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Development of a chromatographic lateral flow immunoassay for detection of African swine fever virus antigen in blood

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Abstract

African swine fever (ASF) is a highly lethal disease of domestic and wild swine caused by African swine fever virus (ASFV). The disease currently circulates in Africa, Europe, Asia and on the island of Hispaniola. The ongoing epizootics in Europe and Asia have produced millions of animal deaths and severe economic losses. No effective vaccine is available for ASF, making rapid and accurate detection of ASFV essential for disease mitigation strategies. Currently available diagnostics for ASFV possess significant limitations related to assay performance, deployability, and/or turn-around time; therefore there is an unmet need for pen-side diagnostic tests with sufficient sensitivity and specificity. A chromatographic lateral flow immunoassay (LFIA) was developed for the detection of ASFV antigen in EDTA-treated whole blood using monoclonal antibodies targeting the viral p30 protein. The assay requires only water to perform and provides results in 25 min, making it well-suited for field use. The LFIA was capable of detecting genotype I and genotype II strains of ASFV in EDTA blood from experimentally infected pigs at varying time-points after infection, though it was unable to detect a genotype X ASFV strain. Diagnostic sensitivity correlated with clinical disease severity, body temperature, and viral DNA levels, and was over 90% in animals showing moderate to severe ASF-related symptoms after challenge with virulent genotype II virus. The LFIA also showed a robust diagnostic specificity of over 98%, which is essential to field testing for a high consequence to foregin animal disease. The LFIA targeting the viral p30 protein can reliably detect ASFV in whole blood from animals showing moderate to severe clinical signs of infection with virulent genotype I and II isolates, making it a promising candidate for use as a field-deployable antigen detection assay. Additional evaluation using field samples and different virus strains is required to further assess the utility of this rapid diagnostic test.

Keywords: African swine fever, Lateral flow immunoassay, Point of care diagnostics

Introduction

African swine fever (ASF) is a transboundary viral disease of domestic and wild pigs, and is one of the most significant threats to domestic and global pork production. The causative agent, African swine fever virus (ASFV), is highly transmissible *via* direct and indirect contact between susceptible swine and is the only known DNA arbovirus, with soft ticks belonging to the genus

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Ornithodoros acting as biological vectors (Costard et al., 2013; Jori et al., 2013). Infection with ASFV produces a range of clinical features depending on virus strain and host susceptibility, with low-virulence and attenuated strains producing minimal or inapparent clinical disease and highly virulent isolates causing a peracute hemorrhagic fever with mortality rates near 100% in susceptible animals (Blome et al., 2013). The disease is endemic throughout much of Southern and Eastern Africa where the virus is maintained in nature though a sylvatic cycle involving two-way transmission between juvenile warthogs and *Ornithodoros porcinus porcinus* soft ticks. Additionally, ASF is endemic on the Mediterranean island of Sardinia, and historical outbreaks have occurred in Western Europe and the Americas (Jori et al., 2013; Cubillos et al., 2013; Jori & Bastos, 2009; Penrith et al., 2013). Domestic pigs become infected *via* feeding by infected soft ticks, contact with wild swine species capable of maintaining sufficiently high viremia levels such as bushpigs and Eurasian wild boar, ingestion of contaminated feed and pork products, and through direct and indirect contact with other infected domestic pigs (Costard et al., 2013; Jori et al., 2013). In 2007, a highly virulent genotype II isolate of ASFV emerged in the Caucasus nation of Georgia and rapidly spread through Central and Eastern Europe and the Russian Federation, and has been detected in Eurasian wild boar as far west as Belgium, Germany, and Italy (Costard et al., 2013; Gogin et al., 2013; Linden et al., 2019; Oganesyan et al., 2013; Rowlands et al., 2008; Sauter-Louis et al., 2021; African Swine Fever (ASF) – Situation Report 3, 2022). In 2018, ASFV was first detected in China, the world's largest pork producer, and its subsequent spread throughout Eastern Asia has inflicted catastrophic animal losses and billions of dollars in economic damages (Gaudreault et al., 2020; Lu et al., 2020; Zhou et al., 2018). No effective vaccine exists for ASF, and control relies on rapid detection, restricting the movement of animals and pork products, and aggressively culling affected herds (Beltrán-Alcrudo et al., 2017).

While a variety of diagnostic assays have been developed for ASF, several limitations hinder the utility of available tests. Quantitative real-time PCR (qPCR) is considered the gold standard for ASFV detection but uses equipment and reagents that are not readily adaptable to a field setting (Gaudreault et al., 2020; Sánchez-Vizcaíno & Heath, 2019). In situations with endemic ASFV infections, antibody testing can be useful for elucidating disease dynamics and detecting the emergence of new genetic variants with reduced virulence but not for early detection purposes. Highly virulent ASFV strains can cause death in infected animals before the induction of detectable levels of anti-ASFV antibodies can occur (Blome et al., 2013; Cubillos et al., 2013; Sánchez-Vizcaíno & Heath, 2019). Laboratory methods for virus isolation require highly specialized facilities and are time-consuming, and currently available antigen capture tests including enzyme-linked immunosorbent assays (ELISAs) and lateral flow immunoassays (LFIAs) have limited field data and are hindered by variable sensitivity and specificity (Gallardo et al., 2015; Oura et al., 2013). Consequently, there is a need for pen-side tests capable of reliably identifying ASFV-infected animals which possess sufficiently high specificity and sensitivity to avoid false negative or false positive results, with the latter able to trigger disruptive countermeasures such as herd culling and export restrictions.

LFIAs, which detect the presence of an analyte via specific antibodies or antigens (Additional file 1), are an attractive platform for on-site diagnostic testing because they are portable, easy to use, reliable, and cost-effective (Wong & Tse, 2009). We developed an LFIA for the detection of ASFV antigen in whole blood using monoclonal antibodies targeting the ASFV-specific p30 protein. The ASFV antigen LFIA was able to detect both genotype I and genotype II ASFV in EDTA blood from experimentally infected pigs, and showed a diagnostic sensitivity greater than 90% for animals displaying moderate to severe symptoms of virulent ASFV infection. Importantly, the assay also demonstrated robust specificity of over 98% using a panel of blood samples from animals demonstrated to be negative for ASFV by qPCR. While the ASFV LFIA was not reactive to a genotype X strain of ASFV, its ability to detect a virulent genotype II ASFV strain genetically related to those circulating in Europe and Asia highlights the potential utility of this assay in combating ongoing ASFV outbreaks. Additional evaluation using field samples will be needed to further discern the viability of the p30-based ASFV LFIA as a pen-side diagnostic test.

Results

Selection and isotyping of anti-ASFV p30 monoclonal antibodies

Three monoclonal antibodies (mAbs), designated 5C1, 2B8 and 1D8, showed specific reactivity against ASFV-infected cells. Indirect immunofluorescence assay (IFA) revealed specific fluorescence associated with the cytoplasm of Vero E6 cells infected with ASFV strain BA71v (Fig. 1A-C) which was absent in uninfected cultures and those which received no primary antibody treatment (Fig. 1D-H). Isotyping identified mAbs 5C1 and 1D8 as IgG1 subclass antibodies and mAb 2B8 as IgG2b, with all three possessing κ light chains. These mAbs were selected for developing the LFIA by the commercial manufacturer.

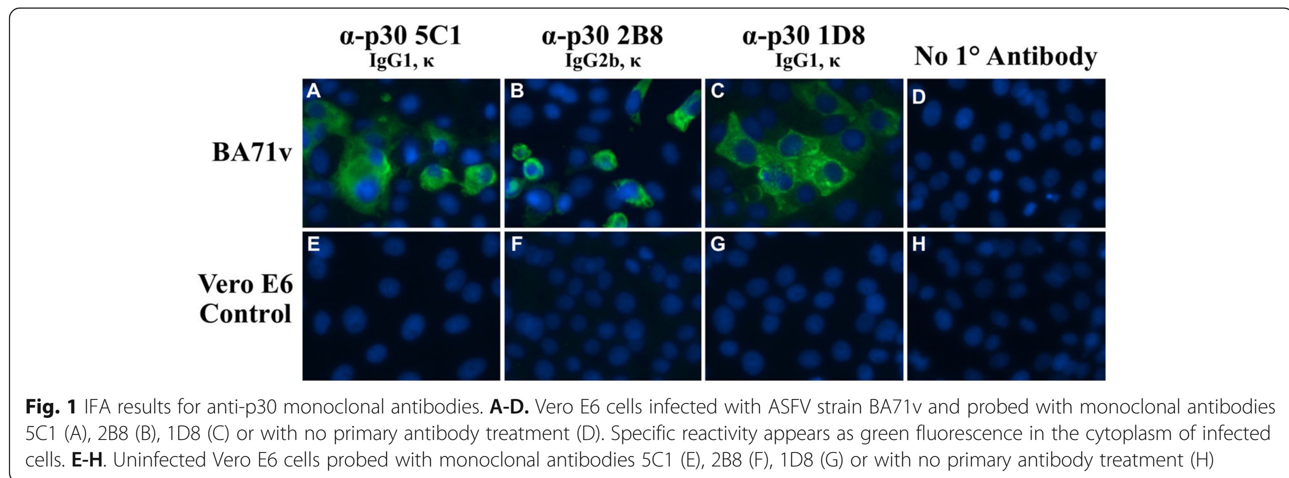

Fig. 1 IFA results for anti-p30 monoclonal antibodies. **A-D.** Vero E6 cells infected with ASFV strain BA71v and probed with monoclonal antibodies 5C1 (A), 2B8 (B), 1D8 (C) or with no primary antibody treatment (D). Specific reactivity appears as green fluorescence in the cytoplasm of infected cells. **E-H**. Uninfected Vero E6 cells probed with monoclonal antibodies 5C1 (E), 2B8 (F), 1D8 (G) or with no primary antibody treatment (H)

Animal experiments, sampling, and qPCR

ASFV-negative whole blood samples were obtained from a total of 79 naïve piglets, including pre-challenge (0 days post-challenge; DPC) samples from 31 animals subsequently infected with ASFV (Table 1). Clinical evaluation of naïve animals at the time of sampling showed 74/79 piglets to be free of any symptoms of disease; five piglets had a clinical score of 1 either due to slight fever (4/5) or mild lethargy (1/5). All 79 blood samples from naïve piglets were negative for ASFV genomic DNA by qPCR.

A total of 31 piglets were challenged with ASFV genotype II strain Armenia 2007 (Arm07), genotype I strain E70, or genotype X strain Ken05/Tk1 as part of six separate animal experiments (Table 2). All 21 piglets challenged with Armenia 2007 ASFV developed clinical disease characteristic of acute ASF and died or were euthanized by 11 DPC (Table 3). In the 56 post-challenge EDTA blood samples obtained from these Armenia 2007-infected animals at various time points post challenge, ASFV genomic DNA levels ranged from undetectable to 1.39×10^9 copy number per milliliter of whole blood (CN/mL), with an overall trend of increasing ASFV DNA levels over time after infection for individual piglets. Symptoms could be observed as early as 5 DPC in some Armenia 2007-challenged animals, and clinical scores for piglets tended to worsen until death. Similar clinical results were seen in piglets challenged with ASFV isolates E70 and Ken05/Tk1 (Table 4).

Sensitivity, specificity, and reproducibility of the LFIA

A total of 79 pre-challenge and 74 post-challenge EDTA blood samples were tested by the p30 ASFV LFIA in duplicate, with results defined as positive (both duplicates show positive results), negative (both duplicates show negative results), or suspect (duplicates show conflicting results). Totally 78/79 pre-challenge samples were LFIA negative, with one pre-challenge sample showing a suspect result, for a specificity with pre-challenge blood of 98.7% (Table 1). The specificity of the ASFV qPCR with pre-challenge blood was 100%, with no false positives or suspect observed.

Of the 74 post-challenge whole blood samples evaluated, 56 were from animals infected with the Armenia 2007 ASFV strain, eight were from animals infected with strain E70, and 10 were from animals challenged with strain Ken05/Tk1. 51/56 Armenia 2007 post-challenge blood samples were positive for ASFV genomic DNA, for an overall post-challenge qPCR sensitivity of 91.1% (Table 5). In comparison, 31/56 Armenia 2007 post-challenge samples were LFIA positive, with 24/56 testing negative and 1/56 testing as suspect, for an overall post-challenge sensitivity of 55.4% when the suspect result is considered a false negative (Table 5). LFIA performance correlated with clinical disease severity, with poor sensitivity (15.4%) observed for samples from asymptomatic animals (clinical score 0) but a sensitivity of 90.9% for samples from animals showing moderate to severe clinical signs of ASF (clinical score > 6) and 100% for post-mortem blood samples (Table 5). A similar correlation is observed with fever scores, with poor LFIA sensitivity for samples from afebrile animals that rises rapidly with increasing fever score. For genotype I E70 post-challenge samples, overall LFIA sensitivity was 75%, with better sensitivity for samples associated with high clinical and fever scores. Interestingly, none of the Ken05/Tk1 post-challenge samples tested positive with the LFIA, despite all samples having detectable levels of ASFV genomic DNA with up to 3.92×10^8 CN/mL, indicating the assay is unable to identify the genotype X ASFV isolate (Tables 4 and 5).

Direct comparison between the ASFV LFIA and ASFV qPCR as a gold-standard reference test shows an LFIA specificity of 98.8% for all 84 samples negative for ASFV genomic DNA by qPCR, including 79 pre-challenge and five post-challenge q-PCR negetive samples, with 83/84

Table 1 ASFV qPCR-negative samples from naïve piglets tested by LFIA ($n = 79$)

Sample ID	Clinical score	LFIA #1/#2	Comments	Sample ID	Clinical score	LFIA #1/#2	Comments
1–1 0DPC[a]	0	−/−		N-10	0	−/−	
1–2 0DPC[a]	0	−/−		N-11	0	−/−	
1–3 0DPC[a]	0	−/−		N-12	0	−/−	
1–4 0DPC[a]	0	−/−		N-13	0	−/−	
2–1 0DPC[a]	0	−/−		N-14	1	−/−	Mild fever 40.6°
2–2 0DPC[a]	0	−/−		N-15	0	−/−	
2–3 0DPC[a]	0	−/−		N-16	0	−/−	
2–4 0DPC[a]	0	−/−		N-17	0	−/−	
2–5 0DPC[a]	0	−/−		N-18	0	−/−	
3–1 0DPC[a]	0	−/−		N-19	0	−/−	
3–2 0DPC[a]	0	−/−		N-20	0	−/−	
3–3 0DPC[a]	0	−/−		N-21	0	−/−	
3–4 0DPC[a]	1	−/−	Mild fever 40.6°	N-22	0	−/−	
4–1 0DPC[a]	1	−/−	Slight lethargy	N-23	0	−/−	
4–2 0DPC[a]	0	−/−		N-24	0	−/−	
4–3 0DPC[a]	0	−/−		N-25	0	−/−	
5–1 0DPC[a]	0	−/−		N-26	0	−/−	
5–2 0DPC[a]	0	−/−		N-27	0	−/−	
5–3 0DPC[a]	0	−/−		N-28	0	−/−	
5–4 0DPC[a]	0	−/−		N-29	1	−/−	Mild fever 40.6°
5–5 0DPC[a]	0	−/−		N-30	0	−/−	
6–1 0DPC[a]	0	−/−		N-31	0	−/−	
6–2 0DPC[a]	1	−/−	Mild fever 40.7°	N-32	0	−/+	Suspect
6–3 0DPC[a]	0	−/−		N-33	0	−/−	
6–4 0DPC[a]	0	−/−		N-34	0	−/−	
6–5 0DPC[a]	0	−/−		N-35	0	−/−	
6–6 0DPC[a]	0	−/−		N-36	0	−/−	
6–7 0DPC[a]	0	−/−		N-37	0	−/−	
6–8 0DPC[a]	0	−/−		N-38	0	−/−	
6–9 0DPC[a]	0	−/−		N-39	0	−/−	
6–10 0DPC[a]	0	−/−		N-40	0	−/−	
N-1	0	−/−		N-41	0	−/−	
N-2	0	−/−		N-42	0	−/−	
N-3	0	−/−		N-43	0	−/−	
N-4	0	−/−		N-44	0	−/−	
N-5	0	−/−		N-45	0	−/−	
N-6	0	−/−		N-46	0	−/−	
N-7	0	−/−		N-47	0	−/−	
N-8	0	−/−		N-48	0	−/−	
N-9	0	−/−					

[a]pre-challenge sample from pigs listed in Tables 2, 3, and 4. LFIA lateral flow immunoassay

Table 2 Pig challenge experiments using three ASFV genotypes and respective EDTA whole blood samples used for testing

Experiment	Animal	ASFV strain	Dose (HAU)	Death (DPC)	Samples tested (DPC)
#1	1–1	Arm07	160	10	0, 5, 7, 10
	1–2	Arm07	160	7	0, 5, 7
	1–3	Arm07	160	7	0, 5, 7
	1–4	Arm07	160	7	0, 5, 7
#2	2–1	Arm07	16	5	0, 5
	2–2	Arm07	16	5	0, 5
	2–3	Arm07	16	7	0, 5, 7
	2–4	Arm07	16	11	0, 5, 7, 10
	2–5	Arm07	16	8	0, 5, 7
#3	3–1	Arm07	360	7	0, 4, 5, 6, 7
	3–2	Arm07	360	7	0, 4, 5, 6, 7
	3–3	Arm07	360	8	0, 4, 5, 6, 7
	3–4	Arm07	360	8	0, 4, 5, 6, 7, 8
#4	4–1	Arm07	100	7	0, 5, 7
	4–2	Arm07	100	11	0, 5, 7, 10, 11
	4–3	Arm07	100	8	0, 5, 7, 8
#5	5–1	Arm07	100	7	0, 5, 7
	5–2	Arm07	100	8	0, 5, 7, 8
	5–3	Arm07	100	7	0, 5, 7
	5–4	Arm07	100	10	0, 5, 7, 10
	5–5	Arm07	100	9	0, 5, 7
#6	6–1	E70	360	7	0, 4, 6
	6–2	E70	360	7	0, 4, 6
	6–3	E70	360	7	0, 4, 7
	6–4	E70	360	8	0, 6
	6–5	E70	360	8	0, 4
	6–6	Ken05/Tk1	360	8	0, 4, 8
	6–7	Ken05/Tk1	360	8	0, 4, 8
	6–8	Ken05/Tk1	360	8	0, 4, 6
	6–9	Ken05/Tk1	360	7	0, 4, 6
	6–10	Ken05/Tk1	360	5	0, 4, 5

HAU hemadsorbing units, *DPC* days post-challenge, *Arm07* Armenia 2007

qPCR-negative samples showing negative LFIA results and 1 sample showing suspect LFIA results (discordant LFIA duplicates). The LFIA was consistently able to detect samples with high levels of ASFV genomic DNA, with a diagnostic sensitivity over 95% for Armenia 2007 samples containing $\geq 10^8$ CN/mL ASFV DNA and of 100% for E70 samples with $\geq 10^8$ CN/mL; LFIA sensitivity was lower for samples containing $<10^8$CN/mL ASFV genomic DNA (Table 6). Overall, a substantial concordance was seen between the ASFV LFIA and ASFV qPCR for blood samples obtained pre-challenge and after challenge with genotype II Armenia 2007 and genotype I E70, with $\kappa = 0.65$ (95% CI, 0.53–0.78). Additionally, near perfect agreement was seen between LFIA duplicates for the samples evaluated. Out of 153 EDTA blood samples tested, discordant LFIA results were only observed for one pre-challenge sample and one post-challenge sample (Tables 2 and 3), with a concordance of $\kappa = 0.97$ (95% CI, 0.92–1.00).

Alignments of p30 amino acid sequences

To better understand the inability of the LFIA to detect the genotype X Ken05/Tk1 ASFV strain, an alignment of the p30 protein from strains Georgia 2007/1, E70, and Ken05/Tk1 was generated and potential linear B cell epitopes within each p30 isolate predicted (Fig. 2). The Georgia 2007/1 strain is genetically highly similar to the Armenia 2007 strain (unpublished results). Both the

Table 3 Armenia 2007 post-challenge samples

Sample ID	p72 qPCR CN/mL	LFIA #1/#2	Clinical score	Fever score	Other signs	Comments
1–1 5 DPC	ND	–/–	0	0	0	
7 DPC	7.69E+ 03	–/–	2	0	2	
10 DPC	6.35E+ 08	+/+	5	3	2	Euthanized
1–2 5 DPC	4.53E+ 08	+/+	0	0	0	
7 DPC	1.39E+ 09	+/+	5	0	5	Euthanized
1–3 5 DPC	4.00E+ 08	+/+	2	2	0	
7 DPC	7.09E+ 08	+/+	9	4	5	Euthanized
1–4 5 DPC	1.51E+ 08	+/+	0	0	0	
7 DPC	7.96E+ 08	+/+	10	4	6	Euthanized
2–1 5 DPC	5.69E+ 08	+/+	5	2	3	Euthanized
2–2 5 DPC	7.32E+ 08	+/+	7	3	4	Euthanized
2–3 5 DPC	2.68E+ 08	–/–	2	2	0	
7 DPC	1.28E+ 09	+/+	12	2	10	Euthanized
2–4 5DPC	ND	–/–	1	0	1	
7 DPC	ND	–/–	0	0	0	
10 DPC	1.89E+ 07	–/–	5	2	3	
2–5 5 DPC	5.27E+ 07	–/–	1	1	0	
7 DPC	9.05E+ 08	+/+	6	3	3	
3–1 4 DPC	3.96E+ 05	–/–	0	0	0	
5 DPC	9.37E+ 06	–/–	3	1	2	
6 DPC	6.43E+ 07	+/+	6	3	3	
7 DPC	4.10E+ 08	+/+	12	3	9	Euthanized
3–2 4 DPC	6.46E+ 05	–/–	0	0	0	
5 DPC	7.75E+ 07	–/–	2	0	2	
6 DPC	5.08E+ 07	+/+	10	1	9	
7 DPC	3.88E+ 08	+/+	N/A	N/A	N/A	Post-mortem
3–3 4 DPC	2.94E+ 04	–/–	0	0	0	
5 DPC	8.34E+ 06	–/–	4	1	3	
6 DPC	5.09E+ 07	+/+	6	3	3	
7 DPC	7.00E+ 08	+/+	11	0	11	
3–4 4 DPC	ND	–/–	0	0	0	
5 DPC	1.94E+ 05	–/–	0	0	0	
6 DPC	4.36E+ 06	–/–	4	1	3	
7 DPC	9.09E+ 07	+/–	7	0	7	Suspect LFIA
8 DPC	3.82E+ 08	+/+	16	3	13	Euthanized
4–1 5 DPC	1.35E+ 08	+/+	4	3	1	
7 DPC	2.63E+ 07	+/+	N/A	N/A	N/A	Post-mortem
4–2 5 DPC	ND	–/–	0	0	0	
7 DPC	1.44E+ 05	–/–	0	0	0	
10 DPC	7.47E+ 07	–/–	3	2	1	
11 DPC	6.74E+ 07	+/+	5	2	3	Euthanized
4–3 5 DPC	8.05E+ 05	–/–	0	0	0	
7 DPC	3.23E+ 08	+/+	7	3	4	
8 DPC	2.42E+ 08	+/+	3	0	3	Euthanized

Table 3 Armenia 2007 post-challenge samples *(Continued)*

Sample ID	p72 qPCR CN/mL	LFIA #1/#2	Clinical score	Fever score	Other signs	Comments
5–1 5 DPC	7.12E+ 07	+/+	2	2	0	
7 DPC	1.48E+ 08	+/+	8	4	4	Euthanized
5–2 5 DPC	1.09E+ 08	+/+	1	1	0	
7 DPC	1.28E+ 08	+/+	5	3	2	
8 DPC	2.52E+ 08	+/+	N/A	N/A	N/A	Post-mortem
5–3 5 DPC	8.72E+ 07	+/+	3	3	0	
7 DPC	2.16E+ 08	+/+	6	3	3	
5–4 5 DPC	4.03E+ 03	−/−	0	0	0	
7 DPC	1.86E+ 07	−/−	5	3	2	
10 DPC	3.30E+ 07	−/−	6	3	3	
5–5 5 DPC	3.38E+ 07	−/−	1	1	0	
7 DPC	1.28E+ 08	+/+	4	3	1	

ND not detectable, *N/A* not applicable, *Other signs* liveliness, body shape, respiratory, neurological, skin, digestive, ocular/nasal signs. *LFIA* lateral flow immunoassay. *DPC* days post-challenge

Georgia 2007/1 and the E70 p30 proteins were highly similar, with a sequence homology of 98%; in contrast, only 89% homology was shared between Georgia 2007/1 and Ken05/Tk1, with a stretch of 7 additional amino acid residues in the Ken05/Tk1 p30 protein that are not present in the Georgia 2007/1 and E70 isolates (Fig. 2A). Linear B-cell epitope prediction based on the primary structure of each p30 isolate revealed this addition occurs in a region of p30 predicted to be highly antigenic in all 3 isolates (Fig. 2A-D). A high density of amino acid substitutions in the Ken05/Tk1 p30 compared to Georgia 2007/1 and E70 p30 is also present within this predicted epitope (Fig. 2A).

Discussion

Accurate and timely identification of ASFV is a mainstay of ASF mitigation. The ideal diagnostic assay for ASF

Table 4 E70 and Ken05/Tk1 post-challenge samples

ASFV Strain	Sample ID	p72 qPCR CN/mL	LFIA #1/#2	Clinical score	Fever score	Other signs	Comments
E70	**6–1** 4 DPC	9.96E+ 07	−/−	0	0	0	
	6 DPC	7.32E+ 08	+/+	0	0	0	
	6–2 4DPC	7.92E+ 07	−/−	2	0	2	
	6 DPC	5.45E+ 08	+/+	8	3	5	
	6–3 4 DPC	2.31E+ 08	+/+	1	1	0	
	7 DPC	4.55E+ 08	+/+	9	3	6	Euthanized
	6–4 6 DPC	6.49E+ 08	+/+	3	3	0	
	6–5 4 DPC	2.03E+ 08	+/+	0	0	0	
Ken05/Tk1	**6–6** 4 DPC	8.08E+ 06	−/−	0	0	0	
	8 DPC	1.86E+ 07	−/−	6	0	6	Euthanized
	6–7 4 DPC	3.37E+ 08	−/−	3	3	0	
	8 DPC	1.16E+ 08	−/−	11	0	11	Euthanized
	6–8 4 DPC	2.13E+ 08	−/−	3	3	0	
	6 DPC	3.90E+ 08	−/−	6	3	3	
	6–9 4 DPC	2.56E+ 08	−/−	0	0	0	
	6 DPC	3.92E+ 08	−/−	2	0	2	
	6–10 4 DPC	8.03E+ 07	−/−	4	3	1	
	5 DPC	1.33E+ 08	−/−	11	3	8	Euthanized

Other signs = liveliness, body shape, respiratory, neurological, skin, digestive, ocular/nasal signs. *LFIA* lateral flow immunoassay. *DPC* days post-challenge

Table 5 Sensitivity for the ASFV LFIA and qPCR in blood samples post ASFV challenge

Challenge Strain	*N*	LFIA				qPCR		
		Pos.	Neg.	Suspect	Sen.	Pos.	Neg.	Sen.
Post-Arm07	56	31	24	1	55.4%	51	5	91.1%
CS = 0	13	2	11	0	15.4%	9	4	69.2%
CS = 1–6	29	16	13	0	55.2%	28	1	96.6%
CS > 6	11	10	0	1	90.9%	11	0	100.0%
Dead	3	3	0	0	100.0%	3	0	100.0%
F = 0	20	5	14	1	25.0%	15	5	75.0%
F = 1	7	2	5	0	28.6%	7	0	100.0%
F = 2	8	5	3	0	62.5%	8	0	100.0%
F = 3	15	13	2	0	86.7%	15	0	100.0%
F = 4	3	3	0	0	100.0%	3	0	100.0%
Post-E70	8	6	2	0	75.0%	8	0	100.0%
CS = 0	3	2	1	0	66.7%	3	0	100.0%
CS = 1–6	3	2	1	0	66.7%	3	0	100.0%
CS > 6	2	2	0	0	100.0%	2	0	100.0%
Dead	0	–	–	–	–	–	–	–
F = 0	4	2	2	0	50.0%	4	0	100.0%
F = 1	1	1	0	0	100.0%	1	0	100.0%
F = 2	0	–	–	–	–	–	–	–
F = 3	3	3	0	0	100.0%	3	0	100.0%
F = 4	0	–	–	–	–	–	–	–
Post-Ken05/Tk1	10	0	10	0	0.0%	10	0	100.0%

CS clinical score, *F* fever score, *Pos.* positive result, *Neg.* negative result, *Sen.* sensitivity

Table 6 Sensitivity of the ASFV LFIA with qPCR-positive samples

qPCR Results	*N*	LFIA			
		Pos.	Neg.	Suspect	Sen.
Pos – Arm07	56	31	24	1	55.4%
$< 10^5$	3	0	3	0	0.0%
10^5–10^6	5	0	5	0	0.0%
10^6–10^7	3	0	3	0	0.0%
10^7–10^8	15	7	7	1	46.7%
10^8–10^9	23	22	1	0	95.7%
$> 10^9$	2	2	0	0	100.0%
Pos – E70	8	6	2	0	75.0%
$< 10^5$	0	–	–	–	–
10^5–10^6	0	–	–	–	–
10^6–10^7	0	–	–	–	–
10^7–10^8	2	0	2	0	0.0%
10^8–10^9	6	6	0	0	100.0%
$> 10^9$	0	–	–	–	–

Pos. positive result, *Neg.* negative result, *Sen.* sensitivity

should be inexpensive, field-deployable, and deliver highly reliable results. Unfortunately, no single assay for ASF is available which meets all three criteria. In this study, we developed and evaluated a rapid LFIA targeting the ASFV p30 antigen which requires only water and can successfully identify ASFV-infected pigs showing moderate to severe ASF-related clinical signs with high sensitivity and specificity. This LFIA requires only non-sterile water, a micropipette, and an EDTA whole blood sample to be performed; it costs approximately US$5 per test strip (E. Serrao, SLRC, personal communication), and can be stored at room temperature up to 2 years from the date of manufacture, making it highly suitable for transport and use in a field setting.

Detecting ASFV antigen is an attractive strategy for field-deployable ASF diagnostics because infection with virulent ASFV produces high levels of viremia (and therefore viral antigens) in domestic pigs and wild boar (Oura et al., 2013). Antigen detection assays, including ELISAs and LFIAs, targeting ASFV soluble antigens and the p72 capsid protein have been previously described (Oura et al., 2013; Hutchings & Ferris, 2006; Sastre et al., 2016). p30 is a viral membrane phosphoprotein which is highly antigenic and abundantly expressed early

A

```
Georgia 2007/1 MDFILNISMKMEVIFKTDLRSSSQVVFHAGSLYNWFSVEIINSGRIVTTAIKTLLSTVKY 60
E70            MDFILNISMKMEVIFKTDLRSSSQVVFHAGSLYNWFSVEIINSGRIVTTAIKTLLSTVKY 60
Ken05/Tk1      MDFILNISMKMEVIFKTDLRASSQVVFHAGSLYTWFSVEIINSGRIVTTAIKTLLSTVKY 60
               *********************:***********.**************************

Georgia 2007/1 DIVKSARIYAGQGYTEHQAQEEWNMILHVLFEEETESSASS-------ENIHEKNDNETN 113
E70            DIVKSAHIYAGQGYTEHQAQEEWNMILHVLFEEETESSASS-------ESIHEKNDNETN 113
Ken05/Tk1      DIVRNARIYAGQGYTEQQAQEEWNMILHVLFEEETESTSSTSLESNHETNGHKADGHETN 120
               ***:.*:**********:*******************::*:        . *: :.:***

Georgia 2007/1 ECTSSFETLFEQEPSSEVPKDSKLYMLAQKTVQHIEQYGKAPDFNKVIRAHNFIQTIYGT 173
E70            ECTSSFETLFEQEPSSEEPKDSKLYMLAQKTVQHIEQYGKAPDFNKVIRAHNFIQTIHGT 173
Ken05/Tk1      ECTSSFETLFEQEPSSETPKDTKLYALAQKAVQHIEQYGKAPDFNKVIRAHNYIQTIYGS 180
               ***************** ***:*** ****:*********************:****:*:

Georgia 2007/1 PLKEEEKEVVRLMVIKLLKKK 194       100% Identity to Georgia 2007/1
E70            PLKEEEKEVVRLMVIKLLKKK 194        98% Identity to Georgia 2007/1
Ken05/Tk1      PLKEEEKEEVRLMVIKLLKKK 201        89% Identity to Georgia 2007/1
               ******** ************
```

B Georgia 2007/1

C E70

D Ken05/Tk1

Score

Residue

*Residue is conserved in all aligned sequences

:Residues are different but groups share strongly similar properties

.Residues are different but groups share weakly similar properties

Fig. 2 Alignments and linear B-cell epitope predictions for p30 from ASFV strains Georgia 2007/1, E70, and Ken05/Tk1. **A.** Primary sequence alignment for ASFV p30 from all three ASFV strains. Predicted linear B-cell epitopes for all 3 isolates are highlighted, and the region containing the greatest sequence diversity is outlined. **B-D**. p30 linear B-cell epitope prediction for Georgia 2007/1 (B), E70 (C), and Ken05/Tk1 (D). Predicted epitopes are defined as sequence regions with values above the threshold score of 0.5 (green dashed line). The region of greatest p30 variability is highlighted in gray for each strain

in the viral infection cycle (Afonso et al., 1992; Prados et al., 1993), making it a viable target for ASFV antigen detection.

The p30 ASFV LFIA was capable of consistently identifying ASFV antigen in genotype II Armenia 2007-challenged pigs with moderate to severe clinical signs or qPCR values of > 10^8 CN/mL, showing a sensitivity of 90.9% (clinical signs) and 96% (qPCR values), respectively (Tables 5 and 6); similar results were seen for genotype I E70-challenged animals. These results are consistent with the trend of higher sensitivity assocaited with progressively increasing levels of viremia and worsening clinical symptomology over time following ASFV challenge observed in this study (Table 3); this explains why the LFIA shows better performance for animals with more severe clinical disease. The ASFV LFIA also showed high specificity with both pre-challenge and qPCR-negative blood samples of 98.7 and 98.8%, respectively, with only 1 suspect test result noted (Tables 5 and 6). Because suspect results cannot be adequately classified as false positives or false negatives, the true specificity of the LFIA is likely to be higher; when duplicate LFIAs run on the same sample are considered separate tests, the specificity of the assay is 99.4% for both, the pre-challenge ($n = 158$) and the qPCR-negative ($n = 168$) samples. A high level of specificity is desirable for a pen-side diagnostic assay. We also used the p30 ASFV LFIA to test 17 blood samples obtained from five pigs experimentally infected with a virulent classical swine fever virus (CSFV) strain, which causes a disease

clinically indistinguishable from African swine fever. All CSFV samples were tested in duplicate and no false positive results were observed, further indicating that the LFIA is highly specific for ASFV (Additional file 2).

While the p30 ASFV LFIA was capable of identifying genotype I and II ASFV strains E70 and Armenia 2007 in the blood of infected animals, it was unable to detect genotype X strain Ken05/Tk1 in any of the samples tested, despite the majority of these samples containing $> 10^8$ CN/mL ASFV genomic DNA as measured by qPCR (Table 4). A likely explanation for this observation is that the monoclonal antibodies used for p30 detection target regions of the p30 protein which are antigenically different between the genotype I/II versus the genotype X virus strains. A previous study evaluating a recombinant p30-based ELISA for detection of ASFV-specific antibodies showed decreased sensitivity associated with samples from animals infected with East African ASFV isolates compared to samples from animals infected with West African or Spanish ASFV isolates (Pérez-Filgueira et al., 2006). Primary sequence alignment and linear B-cell epitope predictions for p30 from the Georgia 2007/1, E70, and Ken05/Tk1 ASFV strains revealed notable diversity in the Ken05/Tk1 p30 protein within a region predicted to be a strong linear epitope in all 3 isolates (Fig. 2). The monoclonal antibodies used for LFIA development in this study were all derived from mice immunized with a recombinant Georgia 2007/1 p30 antigen, and all three monoclonal antibodies were reactive with the p30 antigenin IFAS (Fig. 1) and on western blots (data not shown) generated with genotype I-infected cells, indicating they target linear epitopes. This suggests the p30 ASFV LFIA may not be able to detect ASFV isolates with p30 epitopes which are significantly different from those in the genotype I (E70) or genotype II (Georgia 2007/1; Armenia 2007) p30 proteins. Epitope mapping for each monoclonal antibody to accurately identify which region of the p30 protein they target is needed to evaluate this hypothesis. While this limitation may hinder the utility of the LFIA in regions of East Africa with divergent ASFV strains, the LFIA remains a viable diagnostic test for genotype I and genotype II ASFVs currently circulating in Europe and Asia.

During preparation of this manuscript, a research article (Onyilagha et al., 2022) was published evaluating the performance of the p30 ASFV LFIA, now commercially available as PenCheck® (Silver Lake Research Corporation, Irwindale, CA, USA). The results reported by Onyilagha and colleagues (Onyilagha et al., 2022) showed that the p30 ASFV LFIA has a lower sensitivity than ASFV qPCR but is highly specific and reliably detects ASFV in infected animals with high fever, similar to the results reported here (Table 5); additionally, Onyilagha and colleagues reported that the p30 ASFV LFIA was able to detect ASFV antigen in multiple sample types including whole blood, serum and lymphoid tissue samples (Onyilagha et al., 2022). These authors concluded that the PenCheck® p30 ASFV LFIA "can be used as a herd-level, field-deployable, and easy-to-use diagnostic tool to identify ASF-affected farms when access to portable molecular assays or central laboratories is not possible" (Onyilagha et al., 2022). Taken together, these data support the utility of the p30 ASFV LFIA as a field-deployable rapid antigen detection test.

Whole blood is an ideal diagnostic sample for a pen-side ASFV antigen detection test because it contains significantly higher levels of virus than bodily secretions in infected animals, while still being straightforward to collect in a field setting. ASFV is also present within a variety of body fluids and secretions from infected animals, including saliva, urine, and feces. The ability of the LFIA to detect ASFV antigen in these sample types was not evaluated in this study. While high levels of virus are present within various tissues (e.g. lymph nodes and spleen) of infected swine, these can only be obtained post-mortem and require homogenization and centrifugation before analysis, making them poor choices for testing in the field. Despite these limitations, additional evaluation of the ASFV LFIA with other field sample types (e.g. swine oral fluids) could be useful in expanding the utility of this diagnostic test. Furthermore, this LFIA may be feasible for detecting ASFV antigen in the blood of ASFV-infected Eurasian wild boar, since these animals develop high viremia levels after infection with virulent strains of ASFV (Blome et al., 2013).

Conclusions

The p30 ASFV LFIA evaluated in this study has the ability to detect ASFV p30 antigen in EDTA-treated whole blood from pigs experimentally infected with genotype I and genotype II ASFV isolates with reliable sensitivity and specificity, in a format that is highly portable and easy to use in the field. All blood samples evaluated in this study were obtained from experimentally infected animals and stored under ideal conditions, which may not parallel real-world sampling and testing. Additional testing in the field on samples from domestic and wild swine and with different ASFV genotypes and strains is needed to better elucidate the performance of the p30 ASFV LFIA as a pen-side diagnostic test.

Methods

Viruses and cells

ASFV strain BA71v, a cell-adapted genotype I virus, was propagated and titrated on Vero E6 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Vero E6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM L-

glutamine and 4.5 g/L glucose (Corning, Manassas, VA, USA), supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA) and 1x antibiotic-antimycotic solution (Gibco, Waltham, MA, USA), and grown in a 37 °C humidified incubator with 5% CO_2 atmosphere. BA71v stocks were generated by infecting sub-confluent Vero E6 cells at a MOI of 1. At 24 h post-infection, the cells were lysed using two freeze-thaw cycles and culture supernatant clarified by centrifuging at 3,200 x G for 10 minutes. BA71v stocks were titrated by $TCID_{50}$ based on cytopathic effect and stored at − 80 °C.

Three virus isolates were used for experimental infection of pigs. Viruses were obtained from the European Union Reference Laboratory for ASF [Centro de Investigación en Sanidad Animal, Instituto Nacional de Tecnología Agraria y Alimentaria (CISA-INIA), courtesy of Dr. C. Gallardo]. ASFV strain E70, a highly virulent genotype I virus, was initially isolated in 1970 from the spleen of an infected pig during an outbreak of ASF in Spain and passaged in swine buffy coat culture six times; this virus was used to infect experimental pigs without additional propagation in cell culture. The ASFV strain Ken05/Tk1, a genotype X virus of moderate virulence, was isolated from a soft tick in Kenya and subsequently passaged three times in swine buffy coat culture three times; no additional propagation in cell culture was done before challenging experimental animals. Highly virulent genotype II Armenia 2007 ASFV stock was initially derived from the spleen of an infected pig and passaged six times in swine buffy coat culture. Armenia 2007 ASFV stock was subsequently propagated on primary porcine alveolar macrophages (PAMs) obtained by bronchoalveolar lavage as previously described (Carrascosa et al., 1982). All three viruses used for experimental challenge were titrated as hemadsorbing units (HAUs) as previously described, and viral stocks were stored at − 80 °C (Carrascosa et al., 1982).

Generation of recombinant p30 antigen

Sequence of the CP204L open reading frame (ORF) coding for the ASFV protein p30 was obtained based on the Georgia 2007/1 ASFV genomic sequence (GenBank: FR682468.1) and synthesized into the pUC57 plasmid by a commercial manufacturer (GENEWIZ, South Plainfield, NJ, USA). The full-length CP204L ORF was amplified by PCR using gene-specific primers, with the forward primer (5′ CACCATGGATTTTATTTTAAAT 3′) possessing a 5′ CACC overhang and the reverse primer (5′ GGTGAGATAAAAGCTTAT 3′) possessing a stop codon deletion for c-terminal polyhistidine fusion tag expression. The PCR product was inserted into the pET101/D-TOPO vector (Invitrogen, Waltham, MA, USA) by TOPO® cloning (Invitrogen), then transformed into One Shot™ TOP10 chemically competent *E. coli* (Invitrogen). Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions, and the CP204L sequence and orientation was confirmed by PCR and commercial Sanger sequencing (GENEWIZ). The purified plasmid DNA was then transformed into One ShotTM BL21 Star™ (DE3) chemically competent *E. coli* (Invitrogen).

For recombinant ASFV p30 expression, BL21 *E. coli* were grown in Miller's LB broth (Sigma-Aldrich, St. Louis, MO, USA) containing 100 μg/mL ampicillin (Sigma-Aldrich) until reaching an O.D. of 0.6, then cultures were induced by adding 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG; Sigma-Aldrich). Sixteen h post IPTG stimulation, cultures were harvested by centrifugation and the recombinant p30 protein purified from bacterial pellets via nickel affinity chromatography under native conditions. Pellets were resuspended in pH 8.0 lysis buffer containing 50 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole (Sigma Aldrich), 10% glycerol, and benzonase nuclease (EMD Millipore, Burlington, MA, USA) at 400 U/g cell pellet, then subjected to two freeze-thaw cycles and sonicated using six 10-second bursts. Cellular debris was pelleted by centrifugation at 16,000 x G for 20 min and recombinant p30 protein isolated from the supernatant by batch purification using Ni-NTA Superflow Resin (QIAGEN) with washes and elution using a step-wise imidazole gradient. Purified recombinant p30 protein was dialyzed overnight at 4 °C against pH 7.4 phosphate-buffered saline (PBS; Gibco) containing 150 mM NaCl, 4 mM EDTA, and 10% glycerol, then concentrated using Amicon® Ultra-15 centrifugal filter units (EMD Millipore). Expression of recombinant p30 protein was confirmed by western blot (data not shown) using an anti-His (C-term) HRP monoclonal antibody (Invitrogen), and purity assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with MOPS SDS running buffer (Invitrogen) on NuPage 12% bis-tris gels (Invitrogen) followed by Coomassie blue staining using an eStain L1 Protein Staining System (GenScript, Piscataway, NJ, USA) (data not shown).

Monoclonal antibodies, indirect immunofluorescence assay (IFA) screening, and isotyping

Monoclonal antibodies to the ASFV p30 protein were generated by immunizing BALB/c mice three times with recombinant ASFV p30 antigen, followed by isolation of splenocytes one week after final vaccination and fusion with myeloma cells to generate hybridomas following standard protocols. IFA on ASFV-infected cells was used to screen monoclonal antibodies for specific reactivity to the ASFV p30 protein. Briefly, Vero E6 cells were seeded

at a density of 3.25×10^4 cells/well in 96-well plates with 100 μL/well DMEM supplemented with 2.5% FBS (Atlanta Biologicals) and 1x antibiotic-antimycotic solution (Gibco). Cells were allowed to attach for 2 h in a humidified 37 °Cincubator at 5% CO_2 . Cells were infected with ASFV strain BA71v at a MOI of 0.01 or 0.05, and at 48 h post-infection were washed with PBS pH 7.4 (Gibco), then fixed with 80% acetone (Sigma Aldrich) for 10 min at room temperature. Fixed cells were washed twice with PBS then incubated overnight at 4 °C with 75 μL/well serum-free hybridoma culture supernatant. The following morning, cells were washed three times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) (PBS-T), and then 75 μL/well PBS-T with 1% bovine serum albumin (Sigma-Aldrich) and 1:500 diluted Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) was added. Plates were incubated for 1 h at room temperature protected from light, then washed with PBS-T and counterstained with 75 μL/well 300 nM 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) substrate (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then evaluated for specific fluorescence using an EVOS FL fluorescence microscope (Thermo Fisher Scientific). Monoclonal antibodies demonstrating ASFV-specific reactivity were isotyped using a murine Pro-Detect™ Rapid Antibody Isotyping Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Experimental ASFV infections and blood collection

Thirty-one naive conventional outbred piglets, 8–12 weeks of age, were infected by intramuscular inoculation of ASFV strain Armenia 2007, E70, or Ken05/Tk1 at doses ranging from 16 to 360 HAUs as part of 6 separate animal experiments (Table 2). Anti-coagulated (EDTA) whole blood was collected via jugular venipuncture at multiple days post-challenge and stored at − 80 °C until use. ASFV-negative whole blood samples from a total of 79 piglets, 8–12 weeks of age, including pre-challenge (day 0) samples from 31 animals which were subsequently infected with ASFV, were also collected (Table 2). Pigs were monitored daily for rectal temperature and clinical disease by a veterinarian, and clinical scores were assigned to each animal by evaluating 8 clinical parameters on a scale of 0 (normal/absent) to 3 (most severe) or up to 4 for fever: liveliness (0 = normal; 1 = reduced liveliness but stands without help; 2 = sluggish and does not stand without help; 3 = dormant and refuses to stand with or without help); body shape (0 = normal; 1 = empty stomach/sunken flanks; 2 = empty stomach with indications of weight loss; 3 = wasting with visible ribs and vertebrae); respiratory function (0 = normal; 1 = increased respiratory rate; 2 = significantly increased respiratory rate and abdominal breathing; 3 = severe breathing difficulty including open mouth breathing, wheezing, or severe cough); neurological signs (0 = normal; 1 = stumbling or swaying gait that is quickly corrected; 2 = ataxia/paresis of hindquarters but able to walk; 3 = paralysis of hindquarters and inability to stand, or the presence of convulsions); skin lesions (0 = normal; 1 = erythema or cyanosis over < 10% of the body; 2 = erythema or cyanosis over 10–25% of the body or occasional skin bleeding; 3 = > 25% erythema or cyanosis, large bruising or subcutaneous bleeding, ulceration/necrosis, and cold skin); digestive symptoms (0 = normal; 1 = diarrhea of < 24 hours duration; 2 = diarrhea of > 24 hours duration or occasional vomiting; 3 = bloody diarrhea or frequent vomiting); ocular/nasal discharge (0 = normal; 1 = thin discharge from nose and/or eyes without admixtures; 2 = thick discharge from nose and/or eyes without blood; 3 = bloody discharge from nose and/or eyes); and fever (0 = 37.8–40.5 °C; 1 = 40.6–40.9 °C at one time point; 2 = 40.6–40.9 °C for two sequential days; 3 = ≥41.0 °C; 4 = < 37.8 °C). Moribund animals and those with a total clinical score > 16 were humanely euthanized by intravenous pentobarbital administration. All animal experiments and procedures were performed under high containment BSL-3Ag conditions in the Biosecurity Research Institute at Kansas State University (KSU) following protocol evaluation and approval by KSU's Institutional Biosafety Committee (IBC, protocol #: 850, 1049, 1379) and Institutional Animal Care and Use Committee (IACUC, Protocol #: 3513, 3758, 4363). To evaluate potential cross-reactivity of the p30 LFIA with a viral disease clinically similar to ASF, EDTA blood samples collected from pigs experimentally infected with classical swine fever virus (CSFV) strain Brescia were also tested.

Evaluation of whole blood samples by quantitative real-time PCR

Pre- and post-ASFV challenge, whole blood samples were evaluated for the presence of ASFV-specific genomic DNA by quantitative real-time PCR (qPCR) targeting the viral *p72* gene as previously described (Sunwoo et al., 2019). DNA was isolated from blood by magnetic bead extraction with MagAttract Suspension G beads (QIAGEN) and DNeasy Blood and Tissue Kit (QIAGEN) components using a KingFisher Duo Prime Purification System (Thermo Fisher Scientific) or Taco™ Nucleic Acid Automatic Extraction System (GeneReach, Taichung City, Taiwan), with negative and positive extraction controls included with each run. Real-time PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using PerfeCTa FastMix II (Quantabio, Beverly, MA, USA) and primer and TaqMan probe sequences for detection of the conserved region of the viral *p72* gene according to Zsak et al. (Zsak et al., 2005). Negative controls for qPCR runs were molecular grade water, and positive amplification

controls were quantified using a custom designed plasmid containing the *p72* coding region from a genotype II ASFV isolate (GenScript). Thermocycling parameters included an initial denaturation step at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s then 60 °C for 1 min. A cycle threshold (Ct) of 35 was set as the cutoff for positive reactions. Viral DNA was calculated as copy number (CN) per milliliter whole blood (CN/mL) based on a standard curve generated from serial dilutions of the positive control DNA.

LFIA testing of experimental blood samples

LFIA test strips using anti-ASFV p30 monoclonal antibodies as antigen capture and conjugated detection antibodies were developed by Silver Lake Research Corporation and provided by the manufacturer for testing. The assay consists of a reagent tube containing dried conjugate to which sample is added, and a strip-shaped dipstick through which the sample migrates and color development takes place (Fig. 3A). Whole blood aliquots were thawed at room temperature, and 10 μL blood was added to 200 μL tap water in the LFIA reagent tubes. Tubes were briefly swirled to mix the ingredients and incubated for 5 minutes at room temperature, after which the tubes were swirled again and one LFIA test strip placed in each tube, allowing the sample to migrate up the strip. Strips were visually interpreted as positive or negative after 20 min (Fig. 3B), and all results were photographed. All whole blood samples were tested in duplicate to evaluate concordance between test strip results. All manipulations involving infectious samples were performed under high containment BSL-3 laboratory conditions.

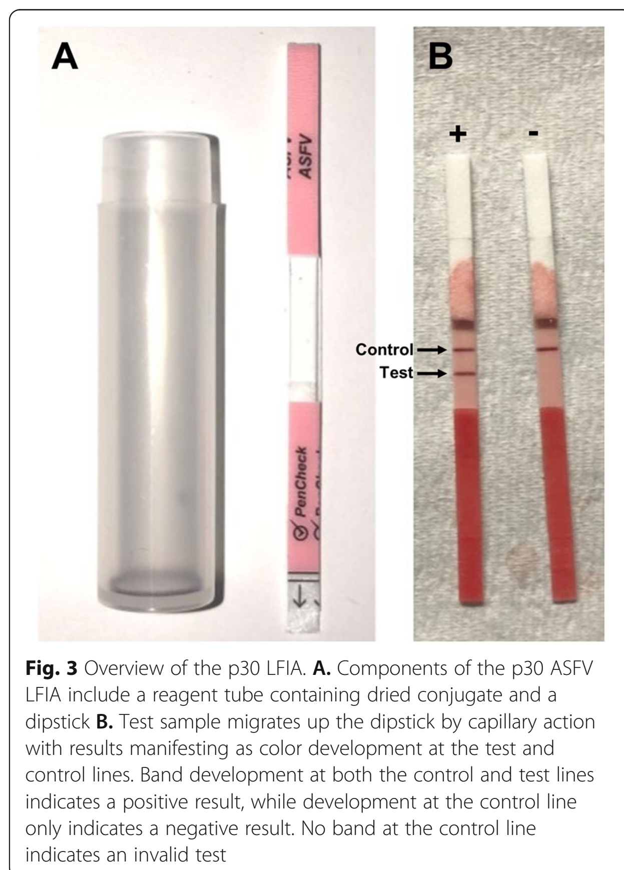

Fig. 3 Overview of the p30 LFIA. **A.** Components of the p30 ASFV LFIA include a reagent tube containing dried conjugate and a dipstick **B.** Test sample migrates up the dipstick by capillary action with results manifesting as color development at the test and control lines. Band development at both the control and test lines indicates a positive result, while development at the control line only indicates a negative result. No band at the control line indicates an invalid test

p30 amino acid sequence alignments and epitope prediction

To evaluate the degree of similarity of the viral p30 protein between ASFV isolates tested by LFIA, the amino acid sequences for the p30 protein from isolates E70 (Genbank: AAL68656), Ken05/Tk1 (Genbank: YP_009702826), and Georgia 2007/1 (Genbank: YP_009927217), a genotype II strain closely related to the Armenia 2007 ASFV strain, were aligned using the Clustal Omega multiple sequence alignment program (EMBL-EBI, Cambridgeshire, UK). The overall percent identity of amino acid sequences compared to the p30 sequence from Georgia 2007/1, which served as the basis for the recombinant antigen used to generate anti-p30 monoclonal antibodies, was determined along with conserved residues, substituted residues which share strongly similar chemical properties, and substituted residues with weak similarity. Linear B-cell epitopes within the p30 protein of each ASFV isolate were predicted using the BepiPred-2.0 web server (Jespersen et al., 2017) accessed through the Immune Epitope Database (IEDB; http://www.iedb.org) (Vita et al., 2019).

Statistical analysis

Sensitivity and specificity of the LFIA were calculated by two methods: 1) using pre- and post-ASFV challenge as the determinant for true infection status, and 2) by using ASFV qPCR as the reference test for virus detection. LFIA results for each sample were interpreted as true positive (TP), true negative (TN), false positive (FP), and false negative (FN) for samples where both LFIA duplicate tests gave the same result (i.e. both positive or both negative), or as suspect when duplicates gave conflicting results (i.e. one positive and one negative). Specificity and sensitivity of the LFIA test were calculated as 100 x TN/(TN + FP) and 100 x TP/(TP + FN), respectively. Concordance between LFIA and ASFV qPCR results, and between LFIA test duplicates, was evaluated using two-by-two contingency tables using the VassarStats website (http://vassarstats.net). Cohen's unweighted kappa coefficient (κ) was calculated to assess the significance of agreement, with κ of 0.00 indicating poor agreement, 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80

substantial agreement, and 0.81–1.00 almost perfect agreement (Landis & Koch, 1977).

Abbreviations
ASF: African swine fever; ASFV: African swine fever virus; LFIA: lateral flow immunoassay; qPCR: quantitative real-time PCR; ELISA: enzyme linked immunosorbent assay; mAbs: monoclonal antibodies; DPC: days post challenge; CN: copy number; CSFV: classical swine fever virus.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s44149-022-00045-9.

Additional file 1. Diagram of lateral flow immunoassay for antigen detection. Top: LFIAs consist of four general components: 1) a sample pad, which takes up the test sample and directs it toward the conjugate pad and test membrane; 2) a conjugate release pad containing analyte-specific antibody conjugated to a label such as colloidal gold; 3) a test membrane with immobilized antibody attached at two regions, with one region containing antibody specific for the analyte at an epitope different than the one targeted by the conjugated antibody, and the second region containing anti-IgG antibody; 4) an absorbent pad which pulls the sample across the membrane. Bottom: the test sample containing the analyte to be detected is taken up by the sample pad and directed to the conjugate release pad, where the conjugated antibody can bind the analyte. Conjugated antibody and antibody-analyte complexes are pulled across the membrane toward the absorbent pad by capillary action and interact with immobilized antibody at the test line and control line. The test line contains antibody targeting a second epitope on the analyte, allowing it to bind analyte complexed to conjugated antibody, resulting in the development of a band at the test line. The control line contains anti-IgG antibody which binds remaining conjugated antibody that is not complexed to analyte and not immobilized at the test line, resulting in the development of a band which indicates the assay is functioning correctly.

Additional file 2. LFIA test strip images for representative samples. Photographs of LFIA results were taken immediately following completion of the assay for each sample evaluated. Top: results for samples from CSFV-infected pigs showing no cross-reactivity or false positives for ASFV. Sample IDs are listed above test strip images. Bottom: representative results for post-challenge ASFV samples collected at multiple time points after infection. Sample IDs are listed above each test strip, and positive (+) or negative (−) results are denoted below each test strip.

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Authors' contributions
DWM performed the IFA screening of monoclonal antibodies, testing of the LFIA with whole blood samples, and the statistical analysis. SYS designed and produced the recombinant p30 antigen and the hybridomas for anti-p30 monoclonal antibody production. NNG produced inoculum for challenge experiments and contributed to animal experiment design and sample processing. JDT developed and performed the qPCR for viral DNA titration. IM participated in animal study design, institutional ethics board approval, and sample collection. CG provided the ASFV strains. JAR was the principal investigator overseeing all experimental work. All authors have read and approved the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval for experimental animals
Experiments involving live animals were approved by the Kansas State University Institutional Biosafety Committee (IBC, protocol #: 850, 1049, 1397) and Institutional Animal Care and Use Committee (IACUC, Protocol #: 3513, 3758, 4363).

Consent for publication
Not applicable.

Competing interests
JAR, IM, DWM, SYS are inventors on patents and patent applications on the use of antivirals, diagnostics and vaccines for the treatment and prevention of virus infections, owned by Kansas State University. The use of the p30 monoclonal antibodies was licensed to Silver Lake Research Corporation, Irwindale, CA, USA, and the product is commercially available as PenCheck® (https://www.penchecktest.com). JDT, NNG, and CG have no competing interests. Jurgen A. richt was not involved in the journal's review or decisions related to this manuscript.

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