Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Analytica Chimica Acta 1140 (2020) 30–40

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Multiplex and visual detection of African Swine Fever Virus (ASFV) based on Hive-Chip and direct loop-mediated isothermal amplification

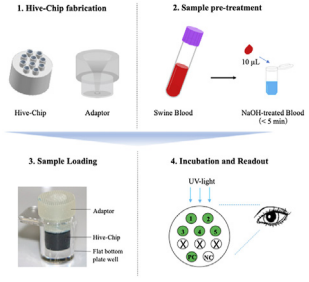
Yuan-Shou Zhu [a], Ning Shao [a], Jian-Wei Chen [a], Wen-Bao Qi [d], Yang Li [a], Peng Liu [e], Yan-Jing Chen [c], Su-Ying Bian [c], Yan Zhang [c, **], Sheng-Ce Tao [a, b, f, g, *]

[a] Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, China
[b] Perfect Diagnosis Biotechnolgoy (ZhenCe) Co., Ltd., Shanghai 200240, China
[c] CapitalBio Corporation, Beijing 102206, China
[d] College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China
[e] Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing 100084, China
[f] School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200240, China
[g] State Key Laboratory of Oncogenes and Related Genes, Shanghai Jiao Tong University, Shanghai 200240, China

HIGHLIGHTS

- Hive-Chip firstly realized simultaneous detection of multiple genes of ASFV, largely avoiding false-negative results.
- Without nucleic acid extraction, direct LAMP was firstly incorporated into the Hive-Chip for visual detection.
- Because very little operation and no complicate instrument is required, on-site detection is possible for this platform.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 July 2020
Received in revised form
26 September 2020
Accepted 5 October 2020

Keywords:
African swine fever virus
Multiplex detection
Hive-chip

ABSTRACT

African swine fever is caused by African swine fever virus (ASFV), and has a mortality rate approaching 100%. It has already caused tremendous economy lost around the world. Without effective vaccine, rapid and accurate on-site detection plays an indispensable role in controlling outbreaks. Herein, by combining Hive-Chip and direct loop-mediated isothermal amplification (LAMP), we establish a multiplex and visual detection platform. LAMP primers targeting five ASFV genes (B646L, B962L, C717R, D1133L, and G1340L) were designed and pre-fixed in Hive-Chip. On-chip LAMP showed the limits of detection (LOD) of ASFV synthetic DNAs and mock samples are 30 and 50 copies per microliter, respectively, and there is no cross-reaction among the target genes. The overall performance of our platform is comparable to that of the commercial kits. From sample preparation to results readout, the entire process takes less than 70 min. Multiplex detection of real samples of ASFV and other swine viruses further demonstrates the

* Corresponding author. Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, China, .
** Corresponding author. .
E-mail addresses: yanzhang_xinlang@sina.com (Y. Zhang), taosc@sjtu.edu.cn (S.-C. Tao).

https://doi.org/10.1016/j.aca.2020.10.011

Direct loop-mediated isothermal amplification

high sensitivity and specificity of Hive-Chip. Overall, our platform provides a promising option for on-site, fast and accurate detection of ASFV.

1. Introduction

African swine fever (ASF), caused by African swine fever virus (ASFV), is a highly contagious and lethal disease which manifests the symptom of acute hemorrhagic fever in domestic pigs and wild boars [1–3]. Since ASF was first reported in Kenya in 1921, it has rapidly spread around many countries of Africa, Europe, America and Asia [4–6]. In August 2018, the first ASF outbreak in China was identified in Shenyang City, Liaoning Province and spread to many provinces in a fast way [7]. By March 5, 2020, 165 outbreaks were reported in 32 Provinces and about 1,193,000 pigs have been culled to halt further expansion, posing a devastating impact on both local and the global swine industry [8].

ASFV is an enveloped double-stranded DNA virus transmitted by soft ticks. ASFV has a giant genome of 170–194 kb and possesses a multilayered structure and overall icosahedral morphology [9,10]. Because of complex genetic composition and high variabilities, ASFV is currently classified into 24 genotypes and more subtypes [11]. There is no effective treatment or vaccine available, thus early and accurate diagnosis of ASFV is crucial for rapid control of outbreaks. More recently, by comparing the genome of ASFV China/2018/AnhuiXCGQ strain and related European genotype II strains, Bao et al. found 54–107 variation sites contributing to the alteration of amino acid residues in 10–38 genes, which put forward the high requirements for accurate and mutation-tolerant detection [12].

The current methods for ASFV detection can be generally divided into two types: 1) The immunology detection methods, including haemadsorption test (HAD) [13], enzyme-linked immunosorbent assay (ELISA) [14], fluorescence antibody test (FAT) [15], and lateral flow assay (LFA) [16]. Although HAD is reliable and effective, they often take several days to get the results and need high-quality tissues for preparing cells. ELISA and FAT are rapid and convenient methods for precisely recognizing viral antibodies, but this serological diagnosis may not suitable for accurate early diagnosis when there is very low level of ASFV antibodies before seroconversion [17]. 2) The molecular biology detection methods, including many kinds of polymerase chain reaction (PCR) [18–20], invader assay [21], loop-mediated isothermal amplification (LAMP) [22,23], and recombinase polymerase amplification (RPA) [24]. As a gold standard for ASFV detection in the laboratory, PCR requires thermal cycling instruments and skilled operators, which is not ideal for resource-limited situations. With no need for well-equipped laboratory and professional operations, isothermal amplification has emerged as a promising on-site detection method. Recently, a variety of novel isothermal amplification assays in combination with clustered regularly interspaced short palindromic repeats (CRISPR) and lateral flow assay (LFA) have been developed for ASFV diagnosis [25–28]. These methods achieved higher sensitivity and specificity, and could be adaptable for point-of-care testing (POCT). Nevertheless, the separation of amplification and detection in open space makes it possible to generate false-positive results due to aerosol contaminations. Without amplification, a high-throughput detection system based on CRISPR-Cas12a is also established for ASFV detection, but it is inconvenient to transfer the Cas12a-cleaved product from a water bath into a disposable cartridge for detection [29]. Last but not least, all methods aforementioned are usually focused on detecting only one gene target at a time, such as B646L encoding viral protein P72 (VP72) [24–29], 9 GL encoding sulfhydryl oxidase [20], P1192R encoding topoisomerase II [22], or K78R encoding DNA binding protein P10 [23], which may lead to false-negative results because of possible mutations on the complex, highly variable genomes of ASFV. For example, Gallardo et al. found the OIE (World Organization for Animal Health)-recommended conventional PCR showed reduced sensitivity when detecting field and experimental samples because of the nucleotide mismatches between the primers and the ASFV target gene [15]. To sum up, the need for rapid, sensitive, integrated, and multiplex detection of ASFV without thermal cycler has not yet been satisfied.

Previously, we have successfully established a capillary array-based LAMP for multiplex visual detection of nucleic acids (CALM) platform for genetically modified organisms (GMOs) monitoring [30]. To incorporate the sample preparation, we subsequently developed a pipette-actuated capillary array comb (PAAC) system with integrated DNA extraction, isothermal amplification, and smartphone detection [31]. Although the integrated detection was accomplished, the fabrication of the chip with embedded glass filter paper is complicated. In comparison, a direct amplification test without DNA extraction is a real need in nucleic acid testing (NAT), such as direct PCR [32], direct LAMP [33], and direct RPA [34], which saves a lot of time and labor. Meanwhile, PAAC system was designed to identify different pathogens, usually with one target gene for each pathogen, which may not suitable to accurately detect variable targets, such as the viral nucleic acid.

Herein, we provide a multiplex and visual detection platform based on capillary array and direct LAMP for rapid and accurate detection of ASFV. Given the complex and variable properties of ASFV genomes, we choose five conserved genes (B646L, B962L, C717R, D1133L, and G1340L) as the target for detection, avoiding the false-negative results in virtue of the mutations of a certain gene. The profile of capillary array looks like a hive, so we named it "Hive-Chip". Different sets of LAMP primers are pre-fixed in each capillary. Without nucleic acid extraction, NaOH-treated swine blood sample is mixed with LAMP reagents directly. Due to the outer surface of the Hive-Chip is hydrophobic and the inner surface of the capillary is hydrophilic, LAMP mixtures can be simultaneously loaded and dispersed into each capillary by capillary forces. Then the Hive-Chip is incubated at 63 °C for 1 h with sealed optical film and the fluorescence signals are visually detected with 365 nm UV-light. To our knowledge, there is no multiplex detection platform employing multiple targets presently available for ASFV detection. Hive chip-based multiplex detection platform greatly enhances detection accuracy and shows superior characteristics, such as high sensitivity, compatibility with isothermal amplification, on-site detection capability, thus possesses the potential to become a major platform for animal or zoonotic epidemics monitoring.

2. Materials and methods

2.1. Chemicals and materials

Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich (MO, USA). Capillaries were purchased from Zhong

Cheng Quartz Glass (Beijing, China). Polydimethylsiloxane (PDMS) precursor was purchased from Dow Corning (MI, USA). Ultra-Ever Dry paint was purchased from UltraTech (FL, USA). Bst DNA polymerase large fragment was purchased from New England BioLabs (MA, USA). Chitosan, dNTP mixture, and agarose B were purchased from Sangon Biotech (Shanghai, China). QuikChange Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (CA, USA). PCR Kit and LAMP Kit for Rapid Detection of African Swine Fever Virus were purchased from Yoyoung Biotech (Guangzhou, China). The genomic DNA samples of ASFV are provided by National African Swine Fever Regional Laboratory, South China Agricultural University (Guangzhou, China). The genomic DNA samples of other swine viruses, including dsDNA virus PRV (pseudorabies virus), ssDNA virus PCV2 (porcine circovirus type 2) and PPV (porcine parvovirus), are obtained from Huazhong Agricultural University (Wuhan, China).

2.2. Preparation of primers and DNA templates

According to the multiple sequence alignment of ASFV China/2018/AnhuiXCGQ (GenBank: MK128995.1) with other genotype II ASFV strains available in NCBI database, we chose five target genes (B646L, B962L, C717R, D1133L and G1340L) based on the function type, conservation, and the length of sequences (data is partially shown in Fig. S1 and Table S1). Meanwhile, we aligned two pairs of OIE (World Organization for Animal Health)-recommended PCR primers (*i.e.*, OIE-F/OIE-R and PPA-1/PPA-2) with the target sequence (data is shown in Fig. S2). To screen more effective primers, we used PrimerExplorer V5 (http://primerexplorer.jp/e/) to design four primer sets for each target gene. All primers were synthesized by Sangon Biotech (Shanghai, China). All gene fragments were synthesized and constructed into pMV vector by Qinglan Biotech (Wuxi, China). The plasmid of mutated B646L gene was constructed using the QuikChange Site-Directed Mutagenesis Kit (primers are listed in Table S4).

2.3. Hive-Chip fabrication

The procedure for fabricating the Hive-Chip mainly consists of three parts: the cleaning of capillaries, the assembly of Hive-Chip, and the super-hydrophobic modification. Firstly, Quartz capillaries with 1.0 mm outer diameter and 0.7 mm inner diameter were cut into 4 mm sections (with a volume of ~1.6 μL) by a diamond wire cutting machine. Then the short capillaries were thoroughly cleaned with piranha solution (H_2SO_4/H_2O_2 = 3:1, v/v) for at least 0.5 h, washed by pure water and ethanol, and dried in an oven. Secondly, seven preprocessed capillaries were inserted into a PDMS support to 3.5 mm deep as demonstrated in Fig. 1, which carried ten arrayed through-holes and was modified by Ultra-Ever Dry Top Coat. According to a previous study [30], the PDMS support was made by a tubular mold with 10 patterned columns of 1.0 mm diameter inside and cut to 4 mm in length. Thirdly, the super-hydrophobic modification was the key step for simultaneously sample loading. To be specific, 15 μL Ultra-Ever Dry Top Coat was loaded carefully on the top surface of the PDMS support and the outer surfaces of the exposed part of capillaries. After that, the modified Hive-Chip was air-dried for use.

2.4. In-tube and on-chip LAMP reaction

All the LAMP primer sets for ASFV detection are listed in Tables S2 and S3. To facilitate the preparation of the reactions, we prepared a 10 × primer mixture containing 16 μM each of inner primer (FIP and BIP), 8 μM each of the loop primer (LF and LB), and 4 μM each of the outer primer (F3 and B3).

In-tube LAMP was carried out in a 25 μL reaction mixture containing 1 × ThermoPol Buffer (containing 2.0 mM $MgSO_4$), totally 8.0 mM $MgSO_4$, 1.4 mM dNTPs, 0.8 M betaine, 25 μM of calcein, 0.5 mM of $MnCl_2$, 0.32 U μL^{-1} Bst DNA polymerase, 1 × primer mixture, and the DNA template. The reaction was performed at 63 °C for less than 60 min in an incubator or thermocycler. After the reaction was completed, the LAMP products were detected in a gel imaging system under the illumination of 365 nm UV-light. To further confirm the results, the LAMP products were also electrophoresed on a 2% agarose gel to generate the predicted fragments.

On-chip LAMP adopted the same reaction system as the protocol described above, except that Bst DNA polymerase was 1.5 × (namely 0.48 U μL^{-1}) and no primers were added, because 1.6 μL of the primer set dissolved in 0.05% chitosan was already individually pre-fixed in the corresponding capillary. One blank capillary with no primers were employed as the negative control (NC). To simultaneously introduce the LAMP mixture into all seven capillaries, an adapter for sample loading was produced as our previously reported [30] (Fig. 1). The adapter had a funnel-shaped inlet connected with a shallow inverted dish, which could cover the exposed parts of all capillaries in the Hive-Chip. When the standard 100 μL pipet tip was inserted into the inlet, the LAMP mixture was gently injected and dispersed into all capillaries by capillary forces. Then the adapter was removed with the locked tip and the plate well was absolutely sealed by an optical film. Finally, the Hive-Chip was incubated at 63 °C for less than 60 min in an incubator and visually detected by a hand-held UV-light device.

2.5. Direct LAMP without nucleic acid extraction

Swine blood samples were collected in anticoagulant (EDTA-coated) tubes, followed by mixing of the samples with 100 mM NaOH in a ratio of 1:2, with a final volume of 30 μL, and incubation at room temperature for 3 min [33]. After that, 1 μL of the mixture was pipetted into LAMP reaction mixtures of 25 μL for subsequent amplification.

2.6. Data analysis

Once the LAMP reactions finished, the fluorescent images of the Hive-Chip were captured by a digital camera or a smartphone. For quantitative analysis, the fluorescent intensity data was extracted by GenePix Pro 6.1 software (Molecular Devices, CA, USA). Signal intensity was set as mean of foreground subtracted by mean of background for each capillary. Signal-to-noise ratio (SNR) was defined as the ratio of signal intensity of the target to signal intensity of the negative control, and the cut-off was set as SNR>1.5. Triplicate tests were performed for samples to verify the reproducibility of the Hive-Chip.

3. Results

3.1. The schematic and workflow for detection of ASFV by combing the Hive-Chip and direct LAMP

To optimize the detection procedure, a direct LAMP method without nucleic acid extraction was adopted [33]. As depicted in Fig. 1, minimal operation and only 3 steps are required. In the first step, swine blood samples were collected in anticoagulant tubes, followed by mixing of the samples with 100 mM NaOH in a ratio of 1:2, and incubated at room temperature. The mixtures were directly used as the template for subsequent amplification. In the second step, the LAMP reaction mixtures containing the pre-processed samples were injected into an inlet of the adapter that connects to the Hive-Chip. The mixtures could be uniformly dispersed into all

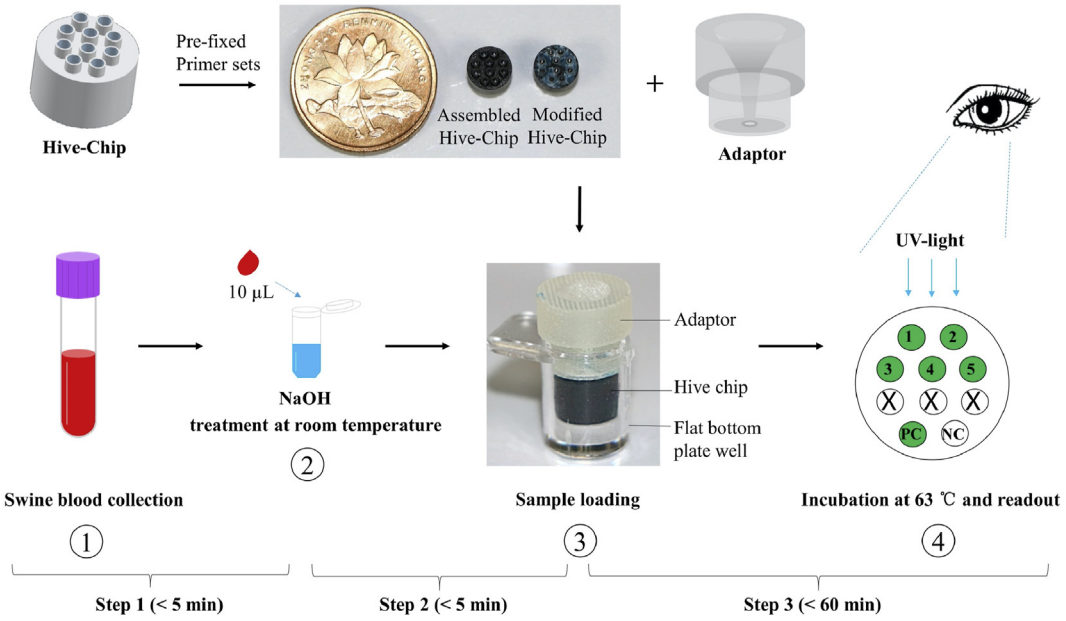


Fig. 1. Schematic illustration of the multiplex detection of ASFV based on Hive-Chip. To detect ASFV in less than 70 min, the blood sample DNA was released in 5 min, followed by 5 min for sample loading and 40–60 min for the LAMP reaction and fluorescent readout. Excited by a hand-held UV-light device, the bright green fluorescence in Hive-Chip indicates the positive LAMP amplification. The symbol "X" indicates no capillaries; NC indicates negative control; PC indicates positive control.

capillaries by capillary forces. The adapter was then removed with the locked pipet tip and the plate was fully sealed by an optical film, providing a closed space for amplification and avoiding the aerosol contamination. In the third step, the LAMP reactions were performed at 63 °C for less than 60 min in an incubator. The fluorescence signals of the amplifications were excited by a hand-held UV-light device and imaged by a digital camera or a smartphone for analysis. Importantly, the entire process from sample collection to the readout of the results takes less than 70 min.

3.2. Validity and specificity tests of in-tube LAMP

The genome comparison of a dominant strain of genotype II ASFV (China/2018/AnhuiXCGQ) with other strains of genotype II (*i.e.*, POL/2015/Podlaskie, Estonia 2014, Russia/Odintsovo_02/14/Boar and Georgia 2007/1) revealed 118 ORFs were identical among all 5 genotype II ASFV strains [12]. The conserved ORFs included 16 structural proteins and proteins involved in morphogenesis; 25 proteins involved in nucleotide metabolism, transcription, replication and DNA repair; 3 proteins acting other enzymatic activities; 4 proteins involved in host cell interactions; 23 members of the multigene family (MGF); and 47 proteins of unknown function.

To design the optimal primer sets for LAMP with high specificity, high sensitivity, and robust for a wide range of detections, we set several principles for target genes selection: (1) Since it is known that most of the variations among ASFV genomes results from gain and loss of the MGF members [35], MGFs were excluded; (2) The target genes were selected from different functional groups as diversified as possible; (3) The length of the target genes was bigger than 500 bp, the purpose is to ensure multiple sequence alignment to obtained conserved sequences in all known strains listed in GenBank database. Next, we performed a multiple sequence alignment analysis of all eligible genes and eventually chose five conserved genes for LAMP primers design, including B646L, B962L, C717R, D1133L and G1340L (data is partially shown in Fig. S1 and Table S1). Although viral protein P72 (VP72) encoded by B646L is the most frequently used gene target for ASFV detection [24–29], many mutations are still found in different strains. Besides, we also aligned two pairs of OIE (World Organization for Animal Health)-recommended PCR primers (*i.e.*, OIE-F/OIE-R and PPA-1/PPA-2) with the target sequence in B646L and found many nucleotide mismatches (Fig. S2), which was consistent with the findings from other studies [15,36]. To avoid the possible false positive detection because of gene mutations in a single gene, it is necessary to perform multi-targets detection. To assure the success of obtaining optimal primer set, we designed four primer sets for each gene (Tables S2 and S3). Evaluation of the primers were divided into two aspects: validity tests and specificity tests. Two detection methods were used to analyze the LAMP products: visual detection with fluorescent reagent and gel electrophoresis.

For validity tests (Fig. 2A), all four primer sets were effective in amplification of the target gene including B646L, B962L, D1133L and G1340L. Both fluorescence detection and gel electrophoresis showed the positive reaction. But only one primer set could successfully amplify the target gene C717R, in return, this prove the necessity of designing multiple sets of primers for a given target. Overall, we obtained at least one set of effective LAMP primers for each of the five ASFV target genes.

To access the specificity, every primer set was used to amplify all the five genes (Fig. 2B and Table S2). The results clearly showed that only when a given set of primers and the corresponding template were added, expected signals were then observed for both fluorescent visual detection and gel electrophoresis, indicating that in-tube LAMP was able to accurately and specifically identify the corresponding ASFV targets.

3.3. Specificity and sensitivity tests of on-chip LAMP

To verify the specificity on chip, LAMP mixtures containing one of the five synthetic DNAs were evenly distributed into capillaries of the Hive-Chip. Further, we set an unrelated LAMP primer set, which is specific for sad1 gene [30], as the positive control in each capillary array to demonstrate the validity of LAMP reaction. All the results were as expected (Fig. 3A). For each synthetic DNA, only the

capillary containing the corresponding pre-fixed primers showed positive signals and the rest were negative. For example, for B646L gene, apart from positive control (PC), the bright green signal was obtained only in capillary "1" pre-fixed with the matched primers. Similarly, the other four genes also showed specific amplification results, which were consistent with in-tube LAMP reactions and demonstrated high specificity.

To evaluate the sensitivity, five DNA templates were mixed in one-pot and loaded at the same time, resulting in positive signals from all the capillaries with ASFV primers in the Hive-Chip. As shown in Fig. 3B, serially diluted DNA mixtures including 880, 88, and 30 copies per microliter were successfully detected. And a sensitivity of lower than 15 copies per microliter was only achieved for the target genes C717R and D1133L, so the limit of detection (LOD) for the current Hive-Chip was around 30 copies per microliter, *i.e.*, 48 copies per reaction. According to the OIE (World Organization for Animal Health) Manual of Standards for Diagnostic Test and Vaccine [37] and the validation of actual samples [36,38], the sensitivity of OIE-recommended PCR is about 600 copies per reaction. In addition, 30 copies per microliter of DNA could be easily obtained from acute infection of ASFV [39]. These facts demonstrating that the sensitivity of Hive-Chip is suitable for ASFV detection in real world. By optimizing the reaction conditions and LAMP primers, we believe that higher sensitivity could be achieved for Hive-Chip.

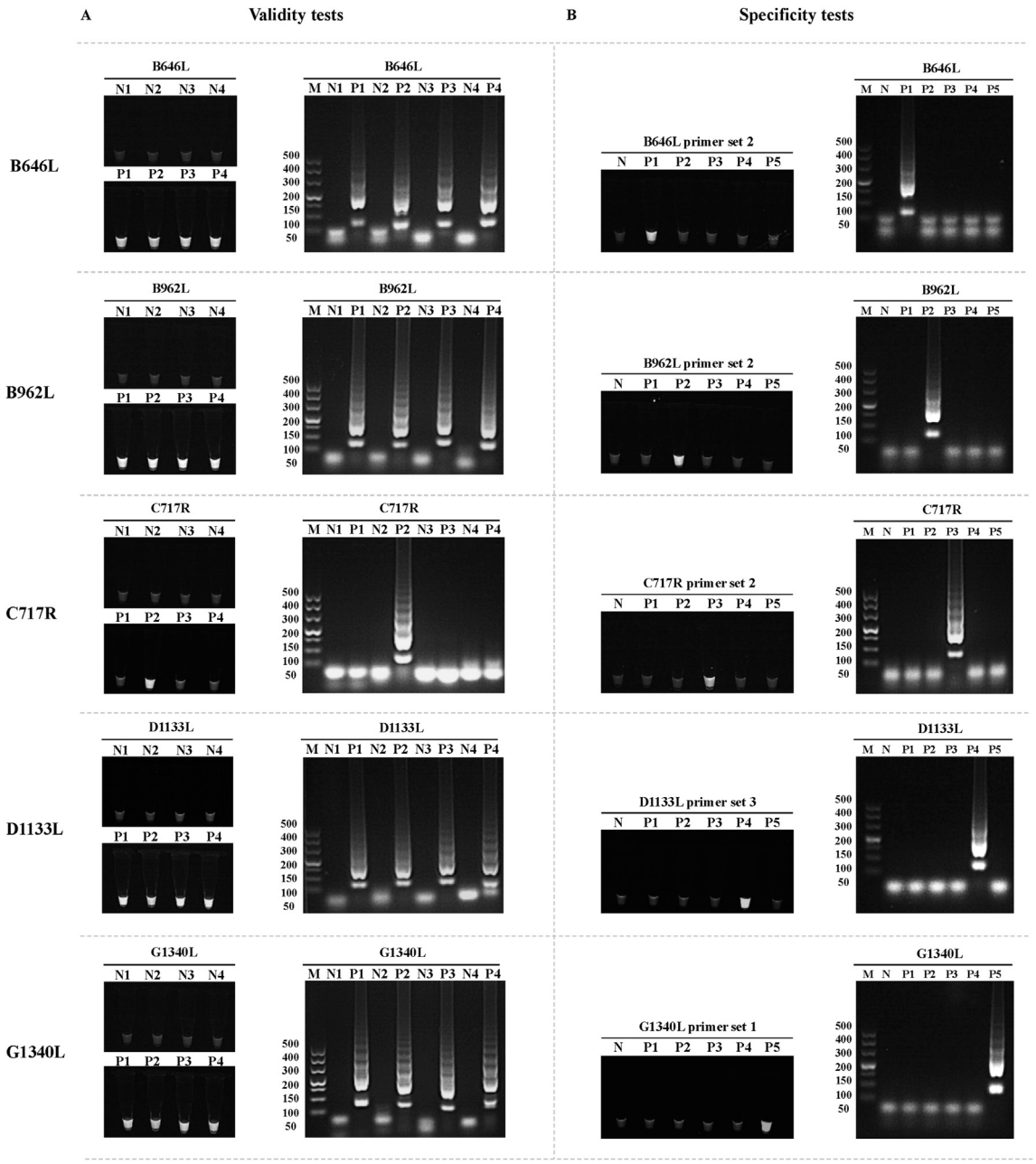


Fig. 2. A set of highly-specific LAMP primers were screened. (A) Validity tests of primers. Left panel: the calcein fluorescence images captured in a gel imaging system, bright white showed the positive reaction; right panel: gel electrophoresis analysis of the products recovered from the corresponding tubes of the left panel; 1–4: different primer sets of each gene; N: no template DNA, ddH_2O only; P: with template DNA. (B) Specificity tests among five primer sets for each target gene in tube. P1, P2, P3, P4 and P5 indicates the template B646L, B962L, C717R, D1133L and G1340L, respectively. N: no template DNA, ddH_2O only; P: with template DNA.

3.4. Testing of mock samples

Sample treatment is one of the key steps in nucleic acid testing (NAT) systems. Although nucleic acid extraction can provide high-quality and relatively pure templates for the down-stream analysis, it also complicates the procedure and enhances the risk of nucleic acid loss and cross-contamination, which is not suitable for point-of-care tests (POCTs). To simplify nucleic acid extraction, Liu et al. [33] developed an accurate, rapid and easy-to-use SNP detection method based on LAMP that do not require DNA extraction from whole blood, dried blood spot, buccal swab and saliva, this method is known as direct-LAMP. According to their results, NaOH treatment could effectively lead to cell lysis, followed by release of DNA for use in LAMP amplification. Meanwhile, various DNA polymerase inhibitors in whole blood, such as hemoglobin, IgG and proteases can be inactivated by the treatment with NaOH solution.

To further verify the applicability of Hive-Chip, the best way is to test deactivated real sample in blood collected from ASFV infected pigs. However, due to safety issue and highly restricted regulation of the government, it is very difficult to collect real samples. Thus, we decided to mimic the real sample. We prepared a set of mock samples by spike all the five synthetic DNA templates into EDTA-blood collected from a healthy pig. The DNA templates were set as 3000, 300, 100, 50, 30 and 0 copies per microliter. Because ASFV is a virus with dsDNA genome, and the viral particle is very easily to be disrupted [25,26], we believe these mock samples are very close to the real sample. By applying the mock samples, we conducted the entire analytical process from sample treatment to fluorescent readout in the Hive-Chip following the protocol shown in Fig. 1. As what we expected, five genes were successfully detected from this blood sample at the low limit of detection (LOD) of 50 copies per microliter (Fig. 4A), which were comparable with the results acquired from the pure plasmid samples. The quantitative analysis of the signal intensity obtained from the corresponding Hive-Chip (*e.g.*, 3000 copies and 30 copies per microliter) further confirmed the reliability of results. It may due to the complex background of swine blood that a sensitivity of lower than 30 copies per microliter was only achieved for the target gene B962L, C717R and D1133L. But from the perspective of detection, as long as the sensitivity of one gene reaches the LOD, ASF will not be missed. Furthermore, to avoid that the complex background of swine blood would generate false-positive, we set the healthy blood sample with no target genes (namely 0 copies per microliter) as a control. According to the results, no capillaries showed bright green fluorescence apart from positive control, which proved the capability of performing analysis of more complicated samples of ASFV. To clearly display the linear detection ranges and detection limits of different genes, we also drew sensitivity plot for each gene (Fig. 4B). Although the signal advancement of the Hive-Chip line charts exhibited positive correlated with the concentration of the targets, they did not show good linear relationship, this could be explained by the fact that LAMP is intrinsically not a linear amplification method. The fluorescence of C717R gene at 30 copies per microliter was a little bit

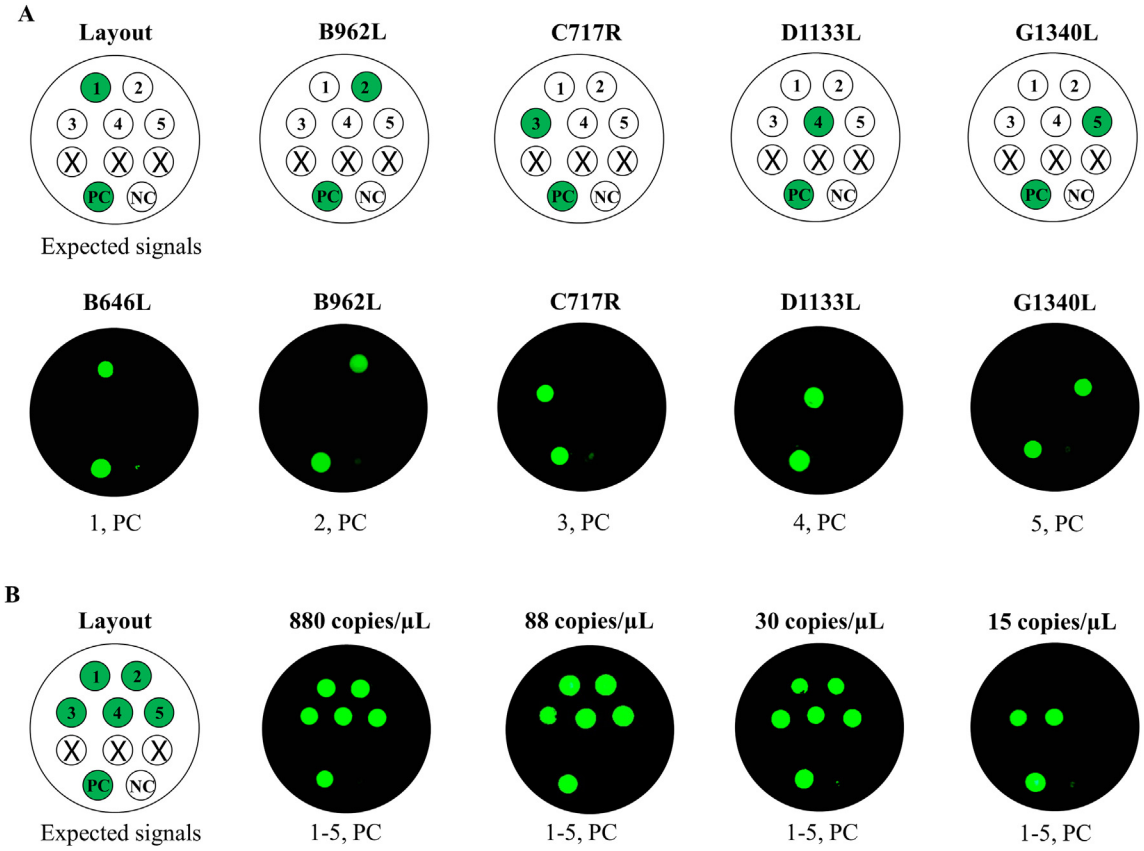


Fig. 3. Specificity and sensitivity tests of LAMP reactions performed in Hive-Chip. (A) The specificity tests with five synthetic DNAs (plasmids) separately. The template of Sad1 gene was also included as a positive control in each test. 1–5: capillaries pre-fixed with LAMP primer set of B646L, B962L, C717R, D1133L, G1340L, respectively; PC: positive control capillary pre-fixed with LAMP primer set of Sad1; NC: negative control capillary with no primer set. **(B)** The sensitivity tests with five synthetic DNAs (plasmids) of different dilutions. The template of Sad1 gene was also added. The fluorescent photographs of the Hive-Chip after LAMP were shown. The bright green color indicated positive LAMP amplification.

higher than that at 50 copies per microliter, which might result from the fluctuation of low copies templates in complex background (Fig. 4B).

Although the relatively conserved sequence (*e.g.*, B646L) of ASFV has been chosen for detection, we can still find that the mutable viral genomes bring many mismatches between the primers and the target sequence (Figs. S1 and S2). In view of the mismatches discovered in two pairs of OIE-recommended PCR primers, Luo et al. also tried to develop an updated PCR assay for detection of ASFV. However, they still target one gene and would face the same

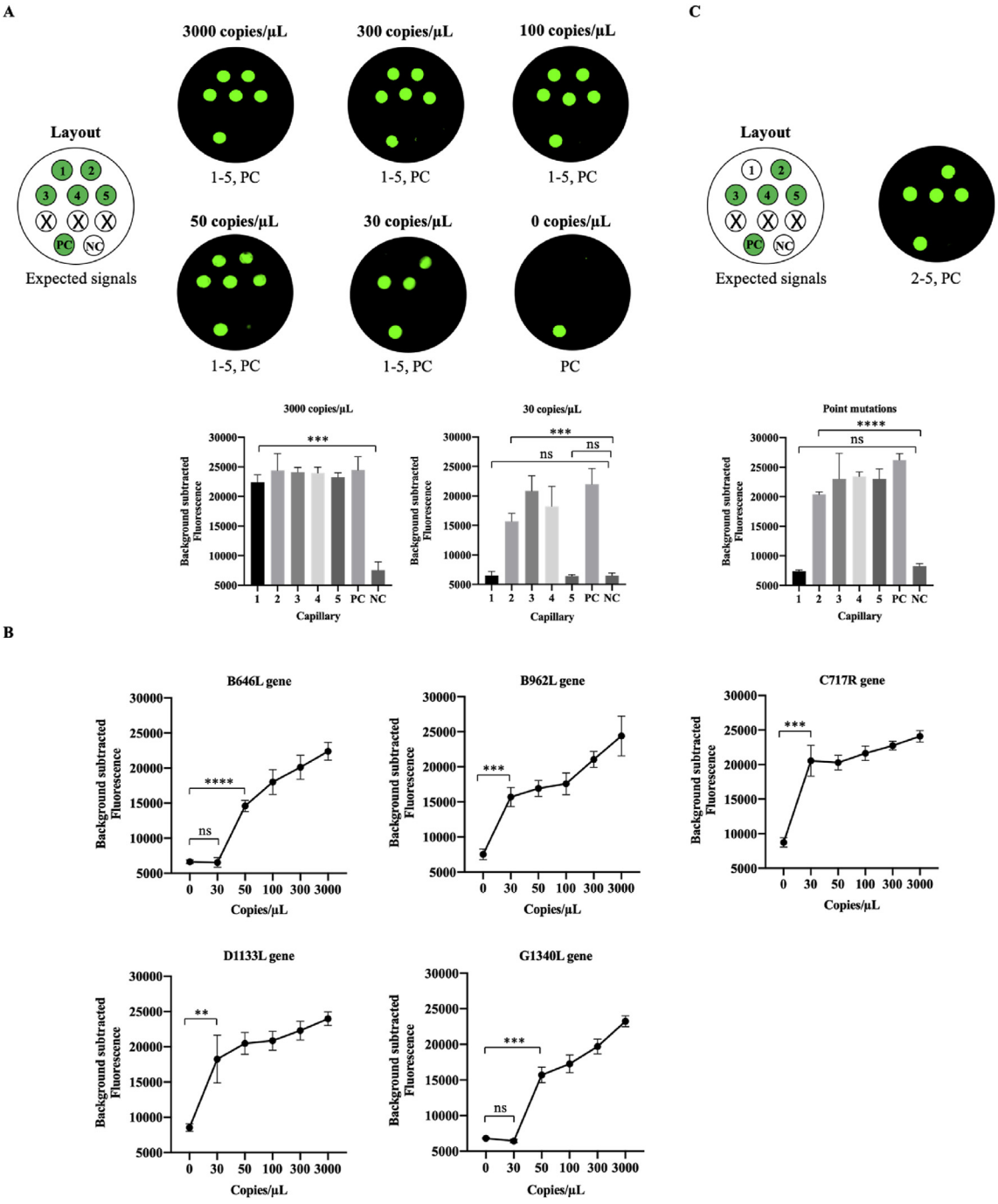


Fig. 4. Multiplex detection of ASFV with mock swine blood sample. (A) Healthy swine blood spiked with five ASFV plasmids of different concentrations. The template of Sad1 gene was included as a positive control in each test. Quantitative analysis of the signal intensity obtained from the corresponding Hive-Chip (*e.g.*, 3000 copies and 30 copies per microliter) confirmed the successful detection.1–5: capillaries pre-fixed with LAMP primer set of B646L, B962L, C717R, D1133L, G1340L, respectively; PC: positive control capillary pre-fixed with LAMP primer set of Sad1; NC: negative control capillary with no primer set. **(B)** The sensitivity plot covering the linear detection ranges and detection limits of different genes by quantitative analysis. The fluorescence of each gene at different concentration obtained from the corresponding Hive-Chip in panel (A). n = three technical replicates, two-tailed Student's *t*-test; $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$; error bars represent the SD of three measurements. **(C)** Healthy swine blood spiked with one mutant plasmid of B646L gene and four wild plasmids of B962L, C717R, D1133L and G1340L gene. The template of Sad1 gene was included as a positive control. Five point mutations (namely C355A, C360T, C365G, C373A and A379G) of B646L plasmid came from four existing ASFV strains (GenBank: EF121429.1, MH025920.1, KM111295.1 and MH025919.1). Quantitative analysis of the signal intensity confirmed the successful detection.

dilemma. Herein, to further demonstrate the necessity of multi-targets detection, we introduced five existing point mutations (namely C355A, C360T, C365G, C373A and A379G) into the locations of the LAMP primers target sequences of B646L according to the results of multiple sequence alignment of different ASFV strains. Then we conducted the Hive-Chip reaction. As shown in Fig. 4C, while "1" capillary lost the positive signal, other capillaries (namely "2–6" capillary) still showed the bright green fluorescence, which guaranteed the accuracy of ASFV detection.

To further prove that the detection performance of Hive-Chip was comparable with commercial kits, PCR kit and LAMP kit (Yoyoung Biotech) approved by China Animal Disease Control Center for ASFV detection were also tested using mock samples of serial dilutions. As shown in Fig. S3, the limit of detection (LOD) of both PCR kit and LAMP kit was about 100 copies per reaction. By contrast, the sensitivity of Hive-Chip was 50 copies per microliter, *i.e.*, 80 copies per reaction, which was comparable with that of these commercial kits. In addition, the sensitivity of our platform meets the international standard, *i.e.*, the recommended OIE Manual of Standards for Diagnostic Test and Vaccine, *i.e.*, approximately 600 copies per reaction [36–38]. As previously reported, Zhao et al. [39] adopted qPCR to quantify VP72 gene copies of the blood samples from three infectious pigs. After 3 days infection, the VP72 gene copies can reach more than 10^7 copies per milliliter (*i.e.* 10^4 copies per microliter), which is far above the limit of detection of Hive-Chip. This also confirmed the practicability of our platform. Actually, we can also increase the input of blood in the LAMP mixtures to improve the sensitivity of detection when it is necessary. Moreover, both OIE PCR and commercial kits (Yoyoung Biotech) can only detect one gene (B646L) at a time and require heavy instrumentation for fluorescence detection. While the LAMP kit needs an extra incubation at 80 °C for 3 min for sample preparation to release the nucleic acids. In the contrast, our platform is capable of monitoring multiple targets in a single test. The results could be directly visualized, and no sophisticated sample preparation is needed.

3.5. Multiplex detection of clinical samples with Hive-Chip

To further demonstrate the capability of Hive-Chip for ASFV detection, we collected three ASFV genomic DNA samples in complex mixtures. As shown in Fig. 5A, all of them were successfully detected by Hive-Chip. Moreover, nine genomic DNA samples of other major swine viruses, including pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), and porcine parvovirus (PPV), were also obtained for specificity testing. As shown in Fig. 5B and S4, the assays exhibited no cross-reaction with ASFV. These results indicate the high specificity of Hive-Chip.

To clearly demonstrate the advantages of our platform, we prepared Table S5 to compare Hive-Chip with other four representative methods for ASFV detection, as well as two commercial kits. Without nucleic acid extraction and heavy instrumentation, Hive-Chip can simultaneously detect five genes related to ASFV in a one-pot reaction, which can largely guarantee the accuracy and avoid the aerosol contamination. The total testing time is less than 70 min and the limit of detection (LOD) is 80 copies per reaction. Moreover, compared with our previous work (Table S6), *i.e.*, CALM platform [30] and PAAC system [31], the most important feature of current study is that we target multiple genes for a single pathogen (especially variable virus). To our knowledge, this concept has not been attempted for LAMP and other isothermal NAT technologies. Meanwhile, direct-LAMP was adopted to simplify the blood sample pre-treatment and facilitate the on-site detection of ASFV. Overall, we provided a total solution based on Hive-Chip for various targets and samples detection.

4. Discussion

Multiplex nucleic acid detection of variable ASFV is critical for accurate and early diagnosis of ASF. However, current methods only target one gene of ASFV, which easily brings missed inspection because of the highly mutable genomes of this virus. Herein, we established a multiplex and visual detection platform based on Hive-Chip and direct LAMP for rapid and accurate detection of ASFV. This platform assured detection accuracy through monitoring several gene fragments in a single test.

There are several advantages of our platform over the existing methods. Firstly, the design of multi-targets detection in a single reaction plays a vital part in accurately diagnosis of the mutable viral nucleic acid. If we consider the singleplex detection as a "Pistol" of shooting a target a time, Hive Chip-based multiplex detection platform may similar to a "Shotgun", which can shoot many "Enemies" (genes of ASFV) a time. In this study, apart from the B646L gene included in OIE-recommended PCR, the other four conserved genes (B962L, C717R, D1133L and G1340L) are also selected as the target for the first time for multiplex detection of ASFV, which largely avoid the false-negative results arising from the possible mutations of a certain gene. Based on this principle, Hive-Chip can serve as a versatile platform for various virus detection. For example, nucleic acid testing (NAT) of the recent outbreak of severe acute respiratory syndrome (SARS)-CoV-2 mainly covers Orf1ab gene, N gene, E gene and human RNase P gene as a control, which requires multiplex detection [40–42]. In this case, Hive-Chip can integrate multiple genes at a time to realize simultaneous detection and subsequently save a lot of time for epidemic control. Moreover, to our knowledge, it has not been reported that there is a significant difference in the expression level of these five genes in different strains of ASFV. Currently, ASFV is usually genotyped using a combination of partial sequencing of the B646L gene and analysis of the central variable region (CVR) of the B602L gene [11]. Meanwhile, other genes like p30, p54, and CP204L gene are also served as a supplementary classification standard [43]. But it is hard to use the difference or ratio of these genes to identify the genotype of samples from different regions. Secondly, multiplex NATs are usually disturbed by cross reaction between different primer sets. Hive-Chip can provide physical isolation of primers by different capillaries, and the hydrophobic modification can avoid the cross contamination of capillaries in sample loading. Meanwhile, the closed space for amplification and visual detection theoretically can prevent 100% aerosol contaminations. Thirdly, nucleic acid extraction is the key step in NATs and it often needs complex operation. Integrated with direct LAMP, the entire process of Hive-Chip from blood sample treatment to detection can be finished within 70 min independent of nucleic acid extraction and sophisticated instruments, which is suitable for on-site detection of ASFV. Besides, other direct amplification methods including direct RPA [34] could also be easily combined with our platform for different types of samples.

Last but not least, Hive-Chip based platform has good flexibility and compatibility. The current version of chip has ten channels for detecting as many as eight targets other than positive and negative control. As for ASFV detection, we only adopt five targets and leave three channels unused. Furthermore, by increasing the size of Hive-Chip, we can increase the channel number and locate more capillaries in a single Hive-Chip unit. Because the current format of Hive-Chip is compatible with standard 96-well plate and it can be assembled into a single well, we can easily perform high-throughput sample loading by means of the multi-channel pipette [30]. Based on this, Hive-Chip can be integrated with the current automated liquid workstation (*e.g.,* Beckman & Biomek 4000) for high-throughput sample loading. Furthermore, to realize

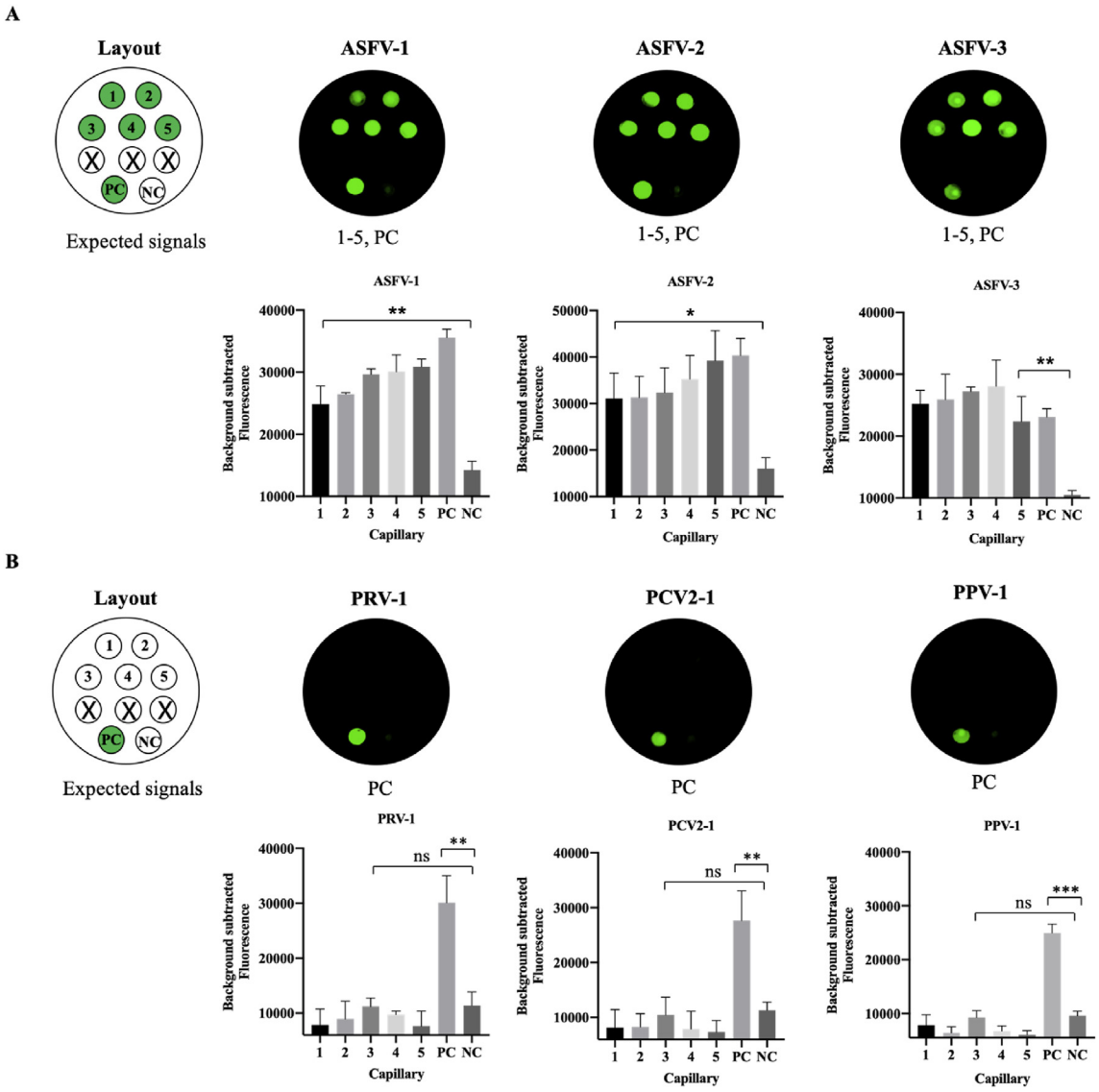


Fig. 5. Multiplex detection of real samples of ASFV and other swine viruses based on Hive-Chip platform. (A) Three genomic DNA samples of ASFV are detected by Hive-Chip. **(B)** The specificity testing against other swine viruses, including PRV (pseudorabies virus), PCV2 (porcine circovirus type 2) and PPV (porcine parvovirus). The template of Sad1 was included a positive control. n = three technical replicates, two-tailed Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; error bars represent the SD of three measurements.

massive detection, we can also employ multi-mode plate reader system (*e.g.,* Biotek & Cytation 5 Cell Imaging Multi-Mode Reader) to acquire fluorescence images for subsequent signal analysis. We believe that Hive-Chip-based platform is suitable for high-throughput screening when combined with widely accessible instruments of 96-well plate operation.

Unfortunately, due to the lack of biosafety laboratory for handling the infectious real samples, we didn't have chance to test swine blood sample obtained from pigs with diagnosed infection. However, sensitive detection of mock blood sample spiked with synthetic ASFV DNAs demonstrated the compatibility of our platform with the multiplex amplifications and the analysis of actual samples. Meanwhile, side-by-side comparison with two approved commercial kits, our platform also showed similar detection performance. Furthermore, multiplex detection of ASFV genomic DNA samples also demonstrate the utility and capability of Hive-Chip. The specificity testing of other swine viruses, including PRV, PCV2, and PPV, showed no cross-reaction with ASFV detection. To upgrade and optimize the Hivfige-Chip platform for ASFV detection, we are developing a portable and high-throughput detection device with integrated isothermal amplification and real-time fluorescence detection.

In summary, we have developed a multiplex visual detection method based on Hive-Chip and direct LAMP with a streamlined operation procedure, which offers a feasible strategy and key reagents for rapid and accurate on-site detection of ASFV. The method that we demonstrated is of high generality, it could be easily adopted for fast development of assays/kits for animal diseases or possible future outbreaks.

CRediT authorship contribution statement

Yuan-Shou Zhu: Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Ning Shao:** Methodology, Writing - original draft. **Jian-Wei Chen:** Methodology, Writing - original draft. **Wen-Bao Qi:** Resources. **Yang Li:** Supervision. **Peng Liu:** Methodology. **Yan-Jing Chen:** Methodology. **Su-Ying Bian:** Methodology. **Yan Zhang:** Methodology. **Sheng-Ce**

Tao: Conceptualization, Supervision, Resources, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sheng-Ce Tao is the founder of Perfect Diagnosis Biotechnolgoy (ZhenCe) Co., Ltd. (Shanghai, China).

Acknowledgements

This work was partially supported by Science and Technology Commission of Shanghai Municipality (No. 19441911900), Interdisciplinary Program of Shanghai Jiao Tong University (No. YG2020YQ10), National Key Research and Development Program of China (No. 2016YFD0500808-4), National Natural Science Foundation of China (No. 31941014, 31970130, 31600672, 31670831, and 31370813). We thank Mr. Jinjun Zhang, Dr. Jikai Wen and Dr. Jiaoyu Deng for their help on collecting DNA samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.10.011.

References

[1] D. Normile, Arrival of deadly pig disease could spell disaster for China, Science 361 (2018) 741.
[2] J. Ma, H. Chen, X. Gao, J. Xiao, H. Wang, African swine fever emerging in China: Distribution characteristics and high-risk areas, Prev. Vet. Med. 175 (2020) 104861.
[3] I. Galindo, C. Alonso, African swine fever virus: a review, Viruses 9 (2017).
[4] R. Eustace Montgomery, On A form of swine fever occurring in British east Africa (Kenya colony), J. Comp. Pathol. Ther. 34 (1921) 159–191.
[5] S. Costard, L. Mur, J. Lubroth, J.M. Sanchez-Vizcaino, D.U. Pfeiffer, Epidemiology of African swine fever virus, Virus Res. 173 (2013) 191–197.
[6] S. Dei Giudici, G. Franzoni, P. Bonelli, D. Bacciu, G. Sanna, P.P. Angioi, M. Ledda, G. Pilo, P. Nicolussi, A. Oggiano, Interaction of historical and modern Sardinian African swine fever viruses with porcine and wild-boar monocytes and monocyte-derived macrophages, Arch. Virol. 164 (2019) 739–745.
[7] X. Li, K. Tian, African swine fever in China, Vet. Rec. 183 (2018) 300–301.
[8] Food and Agricultural Organization of the United Nations, Animal production and Health, ASF situation in Asia update, Available at:: http://www.fao.org/ag/againfo/programmes/en/empres/ASF/situation_update.html, , 2020. (Accessed 5 March 2020).
[9] C. Alonso, M. Borca, L. Dixon, Y. Revilla, F. Rodriguez, J.M. Escribano, Ictv report consortium, ICTV virus taxonomy profile: Asfarviridae, J. Gen. Virol. 99 (2018) 613–614.
[10] N. Wang, D. Zhao, J. Wang, Y. Zhang, M. Wang, Y. Gao, F. Li, J. Wang, Z. Bu, Z. Rao, X. Wang, Architecture of African swine fever virus and implications for viral assembly, Science 366 (2019) 640–644.
[11] C.J. Quembo, F. Jori, W. Vosloo, L. Heath, Genetic characterization of African swine fever virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype, Transbound Emerg Dis 65 (2018) 420–431.
[12] J. Bao, Q. Wang, P. Lin, C. Liu, L. Li, X. Wu, T. Chi, T. Xu, S. Ge, Y. Liu, J. Li, S. Wang, H. Qu, T. Jin, Z. Wang, Genome comparison of African swine fever virus China/2018/AnhuiXCGQ strain and related European p72 Genotype II strains, Transbound Emerg Dis 66 (2019) 1167–1176.
[13] J.M. Rodríguez, R.J. Yáñez, F. Almazán, E. Viñuela, J.F. Rodriguez, African swine fever virus encodes a CD2 homolog responsible for the adhesion of erythrocytes to infected cells, J. Virol. 67 (1993) 5312–5320.
[14] C. Cubillos, S. Gómez-Sebastian, N. Moreno, M.C. Nuñez, L.K. Mulumba-Mfumu, C.J. Quembo, L. Heath, E.M.C. Etter, F. Jori, J.M. Escribano, E. Blanco, African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples, Virus Res. 173 (2013) 159–167.
[15] C. Gallardo, R. Nieto, A. Soler, V. Pelayo, J. Fernández-Pinero, I. Markowska-Daniel, G. Pridotkas, I. Nurmoja, R. Granta, A. Simón, C. Pérez, E. Martín, P. Fernández-Pacheco, M. Arias, Assessment of African swine fever diagnostic techniques as a response to the epidemic outbreaks in eastern European union countries: How to improve surveillance and control programs, J. Clin. Microbiol. 53 (2015) 2555–2565.
[16] P. Sastre, C. Gallardo, A. Monedero, T. Ruiz, M. Arias, A. Sanz, P. Rueda, Development of a novel lateral flow assay for detection of African swine fever in blood, BMC Vet. Res. 12 (2016) 206.
[17] J. Carlson, L. Zani, T. Schwaiger, I. Nurmoja, A. Viltrop, A. Vilem, M. Beer, S. Blome, Simplifying sampling for African swine fever surveillance: Assessment of antibody and pathogen detection from blood swabs, Transbound Emerg Dis 65 (2018) e165–e172.
[18] M. Agüero, J. Fernández, L. Romero, C. Sánchez Mascaraque, M. Arias, J.M. Sánchez-Vizcaíno, Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples, J. Clin. Microbiol. 41 (2003) 4431–4434.
[19] A. Wang, R. Jia, Y. Liu, J. Zhou, Y. Qi, Y. Chen, D. Liu, J. Zhao, H. Shi, J. Zhang, G. Zhang, Development of a novel quantitative real-time PCR assay with lyophilized powder reagent to detect African swine fever virus in blood samples of domestic pigs in China, Transbound Emerg Dis 67 (2020) 284–297.
[20] J. McKillen, M. McMenamy, B. Hjertner, F. McNeilly, Å. Uttenthal, C. Gallardo, B. Adair, G. Allan, Sensitive detection of African swine fever virus using real-time PCR with a 5′ conjugated minor groove binder probe, J. Virol Methods 168 (2010) 141–146.
[21] B. Hjertner, B. Meehan, J. McKillen, F. McNeilly, S. Belák, Adaptation of an Invader assay for the detection of African swine fever virus DNA, J. Virol. Methods. 124 (2005) 1–10.
[22] H.E. James, K. Ebert, R. McGonigle, S.M. Reid, N. Boonham, J.A. Tomlinson, G.H. Hutchings, M. Denyer, C.A.L. Oura, J.P. Dukes, D.P. King, Detection of African swine fever virus by loop-mediated isothermal amplification, J. Virol. Methods. 164 (2010) 68–74.
[23] D. Wang, J. Yu, Y. Wang, M. Zhang, P. Li, M. Liu, Y. Liu, Development of a real-time loop-mediated isothermal amplification (LAMP) assay and visual LAMP assay for detection of African swine fever virus (ASFV), J. Virol. Methods. 276 (2020), 113775.
[24] J. Wang, J. Wang, Y. Geng, W. Yuan, A recombinase polymerase amplification-based assay for rapid detection of African swine fever virus, Can. J. Vet. Res. 81 (2017) 308–312.
[25] X. Wang, E. Xiong, T. Tian, M. Cheng, W. Lin, H. Wang, G. Zhang, J. Sun, X. Zhou, Clustered regularly interspaced short palindromic repeats/cas9-mediated lateral flow nucleic acid assay, ACS Nano 14 (2020) 2497–2508.
[26] C. Yuan, T. Tian, J. Sun, M. Hu, X. Wang, E. Xiong, M. Cheng, Y. Bao, W. Lin, J. Jiang, C. Yang, Q. Chen, H. Zhang, H. Wang, X. Wang, X. Deng, X. Liao, Y. Liu, Z. Wang, G. Zhang, X. Zhou, Universal and naked-eye gene detection platform based on the clustered regularly interspaced short palindromic repeats/cas12a/13a system, Anal. Chem. 92 (2020) 4029–4037.
[27] X. Wang, P. Ji, H. Fan, L. Dang, W. Wan, S. Liu, Y. Li, W. Yu, X. Li, X. Ma, X. Ma, Q. Zhao, X. Huang, M. Liao, CRISPR/Cas12a technology combined with immunochromatographic strips for portable detection of African swine fever virus, Commun Biol 3 (2020) 62.
[28] J. Bai, H. Lin, H. Li, Y. Zhou, J. Liu, G. Zhong, L. Wu, W. Jiang, H. Du, J. Yang, Q. Xie, L. Huang, Cas12a-Based on-site and rapid nucleic acid detection of African swine fever, Front. Microbiol. 10 (2019) 2830.
[29] Q. He, D. Yu, M. Bao, G. Korensky, J. Chen, M. Shin, J. Kim, M. Park, P. Qin, K. Du, High-throughput and all-solution phase African Swine Fever Virus (ASFV) detection using CRISPR-Cas12a and fluorescence based point-of-care system, Biosens. Bioelectron. 154 (2020), 112068.
[30] N. Shao, J. Chen, J. Hu, R. Li, D. Zhang, S. Guo, J. Hui, P. Liu, L. Yang, S.-C. Tao, Visual detection of multiple genetically modified organisms in a capillary array, Lab Chip 17 (2017) 521–529.
[31] J. Hui, Y. Gu, Y. Zhu, Y. Chen, S.-J. Guo, S.-C. Tao, Y. Zhang, P. Liu, Multiplex sample-to-answer detection of bacteria using a pipette-actuated capillary array comb with integrated DNA extraction, isothermal amplification, and smartphone detection, Lab Chip 18 (2018) 2854–2864.
[32] S.E. Cavanaugh, A.S. Bathrick, Direct PCR amplification of forensic touch and other challenging DNA samples: A review, Forensic Sci Int Genet 32 (2018) 40–49.
[33] X. Liu, C. Zhang, M. Zhao, K. Liu, H. Li, N. Li, L. Gao, X. Yang, T. Ma, J. Zhu, W. Hui, K. Hua, Y. Cui, A direct isothermal amplification system adapted for rapid SNP genotyping of multifarious sample types, Biosens. Bioelectron. 115 (2018) 70–76.
[34] C. Myhrvold, C.A. Freije, J.S. Gootenberg, O.O. Abudayyeh, H.C. Metsky, A.F. Durbin, M.J. Kellner, A.L. Tan, L.M. Paul, L.A. Parham, K.F. Garcia, K.G. Barnes, B. Chak, A. Mondini, M.L. Nogueira, S. Isern, S.F. Michael, I. Lorenzana, N.L. Yozwiak, B.L. MacInnis, I. Bosch, L. Gehrke, F. Zhang, P.C. Sabeti, Field-deployable viral diagnostics using CRISPR-Cas13, Science 360 (2018) 444–448.
[35] L.K. Dixon, D.A.G. Chapman, C.L. Netherton, C. Upton, African swine fever virus replication and genomics, Virus Res. 173 (2013) 3–14.
[36] Y. Luo, S.A. Atim, L. Shao, C. Ayebazibwe, Y. Sun, Y. Liu, S. Ji, X.-Y. Meng, S. Li, Y. Li, C. Masembe, K. Ståhl, F. Widén, L. Liu, H.-J. Qiu, Development of an updated PCR assay for detection of African swine fever virus, Arch. Virol. 162 (2017) 191–199.
[37] P.J. Wilkinson, African swine fever, in: Manual of Standards for Diagnostic Test and Vaccines, fourth ed., Office International des Épiz- ooties, Paris, 2000, pp. 189–198.
[38] D.P. King, S.M. Reid, G.H. Hutchings, S.S. Grierson, P.J. Wilkinson, L.K. Dixon, A.D.S. Bastos, T.W. Drew, Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus, J. Virol. Methods. 107 (2003) 53–61.
[39] D. Zhao, R. Liu, X. Zhang, F. Li, J. Wang, J. Zhang, X. Liu, L. Wang, J. Zhang, X. Wu, Y. Guan, W. Chen, X. Wang, X. He, Z. Bu, Replication and virulence in

pigs of the first African swine fever virus isolated in China, Emerg. Microb. Infect. 8 (2019) 438–447.
[40] V.M. Corman, O. Landt, M. Kaiser, R. Molenkamp, A. Meijer, D.K. Chu, T. Bleicker, S. Brünink, J. Schneider, M.L. Schmidt, D.G. Mulders, B.L. Haagmans, B. van der Veer, S. van den Brink, L. Wijsman, G. Goderski, J.-L. Romette, J. Ellis, M. Zambon, M. Peiris, H. Goossens, C. Reusken, M.P. Koopmans, C. Drosten, Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR, Euro Surveill. 25 (2020).
[41] J.P. Broughton, X. Deng, G. Yu, C.L. Fasching, V. Servellita, J. Singh, X. Miao, J.A. Streithorst, A. Granados, A. Sotomayor-Gonzalez, K. Zorn, A. Gopez, E. Hsu, W. Gu, S. Miller, C.-Y. Pan, H. Guevara, D.A. Wadford, J.S. Chen, C.Y. Chiu, CRISPR–Cas12-based detection of SARS-CoV-2, Nat. Biotechnol. (2020) 1–5.
[42] L. Dong, J. Zhou, C. Niu, Q. Wang, Y. Pan, S. Sheng, X. Wang, Y. Zhang, J. Yang, M. Liu, Y. Zhao, X. Zhang, T. Zhu, T. Peng, J. Xie, Y. Gao, D. Wang, Y. Zhao, X. Dai, X. Fang, Highly Accurate and Sensitive Diagnostic Detection of SARS-CoV-2 by Digital PCR (2020), https://doi.org/10.1101/2020.03.14.20036129 medRxiv. [Preprint].
[43] D.A.G. Chapman, A.C. Darby, M. Da Silva, C. Upton, A.D. Radford, L.K. Dixon, Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus, Emerg. Infect. Dis. 17 (2011) 599–605.