



An immuno-chromatographic lateral flow assay (LFA) for rapid on-the-farm detection of classical swine fever virus (CSFV)

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Abstract Classical swine fever (CSF) is a highly contagious and potentially fatal disease of domestic pigs. Classical swine fever is routinely diagnosed by clinical signs, serology, detection of CSF virus (CSFV) nucleic acid by PCR and virus isolation. Most of the current CSF diagnostic methods are expensive and have an extended turnaround time. In the majority of the CSF endemic countries, lack of easy access to diagnostic facilities is a major problem for swine producers trying to obtain early diagnosis and often results in the entire herd being infected. The acute form of CSF can show non-specific signs of illness, leaving CSF often undiagnosed. Hence there is an urgent need for a rapid and reliable pen side diagnostic assay for the better detection and control of this economically important disease of swine. We developed an immuno-chromatographic lateral flow assay (LFA) for on the farm detection of CSFV. A CSFV isolate [CSFV/AP/TRP2/2009 (TS2)] of genotype 1.1 was used for the production of monoclonal antibodies (mAbs) for the LFA's development. The virus detection level of the LFA device was 36.8 TCID₅₀/ml of CSFV. The sensitivity and specificity of LFA in comparison with PCR were 80.36% and 87.10%, respectively. The positive and negative predictive values of the LFA device were 91.84% and 87.10%, respectively. In conclusion, the CSFV-LFA is a reliable

and convenient resource for preliminary on the farm detection of classic swine fever.

Abbreviations

CSF Classical swine fever
CSFV Classical swine fever virus

Introduction

Classical swine fever (CSF) is a highly contagious disease of swine and is a World Organization for Animal Health (OIE)-listed disease due to its huge economic impact on pig production worldwide. CSF is caused by the classical swine fever virus (CSFV), an enveloped virus with single stranded positive-sense RNA genome belonging to the genus *Pestivirus* within the family *Flaviviridae*. There are a number of methods currently being used for the diagnosis of CSF, including viral antigen detection by direct fluorescent antibody test (FAT), nucleic acid detection by reverse transcriptase polymerase chain reaction (RT-PCR) and virus isolation.

Serological diagnosis of CSF can be accomplished by virus neutralization peroxidase-linked assay, fluorescent antibody virus neutralization test (FVNT) or enzyme-linked immunosorbent assay (ELISA). After the first report of CSF in 1962 [1], there were 1308 outbreaks of CSF in India during 1996–2008 [2]. Due to the remoteness of the popular pig producing regions, being distant from diagnostic facilities, as well as the sporadic nature of the disease in India, CSF has not been studied systematically, and therefore the epidemiology of the disease is largely unknown. A major limiting factor in resource—poor settings is that the current CSF diagnostic techniques are expensive, complex, time

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consuming and require equipped diagnostic facilities with trained technical staff. Hence, there is an urgent need for a rapid, sensitive and reliable pen side diagnostic tool for primary screening of CSF at the farm level, prior to confirmation testing at a diagnostic facility.

The Immuno-chromatographic Lateral Flow Assay (LFA) was first described in the 1960's and over the years it has been used extensively for the diagnosis of various infectious diseases [3–5]. A major advantage of LFA is that it can be used as a rapid first-line diagnostic tool without the need for trained personnel or specialized equipment. LFA reagents and materials are usually stable, and relatively inexpensive to produce [6], and the assay is robust, making LFA the preferred pen-side diagnostic tests of choice for use in rural and resource poor settings. Here, we describe the development and evaluation of a LFA for on the farm diagnosis of CSFV.

Materials and methods

CSFV mAbs were produced as previously described [7]. Briefly, a local CSFV isolate of genotype 1.1 [CSFV/AP/TRP2/2009 (TS2)] was used for the production of viral antigen. The virus was propagated in PK-15 cells and purified by sucrose gradient centrifugation. The viral protein concentration was measured by Bradford assay and analyzed by polyacrylamide gel electrophoresis and western blot. Purified viral antigen (62.5 µg) was mixed with equal volume of Freund's complete adjuvant and injected intraperitoneally at a dose of 100 µl/mouse (50 µg of the viral protein per mouse) into 8-10 week old BALB/c mice on day 0. Another 100 µL of the antigen mixed with an equal volume of Freund's incomplete adjuvant was injected at 14, 28, 42, 56 and 70 days post initial injection. Subsequently harvested mouse sera were tested for antibodies at 21 days by in house indirect ELISA.

Cell fusion was carried out following the standard procedure of Galfre and Milstein (1981) with some modifications. Briefly, 1 ml of 50% PEG 4000 was added slowly to the cells (splenocytes; myeloma cells = 5:1) for 30 s with constant flicking, and left undisturbed for 90 s. This was followed by washing with RPMI and centrifugation at 130 g for 5 min. The pellet was suspended in RPMI medium containing 20% FCS and HAT supplement to obtain $3\text{--}5 \times 10^5$ cells/ml. The cells were distributed in 100 µl volumes in to 96 well plates containing feeder cells and screened after 7 days.

In summary, 8 plates (96 well microtitre plates, NUNC, Denmark) were seeded at 50,000 cell/plate using a mixture of 5×10^6 cells/ml of mouse myeloma Sp2/0-Ag cells and 5×10^8 cells/ml of mouse splenocytes. The cell culture supernatant from the hybridoma clones was screened for

the presence of antibodies by indirect ELISA. A total of 37 hybridoma clones secreting anti-CSFV antibody were obtained with a mean fusion efficiency of 20.2%. Two of these hybridoma clones, 3B10 and 2G6, were sub-cloned by limiting dilution. The mAbs were concentrated using the Milipore Centriprep PROSEP G column concentrator and purified by SAS and PEG followed by dialysis. Briefly, solid PEG 6000 (Hi Media, India) at 12.5% (w/v) concentration or saturated ammonium sulphate (SAS) to a concentration of 45% (v/v) was added to the cell culture fluid (CCF) (55 ml) drop by drop with constant stirring at 4 °C for 30 min before being centrifuged at 1000g at 4 °C for 15 min. The pellet was dissolved in a minimum volume of PBS and dialyzed three times in PBS at 4 °C. The dialysate was further affinity purified by using a Milipore Centriprep PROSEP G column.

The detector reagent was prepared by adding affinity purified 3B10 mAbs (1.4 mg/ml) with 10 ml of 40 nm colloidal gold (Sigma, USA). The colloidal gold solution (Sigma, 1%, w/v) was adjusted to pH 9.0 with sodium hydroxide. Anti-CSFV mAb (0.2 ml; 1.4 mg/ml; pH 9.0) was affinity purified and added to 10 ml of pH-adjusted colloidal gold solution. The mixture was gently mixed for 30 min after adding 10% BSA as a blocking solution and finally centrifuged for 30 min at 6000g at 4 °C. After centrifugation, the colloidal gold pellet was suspended in gold conjugation (GC) buffer (0.01 M Tris; 0.08 g NaCl; 2% sucrose, 5% BSA and 0.1% sodium azide). The anti-CSFV mAb coated colloidal gold nanoparticles were ready for use in LFA on the release pad.

Affinity purified 2A7. 3B10 mAb and rabbit anti-mouse IgG (Genei, Bangalore) were applied 0.5 cm apart linearly as test and control lines respectively onto a nitrocellulose membrane (MDI Advanced Diagnostics, Ambala, Haryana, India). The membrane was dried at 37 °C for 2 h and stored at room temperature (RT).

The Easypack lateral flow assembly kit (Advanced Microdevices, India) was used to assemble the LFA device. The conjugate pad containing colloidal gold: 2A7. 3B10 conjugate (20 µl/cm) was overlaid in parallel onto the nitrocellulose membrane containing test and control lines. The sample pad (Type GFB-RTC 15 x 260 nm MDI, Haryana) and the absorbent pad were placed on either end of the nitrocellulose membrane with a 2 mm overlap. The assembled membrane was cut into 3 mm individual strips and stored at RT until further use.

Test antigen was prepared as previously described [8]. Briefly, pig tissue samples (liver, spleen, lymph nodes, tonsils or pooled tissue samples) were homogenized in minimal essential medium (MEM) to prepare a 10% w/v suspension. Homogenates were clarified at 1400xg for 20 min at 4 °C. NP 40 was added to the supernatant to a final concentration of 1% v/v and incubated at 25 °C for

2 h with constant shaking. The tissue suspension was further centrifuged at 1200 \times g for 10 min and the supernatant was collected in LFA buffer.

The LFA device was optimized for sample volume, mAb concentration and pore size of the nitrocellulose membrane. A mAb coating concentration of 40 μ g/device was used with 10 μ m pore sized nitrocellulose membrane. A sample volume of 100 μ l was found to be optimal for the assay. 100 μ l of the test sample was added to the sample pad followed by 100 μ l of the LFA running buffer (13% SDS in PBS). Samples positive for CSFV viral antigen bound with the colloidal gold-2A7.3B10 mAb conjugate and migrated down the device by capillary action and reacted with immobilized 2A7.3B10 mAb to form a red band at the test line. Unbound colloidal gold – 2A7.3B10 mAb conjugate continued to flow along the membrane and reacted with rabbit anti-mouse IgG to produce a red band at the control line. The result was read starting at 1 min and up to 10 min after adding the sample. Distinct red bands at both the test line and the control line indicated a positive sample whereas no band at the test line indicated a negative sample (Figure 1). A serial tenfold dilution of the CSFV stock [CSFV/AP/TRP2/2009 (TS2)-at 15th passage with a titer of 3.68 log₁₀ TCID₅₀.] was used to determine the analytical sensitivity of the test on the LFA. Specificity of the device was demonstrated using CSFV infected and uninfected cell culture supernatant.

A total of 72 CSF suspected samples were tested using the LFA device and the results were compared with those obtained by standard CSF RT-PCR assay. Specific primers described by Lowings *et al.*, 1996 and Ruggli *et al.*, 1996 were used for RT-PCR assay targeting the 5' UTR, E2 and NS5B genes [9, 10]. The RT-PCR positive samples were passaged in PK15 cells. At the 5th and 10th passages the isolates were tested for CSF by indirect FAT and RT-PCR. The lapinized vaccine virus (Mhow vaccine) was used as a positive control for PCR. CSF reference sera numbers 0902, 0277 and 1004 (provided as a kind gift from The European Reference Laboratory, Hannover, Germany) were used as positive control sera for the FAT.

The LFA device was further validated at the College of Veterinary Sciences, Assam Agricultural University by screening 32 tissue antigen suspensions. The results were compared with the results obtained by RT-PCR assay [11] and ELISA (Prionics, Netherlands). The agreement between tests was determined by kappa index.

Results

Serial tenfold dilution of the stock virus isolate [CSFV/AP/TRP2/2009 (TS2)] (3.68 log₁₀ TCID₅₀/ml) was made and dilutions from 10⁻¹ to 10⁻⁵ were tested by LFA. LFA gave

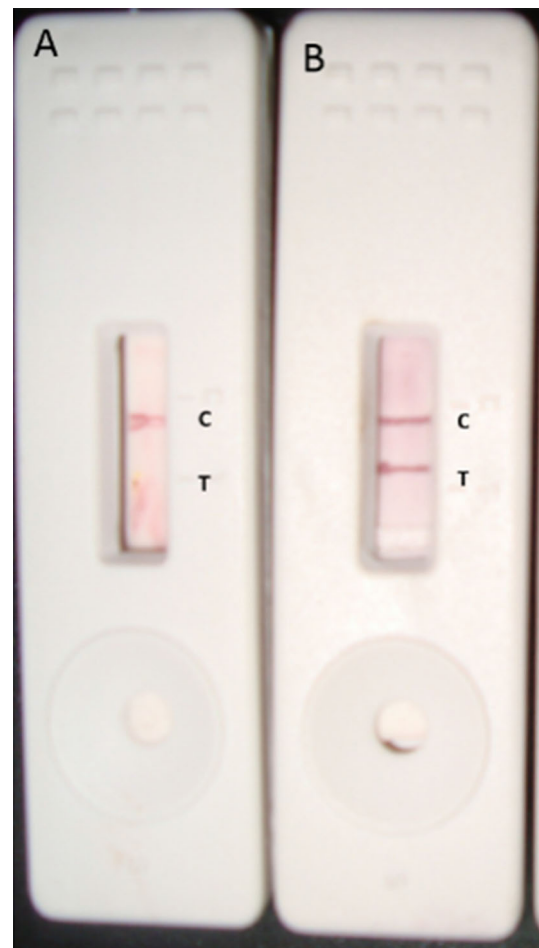


Fig. 1 LFA devices showing (A) negative and (B) positive test results for CSFV antigen. A clear red line at the control line with no color development at the test line indicates a negative test (A) while clear red lines at both the control and test lines indicates a positive test result (B) for CSFV antigen in the sample

positive result until the 10⁻² dilution, which corresponded to a virus concentration of 47.86 TCID₅₀/ml. For determining specificity, the strips were tested with both CSFV and mock infected PK15 cell extract. We found that the LFA gave positive results only with the CSFV-infected culture supernatants while all the mock-infected samples tested negative, with no background color development.

A panel of 72 field samples was simultaneously tested with both the LFA and PCR assay. The sensitivity and specificity of the LFA was calculated using the MedCalc program (MedCalc version 15.8). We found that the sensitivity and specificity of the LFA, when compared to PCR, was 80.36% and 87.10%, respectively. The kappa index for these tests was 0.577 with a 0.95 confidence level. The positive and negative predictive values of the LFA device were 91.84% and 87.10%, respectively. Results from the LFA and standard PCR are provided in Table 1. The LFA was further validated externally at the Assam Agricultural

Table 1 Analysis of the samples tested using the LFA and CSF PCR assay: (A) true positives (B) True Negatives (C) False positives (D) False negatives

Sample Identification	Type of sample	LFA	PCR	Sample Identification	Type of sample	LFA	PCR	Sample Identification	Type of sample	LFA	PCR
Group A				Group B				Group C			
AP/TRP1	Spleen	+	+	URF/LN	Spleen	-	-	Kar2/L	Spleen & Kidney	+	-
AP/TRP4	Spleen	+	+	AP/TRP3	Spleen	-	-	886/LF-Kpm	Whole blood	+	-
Kar1/L	Spleen	+	+	URF/sp	Spleen	-	-	Tnvli/post.vac.	Serum	+	-
Kar4/K	Spleen	+	+	AP/TRP2	Liver	-	-	DPU/Opm13/Tucn	Lymph node	+	-
VVL	Spleen	+	+	MST3/S	Liver	-	-	Group D			
DLF/Tnvli2	Spleen	+	+	CAV/liver	Liver	-	-	Erd2	Spleen	-	+
DPU/Npm/Tucn	Spleen	+	+	URF/MMC	Kidney	-	-	TUCN/Piglet	Spleen	-	+
DPU/Opm12/Tucn	Spleen	+	+	Path/T	Lymph node	-	-	VCRI/MST1/L	Spleen	-	+
TUCN/DP	Lymph node	+	+	MVC/MST1	Lymph node	-	-	DLF/Tnvli4	Kidney	-	+
LF/Kpm	Lymph node	+	+	MVC/MST2	Lymph node & spleen	-	-	DPU/Npm/Tucn	Kidney	-	+
DPU/Npm/Tucn	Lymph node	+	+	URF/P	Lymph node & spleen	-	-	DPU/Opm15/Tucn	Kidney	-	+
DLF/Tnvli3	Lymph node	+	+	VCRI/MST2	Liver & Spleen	-	-	DPU/Opm16/Tucn	Liver	-	+
Erd1/LN	Lymph node & spleen	+	+	Ker1/T	Spleen & Kidney	-	-	LF/Kpm	Whole blood	-	+
DLF	Lymph node & spleen	+	+	Kpm/LWY	Spleen & Kidney	-	-	DPU/Opm13/Tucn	Intestine	-	+
VVL	Tonsil	+	+	Dharm	Spleen & Kidney	-	-	446/LFKpm	Nasal secretion	-	+
DPU/Opm14/Tucn	Tonsil	+	+	Erd5	Whole blood	-	-	LF/886Kpm	Nasal secretion	-	+
DLF/Tnvli1	Liver	+	+	CUL/LF-Kpm	Nasal secretion	-	-				
DLF/Tnvli5	Liver	+	+	LF/446	Nasal secretion	-	-				
DPU/Npm/Tucn	Liver	+	+	DLF/Tnvli6	Intestine	-	-				
Ker1/L/K	Liver, spleen & kidney	+	+	DLF/Tnvli/pre	Serum	-	-				
Tnvli/post.vac.	Serum	+	+	Tnvli/pre.vac.	Serum	-	-				
887/ LF-Kpm	Nasal secretion	+	+	Tnvli/pool.pv.	Serum	-	-				
TS2/WVA/	Antigen crude pellet	+	+	PK15 control TCF	Uninfected CCS*	-	-				
TS2/WVA	Purified antigen	+	+								
VV Ag/CP	Purified antigen	+	+								
VV Ag/P	Purified antigen	+	+								
ASM/FD	CCS*	+	+								
TCF/ASM/ABDH/P3	Assam virus – CCS*	+	+								
TS2/TCF/P5	Passage 5 – CCS*	+	+								
TS2/TCF/P20	Passage 20 – CCS*	+	+								

Table 1 continued

Sample Identification	Type of sample	LFA	PCR	Sample Identification	Type of sample	LFA	PCR	Sample Identification	Type of sample	LFA	PCR
TS1/TCF/P20	Passage 20 – CCS*	+	+								
VVL/TCF/P5	Passage 5 – CCS*	+	+								
KAR/TCF/P10	Passage 5 – CCS*	+	+								
VV/TCF/P10	Passage10 – CCS*	+	+								

* CCS-Cell culture supernatant

University using a separate set of 32 samples comparing LFA with ELISA and RT-PCR, which further confirmed our in-house evaluation.

Discussion

CSFV is antigenically related to bovine viral diarrhoea virus (BVDV) in cattle and Border disease virus (BDV) in sheep; in fact antibodies to BVDV and BDV cross-react with CSFV in serological assays, making CSF diagnosis complex [12]. Although commercial ELISA kits are available for the detection of CSFV antigen, they are time consuming, require laboratory resources and cannot be readily performed on the farm. In this study, an immuno-chromatographic LFA was developed for rapid on-the-farm diagnosis of CSF. This test utilized mAbs produced against a local CSFV field isolate from India for accurate diagnosis. The envelope glycoprotein E2 is the major antigenic protein of CSFV and contains domains which are highly conserved between different strains [13–15]. Cross-reactivity with antibodies directed against BVDV and BDV are likely to be reduced when employing E2 mAbs in a diagnostic assay [16]. This LFA test employs colloidal gold-3B10 mAb conjugate as a detector reagent to capture the viral antigen on the test line.

The detection sensitivity of the LFA device was 47.86 TCID₅₀/ml with a sensitivity and specificity of 80.36% and 87.10%, respectively, when compared to PCR. The amount of CSF viral antigen in various tissues depends on the stage of the disease (i.e. acute, chronic, etc.) as well as the type of tissue. Higher virus concentrations are typically observed in lymphoid organs and liver, with lower concentrations in kidney and intestines of pigs suffering from CSF [17]. In our evaluation of LFA, 11 samples that were PCR positive tested negative with LFA. These samples comprised kidney, intestine, nasal wash and whole blood. It is possible that these samples may have had virus concentrations

below the detection limit of the LFA device, leading to false negative results.

Pigs typically develop antibodies to CSF during the third week of infection, with the antibodies persisting in the surviving animal for years, or even for life [18]. Hence, CSF diagnosis in the early stages of infection should be achieved, preferably through detection of the viral antigen. The LFA device that we have developed can be used in field conditions to screen for viral antigen, with positive samples being confirmed using conventional virus isolation techniques. While the LFA device's sensitivity and specificity can be improved further, it can still be used as an initial on-the-farm screening test. The LFA device is easy to use, and in our study the results could be read in 10 min without the need for any specialized equipment, factors which make this device suitable for field conditions.

We have examined the ability of the LFA to detect CSFV using various ante-mortem samples such as nasal secretions, whole blood and plasma, and post-mortem samples including tonsils, kidney, liver, spleen and lymph nodes to assess the efficiency of the assay with both types of sample. However, the number of ante-mortem samples tested was limited compared to the number of post-mortem samples. It is worth noting that the clinical picture of CSFV can be different, depending on several factors including the age of the pigs. Hence, further in-depth studies involving experimental challenge of pigs to evaluate the amount of virus present in ante-mortem samples is required to establish the most appropriate sample and stage of infection for this assay.

There are several aspects that can be looked into for improving the sensitivity of the LFA device. These include flow properties of the analytical membrane, absorptive capacity of the distal sink, the time the sample spends with the detection analyte in the release pad before coming into contact with the nitrocellulose membrane, the amount of capture antibodies applied to the membrane, pretreatment of the sample pad, and the quality of antibodies used [19].

Further standardization of the test module addressing these aspects could help in increasing the sensitivity of the assay and improving the results.

In summary, we report the development of a simple and reliable immuno-chromatographic LFA for on-the-farm detection of CSFV. This test offers several advantages over the conventional diagnostic tests in that it is cheap, rapid, convenient to use, and is expected to have a good shelf life. This test can serve as a preliminary screening device for the diagnosis of CSF under field conditions, followed by confirmation with conventional laboratory tests. We believe that this assay would be extremely helpful in the diagnosis of CSFV in resource poor and rural pig producing parts of the world.

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Compliance with ethical standards

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Conflict of interest The authors declare no conflict of interest

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Institutional Animal Ethical Committee approval Lr. no. 2182/DFBS/B/2012 dated 31.08.2012).

This article does not contain any studies with human participants performed by any of the authors.

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