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Detection of African swine fever virus by loop-mediated isothermal amplification

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A B S T R A C T

A loop-mediated isothermal amplification (LAMP) assay was developed for the detection of African swine fever virus (ASFV). This assay targets the topoisomerase II gene of ASFV and its specificity was confirmed by restriction enzyme digestion of the reaction products. The analytical sensitivity of this ASFV LAMP assay was at least 330 genome copies, and the test was able to detect representative isolates of ASFV ($n = 38$) without cross-reacting with classical swine fever virus. The performance of the LAMP assay was compared with other laboratory tests used for ASF diagnosis. Using blood and tissue samples collected from pigs experimentally infected with ASFV (Malawi isolate), there was good concordance between the LAMP assay and real-time PCR. In addition to detecting the reaction products using either agarose gels or real-time PCR machines, it was possible to visualise dual-labelled biotin and fluorescein ASFV LAMP amplicons using novel lateral flow devices. This assay and detection format represents the first step towards developing a practical, simple-to-use and inexpensive molecular assay format for ASF diagnosis in the field which is especially relevant to Africa where the disease is endemic in many countries.

1. Introduction

African swine fever virus (ASFV: genus *Asfivirus*, family *Asfarviridae*) is a large enveloped virus with a double-stranded DNA genome of between 170 kb and 190 kb. In sub-Saharan Africa, ASFV is maintained by long-term, inapparent infection of wildlife hosts such as bush pigs (*Potamochoerus porcus*) and warthogs (*Phacochoerus africanus*) which are infected via tick bites of the Argasid tick vector (*Ornithodoros* complex). The virus can also infect domesticated pigs and wild boar where it can cause an acute hemorrhagic fever, which can result in high morbidity and mortality with consequent economic losses. The disease severity ranges from lethal to moderately virulent or even non-virulent according to the host species and strain of the virus involved. Although largely restricted to Africa, outbreaks are also reported regularly in Sardinia. Furthermore, recent outbreaks during 2007 in Georgia, and the subsequent spread to neighbouring countries in the south Caucasus region (Rowlands et al., 2008), demonstrate that the virus can become established and pose a threat to pig industries outside the African continent.

A variety of laboratory tools can be used for the detection of ASFV. The established haemadsorption virus isolation (VI) method (Malmquist and Hay, 1960) can be sensitive, but it takes several days to obtain a result and is reliant upon the regular sourcing of fresh pig tissues for the preparation of primary bone marrow cells. Furthermore, the emergence of virulent non-haemadsorbing, non-cytopathic strains of ASFV (Gonzague et al., 2001) raises the potential of this test to generate false-negative results. In addition to antigen-detection immunoassays (Vidal et al., 1997; Hutchings and Ferris, 2006), a variety of molecular tests including agarose-gel based PCR (Steiger et al., 1992; Agüero et al., 2003, 2004; Basto et al., 2006) and real-time PCR (King et al., 2003; Zsak et al., 2005) assays have also been developed and adopted for routine diagnostic use. Although PCR is a highly sensitive method for the detection of ASFV, it relies on precision thermocycling requiring instrumentation which can be expensive. Loop-mediated isothermal amplification (LAMP), originally described by Notomi et al. (2000), is a novel method that allows rapid amplification of target DNA sequences in a highly specific manner under isothermal conditions. This report describes the development of a one-step loop-mediated amplification (LAMP) assay for the detection of ASFV. After optimisation, the performance of this assay was compared with other laboratory tests used for ASF diagnosis. This assay format provides an alternative to PCR-based detection assays for the detection of ASFV and may be more suitable for use in non-specialised or mobile laboratories.

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Table 1
Details of LAMP assay targets used for the amplification of ASFV.

LAMP assay (gene target)	Primer name	Type	Length	Genome position[a]	Sequence (5′–3′)
VP73 gene	FIP	Forward inner	48	F1: 102224–102248 F2: 102288–102266	GCAGAACTTTGATGGAAACTTATCG-TTAAAAACATTTCCGTAACTGCT
	BIP	Reverse inner	43	B1: 102201–102222 B2: 102148–102168	GCTCTTACATACCCTTCCACTA-CGGTAATCATCATCGCACCCG
	F3	Forward outer	21	102317–102297	CGTTACGTATCCGATCACATT
	B3	Reverse outer	18	102123–102140	TATTCCTCCCGTGGCTTC
Putative DNA primase	FIP	Forward inner	42	F1: 89240–89219 F2 89178–89197	TCGCCCGGATTTACCATTTCCT-GGAAACCAACAAAAGCGAGG
	BIP	Reverse inner	41	B1: 89279–89299 B2: 89340–89321	TTCAGATGACGGCCACCATGG-GTCCGTCGTGTCAATGATGA
	F3	Forward outer	20	89157–89176	GCGGGGATATGGGTACTTTG
	B3	Reverse outer	19	89362–89344	GCAGTCTTCTCCATGTGCC
Topoisomerase II	FIP	Forward inner	39	F1: 148639–148620 F2: 148580–148599	GCAACGTAGCCCCCGAACTG-GAAATGCTTCGCYTCCAACA
	BIP	Reverse inner	40	B1:148654–148674 B2: 148732–148714	ATCACCATGGCGACATGTCGT-GGATAGAGGTGGGAGGAGC
	F3	Forward outer	19	148555–148573	GGCGCAAAATTTTAGCCGG
	B3	Reverse outer	18	148760–148743	GCCGAAGCTTCCTATGCC
	FLoop	Forward loop	20	148619–148600	*[Btn]* [b]-AAAAACCTTTCGTTCACGGT
	BLoop	Reverse loop	20	148691–148710	*[Flc]* [c]-AAAAGCCGCCCAGTATTACC

[a] Genome position according to the ASF virus strain Malawi Lil-20/1 (GenBank accession number AY261361).
[b] Oligo labelled with biotin for lateral flow device studies.
[c] Oligo labelled with fluorescein (FITC) for lateral flow device studies.

2. Materials and methods

2.1. Virus isolates and tissue samples

A representative panel of 38 ASFV isolates was used to assess the performance of the LAMP assay. These materials comprised spleen suspensions, blood or cell culture supernatants taken from the archival material collected at the OIE reference laboratory for ASF (Institute for Animal Health, Pirbright). In addition, two isolates of classical swine fever virus (family: *Flavivirus*; genus: *Pestivirus*) which causes a disease with similar clinical signs in pigs were also tested. Negative controls were prepared from spleen homogenates obtained from uninfected pigs. The validation exercise for the LAMP assay also accessed blood, spleen, kidney, tonsil, mandibular lymph node and mesenteric lymph node samples collected from pigs that had been experimentally infected with ASFV. These 42 samples were collected during post-mortem examination of 7 individual Large White × Landrace pigs sampled at daily intervals (0–6 days) post-inoculation with 1×10^4 HAD_{50} Malawi isolate ASFV. Viral DNA template was prepared using the QIAamp viral RNA mini kit (QIAGEN) as previously described (King et al., 2003) and was stored at −70 °C until required for subsequent testing by ASFV LAMP or real-time PCR assays.

2.2. Primer design

Publicly available sequence data were used to generate consensus sequences by aligning the genomes of ASFV isolates. Initially, three separate LAMP assays were designed: these targeted the VP73 gene, putative DNA primase and topoisomerase II genes of ASFV. Four primers targeting six regions of the genome were designed using a Java-based software located on the Eiken website (http://primerexplorer.jp/e/): primer sequences are shown in Table 1. The two outer primers of each primer set (F3 and B3) assist in displacing the primary strand. The inner primers (FIP and BIP) of each primer set consist of two distinct sequences which correspond to the sense and anti-sense sequence of the target. FIP is made up of F1C which is complementary to F1 (Fig. 1) and the F2 sequence. BIP is made up of B1C which is complementary to B1 (see Fig. 1) and the B2 sequence. The loop primers correspond to the regions between F1 and F2 and B1 and B2.

2.3. Initial optimisation and evaluation of the LAMP assays

Experiments were performed to optimise the $MgSO_4$ and betaine concentrations as well as the amplification temperature for each of the three LAMP assays (VP73 gene, putative DNA primase and topoisomerase II). The $MgSO_4$ concentration was varied from 2 mM to 10 mM in increments of 1 mM and the betaine concentration was varied between 0.8 M and 1.2 M. The reactions were incubated on a thermal cycler (MJ Research) at a single temperature and products were visualized on 1.5% agarose gels. In addition, the LAMP reaction temperature was assessed by testing twelve temperatures between 60.6 °C and 69.2 °C on a gradient PCR thermocycler (Eppendorf, Mastercycler-Gradient).

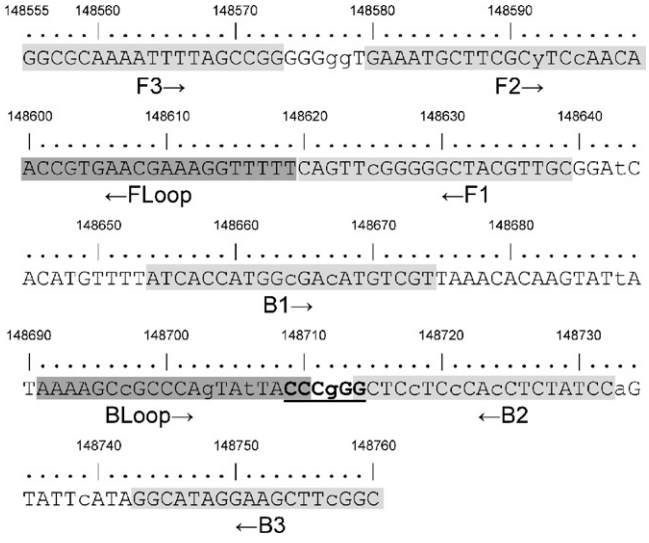

Fig. 1. Schematic showing location of oligo-nucleotide recognition sites within topoisomerase II gene (with reference to Lil-20/1 [AY261361]). Nucleotide positions that were totally conserved between 11 available ASFV isolates are shown in capital letters: consensus sequences are shown in lower case for remaining positions. Underlined sequence represents location of the SmaI restriction enzyme site.