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Protocols

Sensitive detection of African swine fever virus using real-time PCR with a 5′ conjugated minor groove binder probe

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A B S T R A C T

The design of a 5′ conjugated minor groove binder (MGB) probe real-time PCR assay is described for the rapid, sensitive and specific detection of African swine fever virus (ASFV) DNA. The assay is designed against the 9GL region and is capable of detecting 20 copies of a DNA standard. It does not detect any of the other common swine DNA viruses tested in this study. The assay can detect ASFV DNA in a range of clinical samples. Sensitivity was equivalent to the Office International des Epizooties (OIE) recommended TaqMan assay. In addition the assay was found to have a detection limit 10-fold more sensitive than the conventional PCR recommended by the OIE. Linear range was ten logs from 2×10^1 to 2×10^{10}. The assay is rapid with an amplification time just over 2 h. The development of this assay provides a useful tool for the specific diagnosis of ASF in statutory or emergency testing programs or for the detection of ASFV DNA in research applications.

1. Introduction

African swine fever (ASF) is an OIE notifiable infectious disease of domestic and wild pigs and is caused by the African swine fever virus (ASFV), the sole member of the genus *Asfivirus*, family *Asfarviridae*. It is one of the most serious transboundary animal diseases and has the potential for rapid international spread with crippling socio-economic consequences. The disease is enzootic in sub-Saharan Africa, Madagascar and Sardinia where it can exist as a sylvatic cycle between warthogs and ticks of the genus *Ornithodoros*. The disease was first described in Kenya (Montgomery, 1921). The first European outbreak was in Lisbon in 1957 and since then outbreaks have occurred in several European countries, as well as in Cuba and Central and South America. In recent years it has spread through Georgia to several Caucasian countries. Slaughter of swine is the only means of eradication as there is no current treatment or vaccine available.

ASF is characterised by high fever of up to 42 °C, subcutaneous haemorrhage, inactivity, breathing difficulty, loss of appetite and often massive mortality. The mortality can vary as there is a range of severity of disease, from chronic to peracute, depending on virus strain and host.

Deliberate or accidental feeding of pigs with human food waste is considered to be the most likely cause of introduction of the virus, particularly as ASFV can persist in dried meats for long periods (Mebus, 1988; McKercher et al., 1978). It is also known to survive for 18 months in proteinaceous substances at room temperature and for 11 days in non-proteinaceous substances such as faeces.

Diagnosis is complicated by the similarity of a range of other infections, particularly classical swine fever (CSF) (Kleiboeker, 2002) and porcine dermatitis and nephropathy syndrome (PDNS) (Chae, 2005). Rapid and reliable differential diagnosis is critical for the identification and control of the virus in the case of an outbreak and the OIE describes reliable and sensitive virus detection techniques for ASFV, including, real-time PCR and conventional PCR assays, in its Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009, Chapter 2.8.1 (http://www.oie.int/).

Minor groove binder (MGB) probes (Afonina et al., 2002; Belousov et al., 2004) are short oligonucleotides with a minor groove binder molecule and a quencher conjugated to the 5′ end, and a fluorophore to the 3′ end. The minor groove binder molecule functions by intercalating into the minor groove of the DNA α-helix and stabilises the bond, thereby increasing the melting temperature (*Tm*) of the probe and allowing the use of shorter probes. The fluorophore and the quencher are held in close proximity to

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each other due to the 3D conformation of the probe in solution. A fluorescent signal is triggered by hybridisation of the probe to a complementary target due to the resultant separation of fluorophore and quencher.

5′ MGB probes have been used for the detection of hepatitis A virus, enteroviruses, influenza A and B, respiratory syncitial virus and monkeypox (Gersberg et al., 2006; Hymas and Hillyard, 2009; Li et al., 2006). This work describes the development of an MGB probe assay for the rapid and sensitive detection of ASFV. The 9GL region to which it has been designed is a promising target for ASFV detection due to the high levels of nucleotide conservation.

2. Materials and methods

2.1. Virus isolates and clinical samples

DNA from ASFV strain Spain 75 was used to develop the 9GL MGB assay. DNA extracts from 14 other isolates of ASFV were used to evaluate the assay. These isolates represent genogroups I, V, VIII and IX according to p72 subtyping (Gallardo et al., 2009) (Table 2).

Clinical samples originated from six pigs experimentally infected with the ASFV strain Malta/78 (pigs 1–3) or strain MwLil 20/1 (pigs 4–6). These isolates are ASFV p72 genogroups I and VIII respectively. Whole blood was taken from these animals at −1, 2, 5, 8, 9, 11 and 12 days post-infection. The pigs were euthanized between days 5 and 12 post-infection and samples of spleen and musculus quadriceps were taken from each. Meat juice was obtained by gentle freezing and thawing of the meat. Details of these samples are in Tables 4a and 4b.

DNA from a range of Aujeszky's disease virus (ADV), porcine circovirus type 2 (PCV2) and porcine parvovirus (PPV) isolates was selected for specificity testing. In addition, one bovine parvovirus type 1 (BPV1), porcine circovirus type 1 (PCV1), and one swine adenovirus isolate were selected. DNA from fetal calf lung and pig kidney (PK15A) cell lines was used for negative samples. Details of these samples are in Table 2.

2.2. Design of primers and probes

An alignment of 22 Genbank sequences (http://www.ncbi.nlm.nih.gov) from the 9GL region of the ASFV genome was created using the GeneDoc program (www.nrbsc.org/gfx/genedoc/index.html). This area of the genome was chosen as it represented an area of high sequence homology between isolates. These sequences cover a geographical and chronological range of ASFV isolates. GenBank accession numbers and details of these sequences are shown in Table 1.

A number of MGB probes (Quantiprobes) and primer sets were designed using dedicated software (Qiagen, www.qiagen.com/). A suitable 17 mer MGB probe was chosen in an area of complete conservation on the alignment and a compatible primer set was chosen giving an amplicon size of 172 base pairs.

Probe sequence is 5′-CTGAAAGTCCTCCGAGT-3′, forward primer sequence is 5′-GTTGTTATGGAACGCGAAG-3′, and reverse primer sequence is 5′-CGCTCCTAGCTGGAAAGAAAA-3′.

A set of primers spanning the assay region to produce standard DNA for optimisation was designed using Oligo 5.0 (Molecular Biology Insights). Primer and probe sequences were subject to BLAST analysis to determine potential cross homologies with nucleotide sequences in the Genbank database (http://www.ncbi.nlm.nih.gov/). The MGB probe was purchased from Qiagen (Qiagen L.T.D., Crawley, U.K.) and all the primers used in the study were from Eurofins MWG Operon (Eurofins MWG Operon, London, U.K.).

2.3. Extraction of DNA

Nucleic acids from the 14 ASFV strains were extracted by Nucleospin kit (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's instructions.

DNA purification of ASFV from Spain 75, Malta and Malawi clinical samples in GuSCN was performed using neutral phenol to preclean the blood and meat juice samples, followed by Boom silica extraction of DNA as described for RNA extraction (Uttenthal et al., 2003). The DNA was eluted in water and adjusted to 0.3 M sodium acetate; 2 volumes of ethanol were added and the DNA was stored frozen for 24 h before shipment to our laboratories. The samples were precipitated using a standard protocol. ADV, BPV1, PCV1, PCV2, porcine adenovirus and cell culture nucleic acids were extracted from 200 μl amounts using Qiagen DNA Blood Mini Kit (Qiagen, Paisley, U.K.) according to the manufacturers' instructions.

Table 1
Details of GenBank ASFV sequences in the multiple sequence alignment used for MGB assay design.

Accession no.	Isolate	Year	Virulence	Origin	vp72 genotype
AF081173	Wildebeeslaagte/96/1 (M1)	1996	Virulent	South Africa	–
AF081163	Cr96/3	1996	Virulent	South Africa	–
AF081178	Ten62	1962	Virulent	Malawi	–
AF081176	Pretorisuskop/96/5 (Pr5)	1996	–	South Africa	–
AF081174	Mw Lil20/1[a]	1983	Virulent	Malawi	VIII
AF081168	Killean III	1959	–	Kenya	–
AF081165	E75[a]	1975	Virulent	Spain	I
NC_001659	BA71V[a]	1971	–	Spain	I
AF081160	Cameroon 82	1982	–	Cameroon	I
AF081170	Granja63	1963	Virulent	Spain	–
AF081172	Lis60[a]	1960	Virulent	Portugal	I
AF081181	Kat67	1967	Virulent	Democratic Republic of the Congo	–
AF081169	Ken64	1964	Virulent	Kenya	–
AF081180	VF83	1983	Virulent	Zimbabwe	–
AF081171	Ken55	1955	Virulent	Kenya	–
AF081179	Ug61	1961	Virulent	Uganda	–
AF081177	Sp51	1951	Virulent	South Africa	–
AF081166	Haiti 81[a]	1981	–	Haiti	I
AF081164	E70[a]	1970	Virulent	Spain	I
AF081175	Pr4	1996	Virulent	South Africa	–
AF081162	Cr96/1	1996	Virulent	South Africa	–
AF081161	Ch83/1	1983	Virulent	Zimbabwe	–

[a] Isolates available in this study.

2.4. Conventional gel-based PCR

Conventional gel-based PCR for the detection of ASFV nucleic acid was carried out using HotStarTaq Master Mix (Qiagen L.T.D., Crawley, U.K.) according to the manufacturer's instructions. The primers, used at a concentration of 0.5 μM, were those recommended by the OIE, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (ASFV OIE F 5′-ATGGATACCGAGGGAATAGC-3′ and ASFV OIE R 5′-CTTACCGATGAAAATGATAC-3′) and are designed against the p72 region of the ASFV genome. Thermal cycling was carried out on a DNA Engine Dyad thermal cycler (Bio-Rad Laboratories Ltd., Herts, U.K.). Cycling was initiated with a hot start denaturation of 15 min. Forty cycles followed consisting of 30 s each of denaturation at 94 °C, annealing at 50 °C, and extension at 72 °C. Reaction volumes were 25 μl. PCR products were visualized by electrophoresis on 1.5% (w/v) agarose gels (Mast Group L.T.D., Bootle, U.K.) and bands were photographed and annotated using BioRad GelDoc XR and Quantity One software (BioRad, Hemel Hempstead, U.K.).

2.5. Real-time MGB PCR

JumpStart Taq master mix (Sigma–Aldrich, Gillingham, U.K.) was used for the ASFV MGB assay. Probe concentration was 1× as recommended by the manufacturer (no details of actual concentrations are supplied). Primer concentrations of 0.5 μM each were used and Mg^{2+} was used at a final concentration of 3 mM. The assay was run on an MJ Research Opticon 2 real-time thermal cycler (Bio-Rad Laboratories Ltd., Herts, U.K.). The real-time PCR was initiated with a hot start denaturation of 2 min. This was followed by 40 cycles consisting of 30 s each of denaturation at 94 °C, annealing at 53 °C and extension at 72 °C. Reaction volumes were 25 μl including 2 μl of target. The MGB probe was FAM (3′) labeled and quenched with an Eclipse Dark Quencher (5′).

2.6. Real-time TaqMan PCR

Real-time TaqMan PCR was carried out using 2× Gene Expression Mastermix (Applied Biosystems, Warrington, U.K.) according to the manufacturer's instructions. Primers at a final concentration of 2 μM (5′-CTGCTCATGGTATCAATCTTATCGA, 5′-GATACCACAAGATC(AG)GCCGT-3′) and a TaqMan probe at a final concentration of 0.2 μM (5′-[6-carboxy-fluorescein (FAM)]-CCACGGGAGGAATACCAACCCAGTG-3′-[6-carboxy-tetramethyl-rhodamine (TAMRA)]) were used. The reactions were made up to 23 μl with sterile water and 2 μl of target DNA was used as template. Cycling consisted of 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

2.7. Production of dilution series of quantified standard DNA for assay optimisation

Bulk amounts of PCR product were used to produce a dilution series of quantified standard from strain Spain 75 DNA. A 433 base pair PCR amplicon was produced with a set of primers (F 5′-CCGGGAGACGTTGTTTT-3′ and R 5′-TGTTGCATTGGGGACCTA-3′) that spanned the assay region. PCR was carried out with an annealing temperature (*Ta*) of 52 °C and as described for the conventional ASFV PCR. The PCR product was electrophoresed on a 1.5% agarose gel (Mast Group L.T.D., Bootle, U.K.), the bands were excised, purified from the gel using QIAEX Gel Extraction Kit (Qiagen L.T.D., Crawley, U.K.) and quantified using a Jenway Genova UV/VIS spectrophotometer (Barloworld Scientific Ltd., Essex, U.K.). Based on the size of the amplicon and on the average molecular weight of a base pair the estimated number of copies of the amplicon/μl was calculated. From this a dilution series of known amplicon copy numbers from 10^{10} to10^{0} copies/μl was produced.

2.8. Optimisation of the MGB assay and determination of sensitivity and linear dynamic range

The first stage of optimisation involved performing a gradient PCR with *Ta* and Mg^{2+} concentrations between 50–60 °C and 3–7 mM respectively. On determining the optimal *Ta*/Mg^{2+} combination a primer matrix was carried out. This involved varying the forward and reverse primers in all combinations of 0.1, 0.25, 0.5, 0.75 and 1.0 μM concentrations. The MGB probe is supplied by the manufacturer as a 20× solution. Concentrations of 0.25×, 0.5×, 1× and 2× were tested with the optimal *Ta*, Mg^{2+} and primer concentrations. Optimisation was carried out using both 2×10^2 and 2×10^6 copies/μl of target standard DNA. Dynamic range and sensitivity of the optimised assays were determined by running triplicates of the quantified Spain 75 DNA standard material ranging from 2×10^0 to 2×10^{10} copies/μl.

2.9. Specificity testing

To test specificity the ASFV MGB assay was run in parallel to the conventional PCR and TaqMan assays using nucleic acid from the ADV, ASFV, BPV1, PCV1, PCV2, porcine adenovirus and PPV virus isolates previously described (see Table 2). In addition pig kidney (PK15A) and fetal calf lung cell lines were used as controls.

2.10. Comparison of the MGB assay sensitivity to OIE recommended gel-based PCR and TaqMan PCR

In order to compare sensitivity of the MGB assay to gel-based and TaqMan PCR a DNA extract from ASFV isolate Haiti 1981 was diluted 10-fold in DEPC treated water (Ambion, Cambridgeshire, U.K.). Five replicates of each dilution were run with all three assays.

2.11. Comparison of MGB assay and OIE recommended gel-based PCR and TaqMan PCR for the detection of ASFV DNA in clinical samples

Whole bloods, spleen and meat juice samples were tested for the presence of ASFV DNA using the MGB assay and both the OIE recommended conventional PCR and TaqMan assays as described (Tables 4a and 4b).

3. Results

3.1. Optimisation of the MGB assay, determination of sensitivity and linear dynamic range

Optimisation of each assay involved determining the best *Ta*, Mg^{2+} concentration, primer concentrations and probe concentration. In a real-time PCR assay the cycle number at which the fluorescent trace for a particular reaction crosses a pre-determined threshold level is known as the Ct value. Conditions for the MGB assay were chosen that gave the lowest Ct values for a particular known starting concentration of template. In this way an assay of the greatest sensitivity was developed. The assay worked optimally with 3 mM Mg^{2+}, a *Ta* of 53 °C, primer concentrations of 0.5 μM each and a probe concentration of 1×. It was observed that the assay functioned well over a wide range of *Ta*s. Cts for each temperature between 50 and 57 °C were within a one Ct range.

The assay was capable of detecting triplicates of this standard DNA across a ten orders of magnitude range, from 2×10^1

Table 2
Details of virus isolates tested in this study and results of specificity testing using ASFV MGB, TaqMan and conventional PCR assays. All ASFV accession numbers refer to vp72 gene.

	ISOLATE	Origin	Accession no.	p72 genotype	Ct		OIE PCR
					ASFV MGB	ASFV TM	
ADV	Imp 76	England	–	–	No Ct	No Ct	-ve
	Imp 95	Holland	–	–	No Ct	No Ct	-ve
	Imp 156	Denmark	–	–	No Ct	No Ct	-ve
	Imp 186	Belgium	–	–	No Ct	No Ct	-ve
	Imp 187	Belgium	–	–	No Ct	No Ct	-ve
	Imp 188	Belgium	–	–	No Ct	No Ct	-ve
	Imp 209	USA	–	–	No Ct	No Ct	-ve
	Bartha	Hungary	–	–	No Ct	No Ct	-ve
	NIA3	Northern Ireland	–	–	No Ct	No Ct	-ve
	Van Doorn	Holland	–	–	No Ct	No Ct	-ve
ASFV	Moz64	Mozambique 1964	FJ174376	V	31	31	+ve
	Ang72	Angola 1972	FJ174378	I	26	24	+ve
	MwLil 20/1	Chalaswa 1983	AY261361	VIII	26	27	+ve
	CV97	Cape Verde 1997	FJ174380	I	29	32	+ve
	Ug03H	Hoima, Uganda 2003	FJ154428	IX	29	29	+ve
	Ken06.B1	Kenya 2006	FJ154434	IX	31	32	+ve
	Ken07.Eld1	Kenya 2007	FJ154441	IX	26	27	+ve
	BF07	Burkino Faso 2007	–	I	24	25	+ve
	E70	Pontevedra Spain 1970	AY578692	I	23	24	+ve
	Ba71V	Badajoz Spain 1971	FJ174348	I	20	22	+ve
	E75	Lérida Spain 1975	AY578693	I	24	27	+ve
	L60	Lisbon Portugal 1960	AF301539	I	27	29	+ve
	Ss88	Sardinia, Italy 1988	FJ174362	I	26	28	+ve
	Port-au-Prince 81	Haiti 1981	FJ174375	I	20	22	+ve
PCV1	Weybridge	England	–	–	No Ct	No Ct	-ve
PCV2	999	USA	AF055391	–	No Ct	No Ct	-ve
	1010 Stoon	Canada	AF055392	–	No Ct	No Ct	-ve
	1017	Denmark	–	–	No Ct	No Ct	-ve
	1019	Spain	–	–	No Ct	No Ct	-ve
	1206	Belgium	EF990644	–	No Ct	No Ct	-ve
	1306	England	–	–	No Ct	No Ct	-ve
	1247	Sweden	–	–	No Ct	No Ct	-ve
	5549	Northern Ireland	–	–	No Ct	No Ct	-ve
	9367	Italy	–	–	No Ct	No Ct	-ve
	48285	France	AF055394	–	No Ct	No Ct	-ve
	H2755	Hungary	–	–	No Ct	No Ct	-ve
PPV	59e	England	–	–	No Ct	No Ct	-ve
	1005 NADL	Canada	NC_001718	–	No Ct	No Ct	-ve
	1008	France	–	–	No Ct	No Ct	-ve
Swine adeno	Field case	Northern Ireland	–	–	No Ct	No Ct	-ve
BPV1	Haden	–	–	–	No Ct	No Ct	-ve
PK15A	–	–	–	–	No Ct	No Ct	-ve
FCL	–	–	–	–	No Ct	No Ct	-ve

to 2×10^{10} copies/μl, and showed good linearity in this range (Fig. 1).

3.2. Specificity testing

The MGB assay was tested using ADV, BPV1, ASFV, PCV2, PCV1, PPV and porcine adenovirus as well as PK15A and FCL cell line nucleic acids as target and only produced a fluorescent signal with its own specific target (Table 2).

3.3. Comparison of the MGB assay sensitivity to OIE recommended gel-based PCR and TaqMan PCR

Table 3 shows a side-by-side comparison of the three assays on a serial dilution of Haiti ASFV DNA. The two real-time assays showed equivalent sensitivity being able to detect 10^{-4} dilutions in 100% of the five replicates. The MGB assay detected 40% of the five replicates at 10^{-5}, whereas the TaqMan assay detected 20% of the replicates at this dilution. The conventional assay was one log less sensitive, being capable of detecting 100% of the replicates at a 10^{-3} dilution and 20% of the replicates at a 10^{-4} dilution.

3.4. Comparison of MGB assay and OIE recommended gel-based PCR and TaqMan PCR for the detection of ASFV DNA in clinical samples

Data was generated by the ASFV MGB assay run using DNA extracted from whole blood, meat juice and spleen from experimentally infected animals as described. This was compared to data generated using the OIE TaqMan and conventional PCR assays. Tables 4a and 4b show the results. The results from the three assays were extremely consistent. All whole blood samples that were

Table 3
Comparison of the sensitivity of the ASFV MGB, TaqMan and conventional PCR assays using titrated Haiti strain DNA as target.

Dilution	ASFV MGB	ASFV TaqMan	Electrophoresis
Haiti 10^{-2}	+++++	+++++	+++++
Haiti 10^{-3}	+++++	+++++	+++++
Haiti 10^{-4}	+++++	+++++	+————
Haiti 10^{-5}	++———	+————	—————
Haiti 10^{-6}	—————	—————	—————
Haiti 10^{-7}	—————	—————	—————

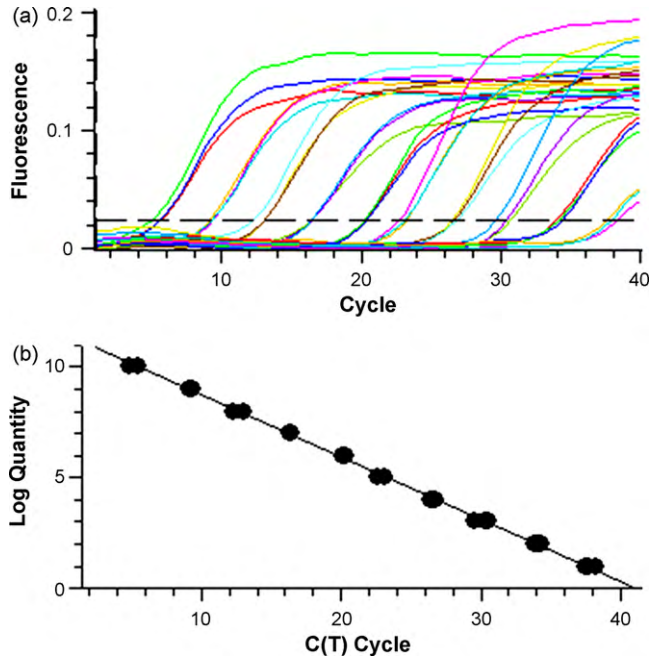

Fig. 1. (a) Fluorescence vs. cycle no. for MGB assay run with triplicates of 2×10^{10} to 2×10^{1} dilutions of standard DNA. (b) Slope and r^2 values from log quantity vs. Ct standard curve for the ASFV MGB assay with dilutions of standard DNA from 2×10^{10} to 2×10^{1}, $y = -0.28x + 11.58$, and $r^2 = 0.99$.

detected by the TaqMan and conventional assays were detected by the MGB assay. Results for the spleen and meat juice samples were also largely consistent across all three assay formats. One spleen sample was negative for the conventional assay (pig no. 3, 11 days post infection) but positive for the two real-time assays.

4. Discussion

African swine fever is considered by the United Nations Food and Agriculture Organisation (FAO) to be a transboundary animal disease of high socio-economic importance based on its mortality, rapid international spread and the according threat to food and trade security. As ASFV is highly contagious and spreads rapidly, with a large amount of shedding (even before the onset of clinical signs) and up to 100% morbidity, it is highly important in the case of an outbreak for laboratories to confirm diagnosis as soon as possible. As such the FAO includes ASF in the emergency prevention of transboundary animal and plant pests and diseases (EMPRES). The OIE classes ASF as a notifiable disease. The OIE prescribed test for international transfer is by enzyme-linked immunosorbant assay (ELISA). Diagnosis can also be by virus isolation (VI) in pig monocytes, fluorescent antibody testing or immunoblotting (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009, Chapter 2.8.1). Several conventional PCR assays have been developed for ASFV detection (Agüero et al., 2003; Steiger et al., 1992) including detection in *Ornithodoros* ticks (Basto et al., 2006). The OIE manual describes conventional and TaqMan ASFV PCR assays (King et al., 2003). Other TaqMan (Zsak et al., 2005) and Molecular Beacon (McKillen et al., 2007) methods have been described. The Invader assay, a novel isothermal amplification method using a cleavase enzyme has been applied to ASFV (Hjertner et al., 2005).

Real-time PCR and isothermal assays are now mainstream techniques for the detection of virus nucleic acids. Their speed, sensitivity and specificity have led to their widespread use for the detection of economically important viruses, including those of swine (Belák, 2005). The closed-tube format of real-time PCR assays such as this MGB assay means that no agarose gel electrophoresis is necessary, run times are shorter, potential for cross contamination is reduced and potential exposure to carcinogenic ethidium bromide is eliminated.

Accurate differential diagnosis of ASF is critical as it may be confused clinically with several other diseases particularly classical swine fever (CSFV) but also bacterial septicemias, fungal poisonings and viral diseases such as porcine reproductive and respiratory syndrome, porcine dermatitis and nephropathy syndrome and Aujeszky's disease (Kleiboeker, 2002). In the event of an outbreak even short delays in determining the correct diagnosis and

Table 4a
Comparison of PCR results on DNA extracted from whole blood samples at various days post-infection (PI) from pigs infected with ASFV Malta/78 (pigs 1–3) or MwLil 20/1 (pigs 4–6) strains.

Pig no.	Days PI	MGB assay Ct	TaqMan assay Ct	OIE PCR
1	−1	No Ct	No Ct	Neg
	2	No Ct	No Ct	Neg
	5	No Ct	No Ct	Neg
	8	No Ct	No Ct	Neg
	12	23	23	+
2	−1	No Ct	No Ct	Neg
	2	No Ct	No Ct	Neg
	5	23	27	+
	8	29	33	+
	9	31	34	+
3	−1	No Ct	No Ct	Neg
	2	No Ct	No Ct	Neg
	5	24	25	+
	8	28	29	+
Pig no.	Days PI	MGB assay Ct	TM assay Ct	OIE PCR
3	11	37	37	+
4	−1	No Ct	No Ct	Neg
	2	No Ct	No Ct	Neg
	5	No Ct	No Ct	Neg
	8	29	30	+
	9	25	26	+
5	−1	No Ct	No Ct	Neg
	2	32	32	+
	5	22	22	+
6	−1	No Ct	No Ct	Neg
	2	No Ct	No Ct	Neg
	5	20	20	+
	8	26	29	+

Table 4b
Comparison of PCR results on DNA extracted from spleen and meat juice samples from the pigs infected with ASFV Malta/78 (pigs 1–3) or MwLil 20/1 (pigs 4–6) strains.

Pig no.	Days PI	Spleen			Meat juice		
		MGB assay Ct	TaqMan assay Ct	OIE PCR	MGB assay Ct	TaqMan assay Ct	OIE PCR
1	12	24	26	+	32	36	+
2	9	24	25	+	34	33	+
3	11	36	35	-ve	33	34	+
4	9	23	23	+	33	34	+
5	5	19	19	+	27	27	+
6	8	20	21	+	29	29	+

initiating the appropriate response can have devastating effects on the abilities of agricultural authorities to curb the spread of infection. The MGB assay described here has a run time of just over 2 h. The speed of the assay is comparable to other real-time chemistries such as molecular beacons or TaqMan probes.

The 5′ conjugated MGB probes (Afonina et al., 2002; Belousov et al., 2004) used in this study are short oligonucleotides with the MGB moiety attached to the 5′ terminus. These are not to be confused with 3′ conjugated TaqMan–MGB probes, which are unconjugated at the 5′ end to allow exonuclease digestion by the polymerase enzyme. The MGB molecule intercalates into the minor groove of the DNA double helix increasing the *Tm* of the complex by up to 30 °C and thereby allowing the design of shorter probes. This could be particularly useful in the design of assays for viruses that have considerable sequence divergence between strains such as many RNA viruses. The marked reduction in *Tm* of the probe/target hybrid when binding is to a mismatched target also confers a high level of specificity on the assay. These MGB probes can also utilise a number of modified bases that confer on them further diagnostic advantages. Both Super A™ and Super T™, when incorporated into AT rich regions cause increased stabilisation. Super G™ bases allow the design of probes in high GC rich sequences. Normally design in such regions would be impossible due to the formation of tetrad aggregates of guanosine causing G–G association and an according reduction in hybridisation efficiency. The conserved nature of the ASFV genome in the 9GL region meant that incorporation of these modified bases was unnecessary for this assay. However, future work will include the evaluation of these probes for RNA viruses.

The ASF MGB assay combines all the necessary characteristics for a modern molecular diagnostic test. The assay is highly sensitive, being capable of detection of as little as 20 copies of standard DNA per reaction. It can detect virus in whole blood, spleen and meat juice. All clinical samples detected by the conventional and TaqMan OIE recommended assays were detected by this test. One of the whole blood sample that was detected by both the TaqMan and the MGB assays was not detected by conventional PCR. This sample was weak (Ct values of 35–37) when run with the MGB and TaqMan assays; so reduced sensitivity of the conventional PCR could explain this. Sensitivity testing showed that the two real-time assays were equally sensitive and that these could consistently detect ASFV Haiti to a dilution of 10^{-4}. The conventional OIE assay was one 10-fold dilution less sensitive. Specificity testing on a range of DNA viruses showed that the MGB assay would only detect specific ASFV DNA and did not detect DNAs from ADV, BPV1, PCV2, PPV and swine adenovirus or from bovine and porcine cell lines. The ideal assay *Ta* was determined to be 53 °C. Optimisation showed little significant difference in functionality between *Ta*s of 50 and 56 °C. As such for the detection of highly variable viral genomes a balance could be found through careful selection of *Ta* that allows tolerance of some mismatches whilst still retaining high specificity.

In many diagnostic situations high throughput pathogen detection is important. Real-time PCR methods allow such high throughput application. Real-time assays can be carried out in 96 well plate formats and this allows them to be adapted easily to automated platforms. Automation of both reaction set-up and nucleic acid extraction now allows for highly reproducible high throughput pathogen detection. MGB assays could readily be adapted for such automated processes. Cost of the assay probe is equivalent to other real-time reporter chemistries.

In summary we have developed an assay suitable for the rapid, reproducible and sensitive differential detection of ASFV DNA using MGB technology. This assay is suitable for applications in research into ASFV and could be used in emergency outbreak situations if validated further. The MGB technology was easily applicable to the molecular detection of ASFV and has unique qualities that could lend themselves to improved detection of more difficult viral targets.

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