear Epitope Prediction. The immunogenic regions were highlighted with yellow color and the region predicted to contain an intrinsically disordered region is marked with dashed vertical bars. **(D)** A schematic diagram of constructs expressing EGFP-p30 fusion proteins and a summary of epitope mapping by IFA. White bars denote truncated p30, while dashed lines denote truncation regions. **(E)** P30 fragments recognized by mAbs (47-3, 62-35, and 142-4) determined by IFA. The EGFP-p30 fusion proteins detected by mAbs and Alexa-Fluor-594 conjugated goat anti-mouse IgG (red fluorescence) in Vero cells, and green fluorescence signal (EGFP) indicated the expression of fusion proteins.

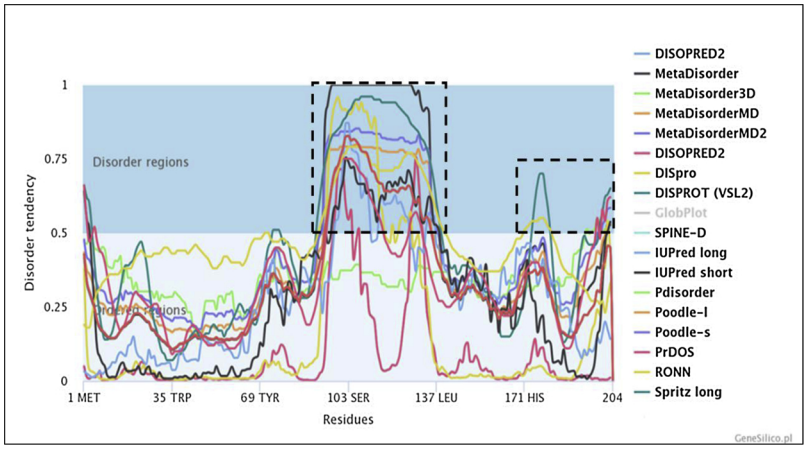

Fig. 6. Predicted disorder profile of p30 from the BA71 V strain. Sequence-based disorder predictions were carried out using the GeneSilico MetaDisorder server. The x-axis shows the residues from 1 to 204, and the y-axis shows the disordered tendency ranging from 0 to 1. All residues with a disorder probability of more than 0.5 were considered to be disordered.

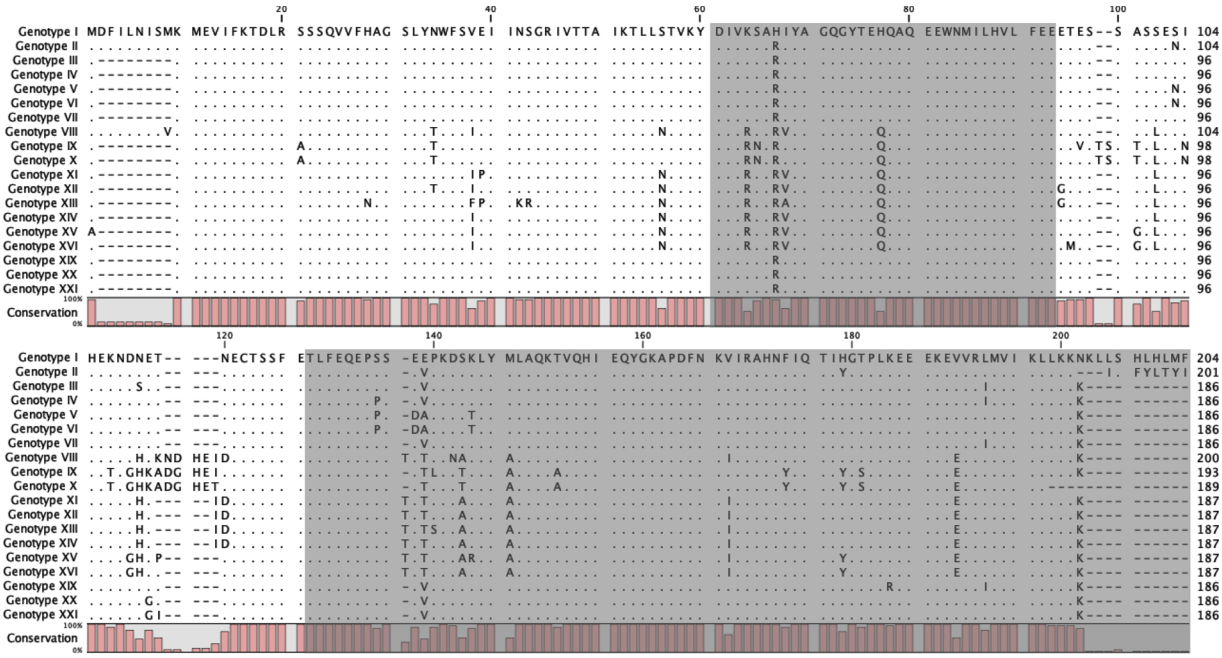

Fig. 7. Sequence alignment of p30 proteins of 19 ASFV genotypes. Sequence alignment was performed with CLC Genomics Workbench 11 (QIAGEN, Hilden, Germany). Matching residues are denoted with a dot, whereas gap regions are denoted with dash line. The numbers on the top indicate position on p30 of Genotype I, and the coordinate of amino acid in the alignment is specified on the right terminus for each sequence. Epitope regions of amino acid 61~93 recognized by monoclonal antibody 47-3 and amino acid 120~204 recognized by monoclonal antibodies 62-35 and 142-4 are highlighted with grey color. The GenBank accession numbers of 19 ASFV p30 protein sequences are: Genotype I (BA71 V; U18466.2), Genotype II (Georgia 2007/1; FR682468.1), Genotype III (SPEC/257; EU874265.2), Genotype IV (RSA/04/3; EU874308.1), Genotype V (MOZ/1960; EU874309.1), Genotype VI (SPEC/265; EU874264.2), Genotype VII (SPEC/154; EU874291.2), Genotype VIII (PHW/88/1; EU874257.2), Genotype IX (KEN/05/1; EU874301.1), Genotype X (ken09Tk.13/1; HM745382.1), Genotype XI (KAB/62; EU874289.1), Genotype XII (MZI/92/1; EU874288.2), Genotype XIII (SUM/1411; EU874287.1), Genotype XIV (NYA1/2; EU874302.1), Genotype XV (TAN/01/1; EU874303.2), Genotype XVI (TAN/03/1; EU874304.1), Genotype XIX (RSA/96/2; EU874281.2), Genotype XX (RSA/95/4; EU874295.1), and Genotype XXI (RSA/96/1/P; JQ745031.1).

contains aa 61-93 was also recognized by a serum antibody generated from ASFV-infected pigs (Murgia et al., 2019). This recent study used a vaccination strategy incorporating priming with a vector-expressed antigen, followed by boosting with an attenuated live virus. The major effect of the prime boost was to enhance recognition of an epitope within the sequence aa 61–110, suggesting this region contains an epitope with important immunological function (Murgia et al., 2019).

In contrast, mAbs 62-35 and 142-4 only recognized a large polypeptide fragment in the C-terminal half of p30 (120-204 aa). This fragment overlaps with a region (91-143 aa) that has a high predicted propensity to be flexible and potentially intrinsically disordered (IDP).

A similar phenotype was observed in the nucleocapsid of porcine epidemic diarrhea virus (PEDV), in which an epitope targeted by a mAb is located within a relatively large intrinsically disordered region (Wang et al., 2016). In SDS-PAGE analysis, the full-length ASFV p30 and the fragment covering amino acids 50-150 migrated aberrantly, at a higher molecular weight than the predicted size (Fig. 5A). The Ebola virus nucleoprotein (NP) was previously observed to exhibit similarly aberrant migration; significantly, the C-terminus of NP contains a highly acidic region that is predicted to be disordered. In SDS-PAGE analysis, NP migrated with an apparent molecular mass larger than expected based on the predicted size of the protein (Shi et al., 2008).

Another property potentially related to intrinsic disorder is the highly immunogenic nature of the C-terminal region, which could be due to the exposure of acidic residues. A recent animal study revealed that the C-terminal part of p30 is immunodominant; the region that conferred immune recognition in ASFV-infected pigs was determined to be predominantly located between aa 111-130 (Murgia et al., 2019). An interesting observation in our study is that a higher degree of variability exists between different ASFV isolates within this potentially poorly structured but highly antigenic region of p30 (Perez-Filgueira et al., 2006). The variability does not affect the prediction of disordered regions for isolates belonging to both genotypes I and II. However, the implications of disordered or unstructured regions for the function of ASFV p30 protein need to be further studied. In particular, identification of the specific mAb binding site would allow for development of genotype-specific diagnostic assays, as well as assays applicable to genetically diversified field strains.

In summary, we generated a panel of anti-p30 mAbs. These mAbs were characterized on three important properties: (i) reactivity on ASFV-infected cells with BA71 V and Georgia/2007 strains; (ii) immunohistochemical analysis in tissues from pigs infected with Georgia/2007 strain; and (iii) specific epitope region recognized by a specific mAb. This panel of mAbs and mAb-based diagnostic assays would be important tools to aid in ASF disease control and prevention.

Acknowledgements

This project was funded by the Kansas National Bio and Agro-Defense Facility Transition Fund and the Kansas Bioscience Authority through a matching grant to Kansas State University's Center of Excellence for Emerging and Zoonotic Animal Diseases (to R. R. R. Rowland); and Kansas State University College of Veterinary Medicine research startup fund (to Y. Fang). We would like to thank Dr. RT Baker (Clinical Genomics Pty Ltd., Australia) for the contribution of the pHUE expression vector, and Dr. Jerome Nietfeld for the IHC analysis.

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