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Development of a TaqMan® PCR assay with internal amplification control for the detection of African swine fever virus

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Abstract

A closed-tube polymerase chain reaction (PCR) was developed to allow the rapid detection of African swine fever virus (ASFV) DNA. This assay targets the VP72 gene of ASFV and uses the 5′-nuclease assay (TaqMan®) system to detect PCR amplicons, avoiding tube opening and potential cross-contamination of post-PCR products. An artificial mimic was engineered with the TaqMan® probe site replaced by a larger irrelevant DNA fragment allowing discrimination from ASFV by using two-colour TaqMan® probe reporters. When added to the samples, successful amplification of this mimic demonstrated the absence of substances inhibitory to PCR, thereby validating negative results. Assay sensitivity was confirmed by obtaining positive signals with a representative selection of ASFV isolates. Many of the clinical and post-mortem features of ASF resemble those of classical swine fever (CSF) and porcine dermatitis and nephropathy syndrome (PDNS). Therefore, fast and reliable detection of ASFV is essential not only for the implementation of control measures to prevent the spread of ASF, but also in the differential diagnosis from CSF and PDNS. This assay should prove to be a valuable tool in the laboratory diagnosis of ASF and will complement existing molecular methods to provide rapid differential diagnosis in cases of suspected swine fever.

Keywords: African swine fever virus; Diagnosis; Polymerase chain reaction; Fluorogenic PCR assay; TaqMan®

1. Introduction

African swine fever virus (ASFV) infects domestic pigs causing disease, which can result in high morbidity and mortality with associated economic losses. The virus was first described in Kenya in 1921 (Montgomery, 1921) and was subsequently found to exist in many countries in southern and eastern Africa. Although having some characteristics similar to Iridoviruses and Poxviruses, ASFV has been formally classified as the prototype member of the genus *Asfivirus* within the family Asfarviridae (Dixon et al., 2000). ASFV has a double stranded DNA genome of between 170 and 190 kb in size. It is unique as the only known DNA arbovirus, and is capable of infecting members of the vertebrate family Suidae (domestic and feral pigs, wild boars, bush pigs, warthogs and the giant forest hog) and the Argasid tick vector (*Ornithodoros* complex). Tick-borne transmission of ASFV is considered to be the major route of infection in regions of Africa where the disease is endemic. However, in southern Europe and Latin America major ASF outbreaks have occurred that are associated with import of contaminated meat products and the subsequent introduction of ASFV into native tick vectors (*Ornithodoros marocanus*).

The clinical features of ASFV infection are high fever, diarrhoea, haemorrhage and generalised reddening of the skin. These clinical signs and additional post-mortem findings such as splenic enlargement and haemorrhages in lymph nodes and kidney are indistinguishable from those seen during CSFV infection. More recently the differential diagnosis between ASF

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and CSF has been further complicated by the emergence of porcine dermatitis and nephropathy syndrome (PDNS), which can also have a similar clinical presentation (Done et al., 2001). Therefore, rapid and reliable detection of ASFV is essential, not only for the implementation of control measures to prevent the spread of ASF, but also in the differential diagnosis from other pig diseases with similar clinical presentation.

Laboratory diagnosis of ASFV is routinely performed by virus isolation (VI) in porcine bone marrow (PBM) cell cultures but although this is a reliable and sensitive method, it takes 6 days to declare a negative result. Polymerase chain reaction (PCR) has been described as a suitable rapid alternative to VI for the detection of ASFV (Steiger et al., 1992) and may be particularly useful for screening poor quality or degraded samples with non-recoverable virus. This would allow the diagnosis of ASFV to be made within hours of sample receipt so that control measures and restrictions could be implemented or lifted in a much shorter time-scale than if VI was employed as the sole diagnostic method. The aim of this study was to improve the reliability and ease of use of PCR for the detection of ASFV by using sequence data from diverse ASFV isolates to design a primer set for use in a closed-tube assay with a fluorescent probe. The incorporation of a probe confirms the specificity of the PCR product and allows automated reading of the test. The presence of PCR inhibitors in some samples can result in false negative results through failure of the PCR reaction (Belak and Thoren, 2001). To measure this, a DNA internal standard or 'mimic' was constructed with the same primer recognition sequences as the wild-type virus but with a heterologous DNA fragment inserted between the primer sites to make a larger-sized amplicon. The amplicon of the mimic can easily be distinguished from that of the virus by its larger size, using agarose gel electrophoresis or, as described here, by the use of a separate fluorescent probe specific for the insertion.

2. Materials and methods

2.1. Preparation of viruses

In order to assess the sensitivity of the TaqMan® PCR assay, a panel comprising 25 ASFV isolates was assembled, representative of the diversity revealed by phylogenetic analysis (Table 1: Bastos et al., 2002 unpublished data). These viruses were propagated by in vivo passage in susceptible pigs or in peripheral blood mononuclear (PBL) or PBM cells (Malmquist and Hay, 1960); samples taken for PCR analysis were spleen tissue taken from infected pigs, at or just after the onset of clinical disease, PBL or PBM cell harvests. Negative controls consisting of spleen homogenates from uninfected pigs and uninfected PBM and PBL cell culture harvests were also prepared. The DNA from 11 representative isolates from this panel was titrated for further study. Sixteen unclassified African and European tick ASFV isolates (OURT 88/1, 88/2 and 88/3 from Portugal, TIK/82, KON/83, LIL 20/1 and LIL 90/1 from Malawi, KIRT 89/2, 89/3 and 89/4 from Tanzania, and LIV 5/40, LIV 13/33, MFUE 6/1, SUM 14/11 KAB 6/2 and CHGT 88/1 from Zambia), which had been passaged in susceptible pigs were also tested.

2.2. Template extraction

DNA was purified from the representative ASFV isolates and uninfected spleen, PBM and PBL negative control samples to a final elution volume of 60 μl by a viral RNA kit (QIAgen, UK) according to the manufacturer's instructions. This method does not discriminate in its ability to purify RNA from DNA and has proved a valuable method to prepare template for the laboratory diagnosis of CSF (McGoldrick et al., 1998). Additional extraction negative controls were prepared at this stage for each ASFV isolate and uninfected negative material by running parallel extractions of nuclease-free water with the kit. The extracted samples and controls were stored at −20 °C until they were used in the TaqMan® PCR and in the PCR protocol outlined in the 4th Edition of the Office International des Epizooties (OIE) manual of standards, diagnostic tests and vaccines (Wilkinson, 2000). In order to assess the specificity of the TaqMan® PCR, samples from pooled EDTA blood samples ($n = 50$) collected during the 2000 UK classical swine fever (CSF) outbreak were tested. These samples were also tested for CSFV by reverse-transcriptase nested TaqMan® PCR (McGoldrick et al., 1999) and for porcine circovirus-2 by TaqMan® PCR (Grierson et al., 2002 unpublished data).

2.3. Construction of an artificial template (mimic)

A 537 bp fragment of ASFV encompassing the proposed diagnostic VP72 PCR amplicon, was amplified from a plasmid containing the *EcoR I* B fragment of the ASFV BA71V isolate (Almendral et al., 1984) using primers 5′-ATA GGA TTA AAA CCT ACC TGG AAC ATC TCC G-3′ and 5′-GGT ACT GTA ACG CAG CAC AGC TGA ACC GTT CTG-3′ and cloned into pGEM-T easy (Promega). Plasmid DNA (pASFV-VP72) was purified from DH5α cultures using QIAprep spin minikit (QIAgen) and the presence of inserts of the appropriate size were confirmed by restriction enzyme digestion and by sequencing (Big Dye, Applied Biosystems). The strategy for the mimic construction is outlined in Fig. 1. Briefly, two independent VP72 fragments of 364 bp (fragment A) and 119 bp

Table 1
Details of the representative ASFV and CSFV isolates used in the study

Isolate	Location	Genotypic group[a]	Species of origin	Year collected	Source of PCR template	C_T values	OIE PCR
DAKAR/59	Dakar, Senegal	1	Pig	1959	Pig spleen	23.60	+
LIS/60	Lisbon, Portugal	1	Pig	1960	Pig spleen	24.25	+
MALTA/78	Malta	1	Pig	1978	Pig spleen	30.40	+
BEL/85	West Flanders, Belgium	1	Pig	1985	Pig spleen	21.79	+
BEN 97/1	Ladji, Cotonou, Benin	1	Pig	1997	PBM	32.02	+
BRAZIL/79	Brazil	1	Pig	1979	PBL	33.05	+
DOM REP	Dominican Republic	1	Pig	1979	Pig spleen	28.67	+
CAM 1/86	Limbe, Cameroon	1	Pig	1986	Pig spleen	26.16	+
NUR 95/1	Orgosolo, Nuoro, Sardinia	1	Pig	1995	Pig spleen	25.53	+
RSA 1/93* (SPEC 257)	Ellisras, Northern Province South Africa	3	Pig	1993	PBM	30.15	+
BOT P1/99*	Sherwood, Botswana	3	Pig	1999	PBM	30.08	+
RSA 1/92* (SPEC 251)	Rustenburg, North West Province, South Africa	4	Pig	1992	PBM	32.44	+
RSA W/1/99*	Northern Province, South Africa	4	Warthog	1999	PBM	30.59	+
TENGANI	Tengani, Malawi	5	Warthog	1960	Pig spleen	25.34	+
MOZ/79	Beira, Mozambique	5	Pig	1979	Pig spleen	28.10	+
MOZ/60	Mozambique	5	Pig	1960	Pig spleen	29.69	+
MOZ 94/8	Manica Province, Mozambique	6	Pig	1994	Pig spleen	26.09	+
NAM P1/95*	Windhoek, Namibia	7	Pig	1995	PBM	27.69	+
DEDZA	Chilikumwere, Dedza, Malawi	8	Pig	1986	Pig spleen	25.69	+
KAL 88/1	Kaliyoyo, Eastern Province, Zambia	8	Pig	1988	Pig spleen	25.35	+
UGA 1/95	Mulindue, Uganda	9	Pig	1995	PBM	28.92	+
HINDE II	Nanyuki, Kenya	10	Warthog	1959	Pig spleen	23.48	+
KWH/12	Kiriwira, Tanzania	10	Warthog	1968	PBM	28.28	+
BUR 84/2	Mushasha-Gitega, Burundi	10	Pig	1984	Pig spleen	23.75	+
KAB 94/1	Kabete, Kenya	10	Pig	1994	Pig spleen	28.02	+
CSF	East Anglia, UK	-	Pig	2000	19 EDTA bloods	>40.00	-

TaqMan® (expressed as C_T values) and OIE PCR results are shown. All strains were obtained from IAH Pirbright except isolates marked * which were obtained from ARC-Onderstepoort Veterinary Institute, Pretoria, South Africa.

[a] Genotype groupings assigned according to Bastos et al., 2002, submitted.

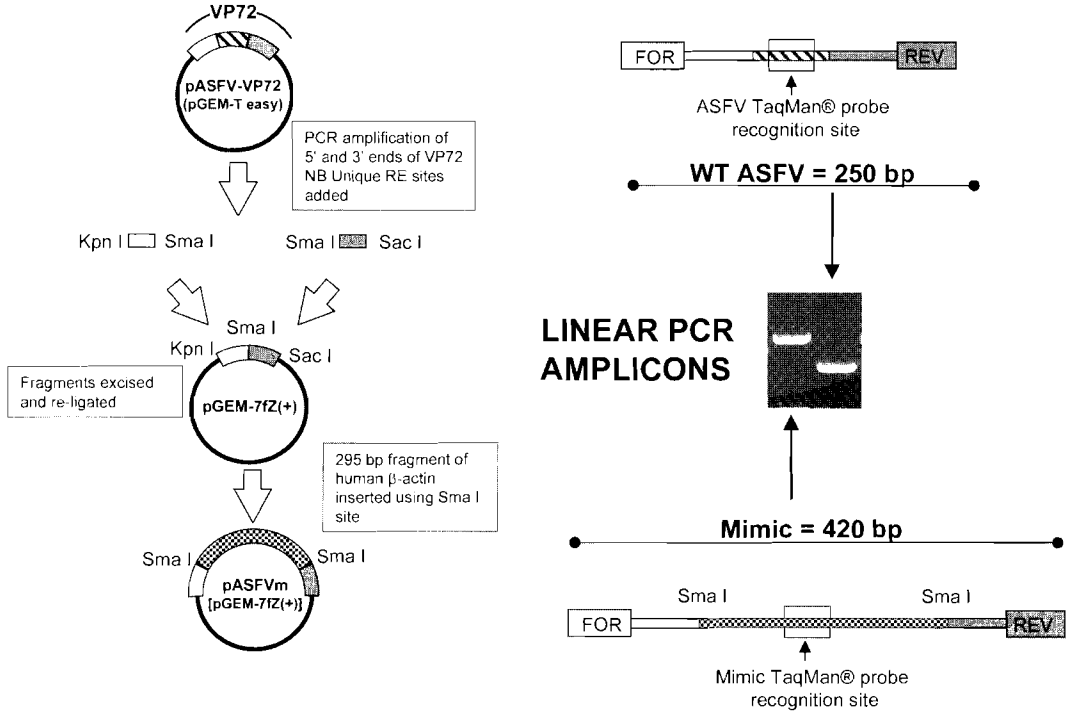

Fig. 1. Schematic overview of the strategy used to construct a plasmid (pASFVm) for use as an artificial template (mimic) for the TaqMan® PCR. The resulting mimic amplicon (420 bp) is larger than that produced by wild-type ASFV (250 bp) as shown in the 2% agarose gel.

(fragment B) containing the proposed ASFV sense and anti-sense primer sites, respectively, but excluding the ASFV TaqMan® probe site were amplified by PCR. The primers were modified at the 5′-ends to provide cleavage sites for *Kpn I* (sense) and *Sma I* (anti-sense) for fragment A and *Sma I* (sense) and *Sac I* (anti-sense) for fragment B. These PCR fragments were subjected to restriction digestion with the relevant enzymes (Promega) and subsequently cloned into pGEM-7Zf(+) (Promega) prepared by *Kpn I* and *Sac I* digestion. The resulting plasmid contained a 489 bp insert of VP72 with a deletion of 136 bp that was marked by the addition of a unique *Sma I* site. After the linearisation with *Sma I*, the plasmid was incubated with calf alkaline phosphatase (Promega) to prevent recircularisation. A 295 bp fragment of human beta-actin gene generated by PCR using primers with 5′-*Sma I* recognition sites: 5′-TTT CCC GGG TCA CCC ACA CTG TGC CCA TCT ACG A-3′ and 5′-TTT CCC GGG CAG CGG AAC CGC TCA TTG CCA ATG G-3′ was ligated into the

Table 2
Multiple alignment for available ASFV sequences highlighting the mismatches with the TaqMan® PCR primer and probe recognition sites

ASFV isolate (or GenBank accession number)	**SENSE PRIMER** CTGCTCATGGTATCAATCTTATCGA	**TaqMan® PROBE SITE** CCACGGGAGGAATACCAACCCAGTG	**ANTI-SENSE PRIMER** GATACCACAAGATCRGCCGT
42 ASFV isolates	----------	----------	----------A-----
RSA 1/98	----------	----------T----------	----------A-----
SPEC 260	----------	----------T----------	----------A-----
Uganda (L27499)	----------	----------T----	----------G-----
DR-1 (L27498)	----------	----------T----	----------G-----
Hinde II	----------C---	----------T----	----------G-----
KWH/12	----------	----------	----------G-----
UGA 3/95	----------	----------	----------G-----
BUR 1/90	----------	----------	----------G-----
BUR 2/84	----------	----------	----------G-----
BUR 1/84	----------	----------	----------G-----
UGA 1/95	-------C----------	----------C-	----------A-----
NG/98	----------	----------	--A----------A-----

plasmid, and transfected into *E. coli* DH5α. The sequence of the mimic plasmid (pASFVm) was confirmed by sequencing (Big Dye, Applied Biosystems) after which purified plasmids (QIAprep spin minikit, QIAgen) were linearised with *Kpn I* for use as an artificial template (mimic) in the single tube PCR. Successful amplification of this mimic in the samples was detected using a TaqMan® probe (Applied Biosystems) containing a different fluorochrome to that used for detection of the viral amplicon (5′-VIC™-CAT GCC ACC CTG CGC CTA GAC CT-3′ TAMRA). The appropriate concentration of the mimic DNA used for the TaqMan® assay was optimised by serial dilution so that assay sensitivity was not reduced.

2.4. Closed-tube fluorogenic PCR

2.4.1. Design of probe and primers

A TaqMan® probe (5′-[6-carboxy-fluorescein (FAM)]-CCA CGG GAG GAA TAC CAA CCC AGT G-3′-[6-carboxy-tetramethyl-rhodamine (TAMRA)] from Applied Biosystems) was designed from an alignment of 54 available ASFV sequences for the 3′-end of VP72 (sequences from GenBank and Bastos et al., 2002, unpublished data) using criteria outlined in PRIMER EXPRESS software (version 1.5, Applied Biosystems). PCR primers (Genosys Biotechnologies): sense primer (5′-CTG CTC ATG GTA TCA ATC TTA TCG A-3′) and anti-sense primer (5′-GAT ACC ACA AGA TC(AG) GCC GT-3′ were also designed to target conserved regions of the 3′-end of the VP72 gene (Table 2). Assay validation was performed at two independent laboratories (Veterinary Laboratories Agency [VLA], Weybridge and Institute for Animal Health [IAH], Pirbright) using end-point (VLA) and quantitative (IAH) TaqMan® reader instrumentation.

2.4.2. End-point detection of ASFV

Single-tube PCR reactions were prepared containing 50 mM KCl, 10 mM Tris–HCl pH 9.0, 2 mM $MgCl_2$ (buffers supplied with *Taq* DNA polymerase [Promega]), 0.4 mM of each dNTPs, 2.5 U *Taq* DNA polymerase (Promega), 50 pmol of each primer, 5 pmol ASFV probe (FAM reporter) and 5 pmol mimic probe (VIC reporter). HPLC water was added to make the final reaction volume up to 50 μl. This mastermix was dispensed into a MicroAmp® optical 96-well reaction plate (Applied Biosystems). Four no template control (NTC) wells without either mimic or sample DNA were prepared to establish baseline fluorescence. Test samples (5 μl of prepared template) and mimic DNA (5 μl of lincarised plasmid) were then carefully added to the plate in a separate work-area. Negative control (NEG) wells containing only mimic template and with HPLC water substituting for sample DNA were interspersed between each sample well on the 96-well plate. All wells were sealed with optical lids (Applied Biosystems). Amplification conditions (GeneAmp® PCR system 9700, Applied Biosystems) used were an initial denaturation step of 94 °C for 120 s followed by 35 cycles of 94 °C for 30 s, 58 °C for 60 s and 72 °C for 45 s. Chain elongation at 72 °C was extended to 420 s for the final cycle after which the plate was held at 4 °C until the fluorescence signal was measured (ABI Prism 7200 sequence detector, Applied Biosystems). Statistical analysis of the FAM and VIC fluorescence signals was performed using 2D scatter-plots of the multicomponent values (SEQUENCE ANALYSER ver 1.6, Applied Biosystems) imported into STATISTICA (ver 6, Statsoft). A 99% confidence ellipses were drawn around the water control (NEG) and NTC wells. These confidence ellipses were used to define ASFV positive samples, ASFV negative samples and samples where the PCR had failed. ASFV negative samples were grouped with the water control wells, while ASFV positive samples were identified as points lying outside of the water control 99% confidence ellipse. Sample failures were grouped with readings from NTC wells.

2.4.3. Quantitative determination of ASFV

PCR mixes containing 50 pmol of sense primer, 50 pmol of anti-sense primer, 5 pmol of probe and 1 × TaqMan® master mix (Applied Biosystems) were made up in nuclease-free water (Promega) and 22 μl of the mix added to a MicroAmp® optical 96-well reaction plate followed by 3 μl of extracted template or blank extraction control. The plate was spun for 60 s at 300 × *g* in a centrifuge (Sorvall) prior to amplification in GeneAmp® 5700 Sequence Detection System (Applied Biosystems). The samples were amplified with the following programme using the following conditions: 50 °C for 120 s, one cycle (uracil N-deglycosylase digest); 95 °C for 10 min, one cycle (activation of the *Taq* Gold thermostable DNA polymerase present in the master mix); 95 °C for 15 s, 58 °C for 60 s, 40 cycles. After amplification, a threshold cycle (C_T) value was assigned to each PCR reaction as previously described (Oleksiewicz et al., 2001). The absolute negative for any test sample, uninfected negative or extraction blank control corresponded to a C_T value of >40.0. Fifty cycles of replication were also used in some experiments (absolute negative values then corresponded to a C_T value of >50.0).

2.5. OIE-reference PCR method for the detection of ASFV

The TaqMan® PCR method was compared with the PCR protocol outlined in the current OIE manual (Wilkinson, 2000). This PCR targets a 278 bp fragment of the middle portion of VP72 and has no overlap with the TaqMan® PCR amplicion. For these experiments,

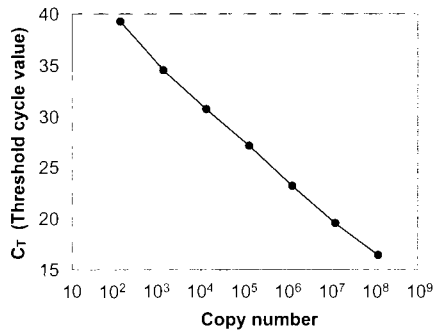

Fig. 2. Analytical sensitivity of the ASFV TaqMan® PCR determined using serial $\log_{10}$ dilutions of linearised pASFV-VP72.

the primers (5′-ATG GAT ACC GAG GGA ATA GC-3′ and 5′-CTT ACC GAT GAA AAT GAT AC-3′) and cycling conditions outlined in the OIE manual were followed. Briefly, PCR mix was added to the wells of a PCR plate (ABgene) and consisted of the following reagents: nuclease-free water (30.5 μl, Promega), 50 mM KCl, 10 mM Tris–HCl pH 9.0 (Promega), 2.5 mM $MgCl_2$ (Promega), 10 mM (each) dNTPs, 50 pmol of each primer and 2.5 U *Taq* DNA polymerase (Promega). The final reaction volume was made up to 50 μl in each well by adding 3.0 μl of extracted template or blank extraction control. Amplification was carried out in a PTC-100™ thermal cycler (MJ Research, Inc.) and the products visualised by elecrophoresis on a 1.5% agarose gel stained with 1.0 μg/ml ethidium bromide.

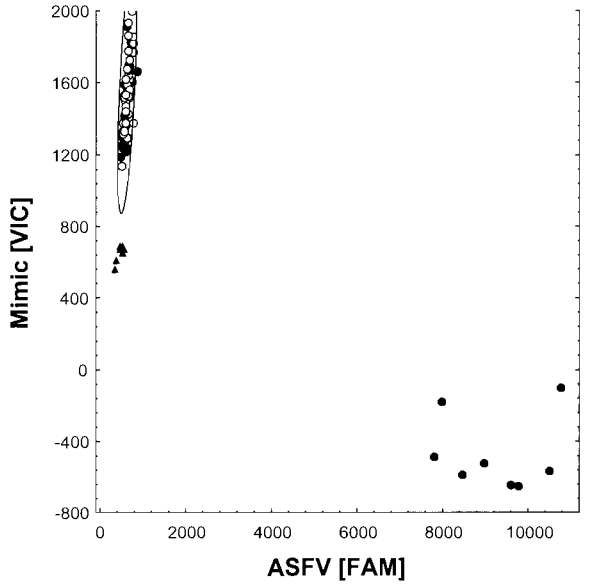

Fig. 3. Detection of eight samples spiked with ASFV template by TaqMan® PCR. Two-dimensional (2D) scatter-graph (STATISTICA) shows ASFV-FAM and MIMIC-VIC signals for samples ($n = 47$, ●), PCR controls (NEG: containing mimic template: $n = 39$, ○) and NTCs ($n = 8$, ▲). A 99% confidence ellipse drawn around the NEG controls is used to assign results for positive and negative samples.

3. Results

3.1. Optimisation of TaqMan® PCR

The analytical sensitivity of the TaqMan® PCR method was determined using a $\log_{10}$ dilution (10^{-5} 10^{-12}) series of a *Sal I* linearised plasmid (pASFV-VP72). Using both the end-point and quantitative TaqMan® approaches, the template was detected to 10^{-9} dilution, corresponding to a lower limit of sensitivity of between ten and 100 molecules (Fig. 2). To establish the optimum dilution of mimic to be added to the TaqMan® assay, a chequer-board titration experiment was performed to investigate the effects of increasing mimic template on the assay sensitivity. High concentrations of mimic (in excess of 10^{-7} dilution) reduced assay sensitivity by 10-fold. However, it was found that by using a 10^{-9} dilution (corresponding to 40 molecules) of *Kpn I* linearised plasmid (pASFVm), a consistent mimic signal was produced without affecting the sensitivity of the TaqMan® assay (data not shown).

The size of the PCR product for ASFV was 250 bp in comparison to the larger mimic amplicon of 420 bp. A direct consequence of this difference in size was that in the presence of wild-type ASFV, amplification of the larger mimic template was inhibited. Discrimination of the two amplicons was achieved using two-colour FAM and VIC reporters for ASFV and mimic, respectively. Fig. 3 shows a 2D scatter-plot for the results of a

Table 3
Results showing the limit of detection achieved by the TaqMan®, OIE PCR and VI procedures using (A) ASFV diluted into uninfected spleen material and (B) serial $\log_{10}$ dilutions of the DNA of 11 selected ASFV isolates

Isolate	Detection limit		
	TaqMan® PCR (7 μl)[a]	OIE PCR (7 μl)[a]	VI (300 μl)[a]
A			
LIL 20/1	10^{-5}	10^{-2}	10^{-6}
MALTA/78	10^{-5}	10^{-5}	10^{-6}
B			
CAM 1/86	10^{-4}	10^{-3}	n.d.
HINDE II	10^{-5}	10^{-2}	n.d.
KAB 94/1	10^{-4}	10^{-1}	n.d.
DEDZA	10^{-5}	10^{-2}	n.d.
KAL 88/1	10^{-6}	10^{-3}	n.d.
UGA 1/95	10^{-4}	10^{-1}	n.d.
RSA 1/92	10^{-2}	10^{-1}	n.d.
RSA W/1/99	10^{-2}	10^{-1}	n.d.
TENGANI	10^{-4}	10^{-3}	n.d.
MOZ 94/8	10^{-4}	10^{-2}	n.d.
NAM P1/95	10^{-3}	10^{-1}	n.d.

n.d., Not determined.

[a] Relative volume of diluted starting material tested by the different assays. For the PCRs, this calculation accounts for a concentration due to the extraction methods used.

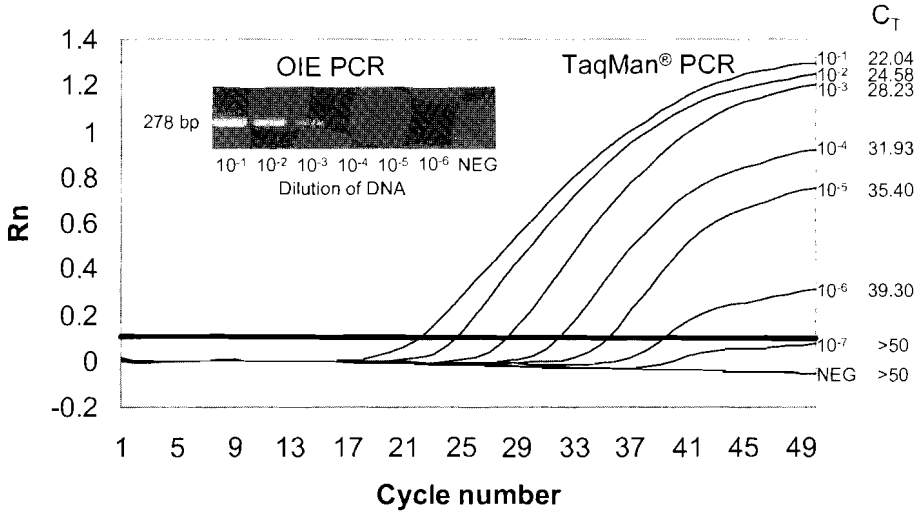

Fig. 4. Comparison of the results obtained by the TaqMan® PCR (amplification plots of fluorescence signal) and the OIE PCR method (bands obtained by gel electrophoresis are shown in inset) on the dilution series of the virus KAL 88/1. TaqMan® C_T values for the respective dilutions of DNA are shown.

representative assay. ASFV positive samples were clearly distinguished from negative samples as they had a high FAM and low VIC fluorescence. ASFV negative samples were grouped within the 99% confidence ellipse for the negative controls (NEG).

3.2. Performance of the ASFV TaqMan® PCR assay with diverse ASFV isolates

The TaqMan® PCR successfully detected DNA extracted from 25 diverse ASFV isolates tested (Table 1). The OIE reference PCR method also detected all 25 isolates. All 16 African and European tick ASFV isolates were also detected by the TaqMan® assay with all C_T values below 30.0 (data not shown). Serial $\log_{10}$ dilutions were made from the DNA of 11 selected ASFV isolates, which included at least one representative virus from each genotypic group. These dilution series were tested both by the TaqMan® PCR assay and the OIE method and the limit of detection by each procedure is shown in Table 3. Fig. 4 illustrates the fluorescence values obtained by the TaqMan® PCR and the bands obtained by gel electrophoresis using the OIE PCR method, respectively, on the dilution series of the virus KAL 88/1. The TaqMan® PCR clearly had a higher limit of detection than the OIE PCR on each of these isolates ranging from one to three $\log_{10}$ of dilution.

The sensitivity of the TaqMan® PCR assay was compared directly to that of VI by testing a dilution series of two ASFV isolates (LIL 20/1 and Malta/78). In these experiments, the material containing ASFV was diluted in normal spleen cells taken from an uninfected pig. VI was shown to be approximately a single $\log_{10}$ more sensitive than the TaqMan® PCR assay when 3 μl of the template produced using the QIAgen kit (this volume corresponds 7 μl of the original material when the extraction method is taken into account) was used for the TaqMan® PCR in contrast to 300 μl of material for VI (Table 3). It was subsequently shown that an increase in sensitivity of 2 $\log_{10}$ was achieved using a nested PCR TaqMan® approach (data not shown).

3.3. Specificity of the ASFV TaqMan® assay

Fifty EDTA bloods (pools of ten animals) collected from pigs during the United Kingdom 2000 CSFV outbreak were tested by the ASFV TaqMan® PCR. Of these samples, 19 had been previously confirmed as positive for CSFV (McGoldrick et al., 1999), while ten were strongly positive by TaqMan® PCR specific for porcine circovirus-2 (Grierson et al., 2002 unpublished data). These samples were all negative with the ASFV TaqMan® assay.

4. Discussion

This report describes the development of an improved molecular test to detect ASFV. This assay uses the closed-tube TaqMan® format that has recently been successfully applied for the molecular diagnosis of many other viruses of veterinary importance (Belak and Thoren, 2001). This approach has several advantages over gel-based PCR methods such as increased speed as well as the provision of an objective and quantitative result. Furthermore, the inclusion of a TaqMan® probe confirms the identity of the PCR product; and since it is not necessary to open the reaction tube post-PCR, it is possible to process large numbers of samples rapidly without significant risk of contamination. During vali-

dation of this assay, PCR amplicons were also routinely visualised on agarose gels in addition to fluorescence determination with the TaqMan® instrumentation. There was broad agreement between these two methods, suggesting that gel-based methods may provide a useful alternative for detection of the PCR product where it is not possible to access TaqMan® equipment. In some circumstances it may be necessary to transport samples to ASFV reference laboratories for molecular analysis. We would recommend similar collection and transport protocols to be used as currently employed for traditional VI methods.

The performance of the ASFV TaqMan® assay was assessed at two independent sites; The VLA (OIE Reference Laboratory for CSF) and the IAH at Pirbright using end-point (ABI 7200) and real-time (ABI 5700) instrumentation, respectively. Only minimal modifications to the PCR buffer and cycling parameters were required to achieve an assay with similar analytical sensitivity at the two sites. The two different TaqMan® readers each have advantages for specific laboratory applications. For example, a large number of samples can be screened for the presence of ASFV using an end-point detector, whereas the assessment of viral load in clinical and experimental samples requires the use of real-time or quantitative instrumentation.

In order to ensure that the assay was able to detect a wide range of ASFV isolates, the PCR primers and a TaqMan® probe were designed to recognise conserved regions identified in an alignment of VP72 sequences. Only a limited number of ASFV VP72 sequences are currently available on GenBank. In this study, the sequence of 3′ fragments of VP72 from 48 additional ASFV isolates were included (Bastos et al., 2002, unpublished data) allowing greater confidence in the ability of the assay to detect diverse ASFV isolates. An additional benefit of targeting the 3′-end of VP72 is that this PCR amplicon can be sequenced in order to provide phylogenetic classification in the event that positive samples are obtained. Comparative experiments using DNA extracted from 12 selected ASFV isolates demonstrated that although the gel-based assay outlined in the current OIE manual was able to detect all the diverse ASFV isolates, it was consistently less sensitive than the TaqMan® assay. For compatibility with the TaqMan® RT-PCR for CSFV, template extraction for this study was carried out with the QIAgen viral RNA kit. This method yielded satisfactory DNA from the panel of ASFV isolates tested. Comparison with VI suggested that if exquisite sensitivity of the TaqMan® PCR is required then improvements to the assay, such as testing a greater volume of template, using an alternative DNA extraction method, or even use of a nested format could be employed.

The end point assay complements the existing RT-nPCR TaqMan® assay for CSFV (McGoldrick et al., 1999) which was shown to be robust and reliable during the 2000 CSF outbreak in the United Kingdom (Drew, 2001). In contrast to CSF, there has never been a documented case of ASF in the United Kingdom. To provide in-tube automated validation of the large number of anticipated negative results, an artificial template (mimic) was engineered. Successful amplification of the mimic ensures that the correct PCR components have been added to the well. This mimic may also be a valuable tool when testing DNA extracted from difficult biological matrices such as degraded samples and ticks where the presence of substances inhibitory to PCR can be a common problem (Rijpkema et al., 1996; Nicolas and Plichart, 1997).

In summary, a sensitive TaqMan® PCR was developed for the detection of ASFV. Using this method it is possible to obtain a reliable laboratory diagnosis within 24 h of sample receipt. This method is much faster than VI, which can take up to 6 days to declare a negative result. Such a reduction in time is not trivial since it will shorten the period of time that a farm or slaughterhouse is held under movement restrictions, allowing rapid return to normal trading and a significant reduction in animal welfare problems arising from overcrowding. In light of the acceptance of molecular assays to provide the confirmatory laboratory diagnosis for CSFV, this ASFV TaqMan® PCR assay will be a valuable tool for rapid differential diagnosis in cases of suspected swine fever.

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