

Molecular beacon real-time PCR detection of swine viruses

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Abstract

Rapid and reliable detection of viral pathogens is critical for the management of the diseases threatening the economic competitiveness of the swine farming industry worldwide. Molecular beacon assays are one type of real-time polymerase chain reaction (PCR) technology capable of fast, specific, sensitive, and reliable viral detection. In this paper, the development of molecular beacon assays as novel tools for the rapid detection of Aujeszky's disease virus, African swine fever virus, porcine circovirus type 2 and porcine parvovirus is described. The assays are capable of rapidly detecting 2×10^1 copies of target and are linear between 2×10^9 and 2×10^2 copies. They can detect virus specifically in clinical samples such as whole blood, serum and tissue. In comparison to conventional PCR they are either as sensitive or more sensitive. As such these molecular beacon assays represent a powerful tool for the detection of these viruses in swine.

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1. Introduction

All four of the viruses for which molecular beacon assays have been developed in this study represent a threat to the competitiveness of swine farming worldwide, through reduced growth, mortality, enforced slaughter or movement and trade restrictions. As such development of rapid, easy to use, reliable, sensitive and specific methods for detection of these viruses is critical, in order to monitor viral epidemiology and to understand viral pathogenesis. The aim of this study was to develop such tests using molecular beacon technology, a powerful variant of real-time polymerase chain reaction (PCR).

Aujeszky's disease virus (ADV) or pseudorabies disease virus is a member of family *Herpesviridae*, subfamily *Alpha-herpesvirinae*. The genome is approximately 150 kilobases (kb) in length and consists of double-stranded linear DNA (Mettenleiter, 1991). Pigs are the only natural host for ADV, but it can infect most mammals except for the tail-less primates (Sawitzky, 1997). In young piglets the central nervous system

is affected and mortality may reach 100%. Typical symptoms include twitching, muscle tremor, fever and vomiting (Sabo et al., 1969). Infected gilts tend to show respiratory symptoms such as coughing, sneezing along with fever, central nervous system disorders, vomiting and pruritus. In uncomplicated cases clinical symptoms will disappear after about 10 days and the animals can recover, although recovery rates do increase with age. Infection during pregnancy can cause stillbirth, abortion and mummified foetuses (Kluge and Mare, 1974). Aujeszky's disease is an Office International des Epizooties (OIE) notifiable disease.

African swine fever virus (ASFV) is a large enveloped virus, 175–215 nm in diameter with a variable length double-stranded DNA genome of 170–190 kb (Yanez et al., 1995). It is assigned as the only member of the genus *Asfivirus*, family *Asfarviridae* (Murphy et al., 1999). ASFV naturally infects all suids including warthogs, bushpigs and the domestic pig. The disease is endemic in Sardinia and in sub-Saharan Africa where transmission is facilitated by ticks of the genus *Ornithodoros*. Symptoms vary considerably depending on strain and host. Severe symptoms can include fever, haemorrhaging of the skin and internal organs, with death in 2–10 days and up to 100% mortality. Less virulent strains may cause slight fever, loss of appetite and depression (Mebus, 1988). African swine fever is an OIE notifiable disease.

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Porcine circovirus type 2 (PCV2) is a small, non-enveloped virus with a circular, single stranded DNA genome of approximately 1800 nucleotides (Meehan et al., 1998) belonging to the family *Circoviridae*, genus *Circovirus*. PCV2 is different antigenically and genomically from the non-pathogenic porcine circovirus type 1 (PCV1) (Tischer et al., 1982). It is associated with a number of clinical conditions including porcine multi-systemic wasting syndrome (PMWS) (Allan et al., 1998) and porcine dermatitis and nephropathy syndrome (PDNS) (Meehan et al., 2001). PMWS affected piglets are typically 6–16 weeks of age and along with severe wasting exhibit enlarged lymph nodes, pale skin colour and jaundice, unthriftiness, dyspnoea, pallor and diarrhoea. Due to the strong association between PCV2 and PMWS it is now believed that PCV2 is the causative agent in this disease complex. However, although PCV2 fulfils Koch's postulates as the causative agent of PMWS and disease symptoms can be reproduced by infection with this virus alone (Allan et al., 2003), its presence alone will not necessarily cause onset of disease.

Porcine parvovirus (PPV) is a small non-enveloped member of the family *Parvoviridae*, genus *Parvovirus*. The genome consists of a single molecule of linear single stranded DNA, approximately 5 kb in size (Bergeron et al., 1996). Infection in sows typically causes reproductive problems such as small litters, mummified foetuses, stillbirths, abortion, and neonatal deaths (van Leengoed et al., 1983). Although PPV is not shed over a long period it is capable of surviving outside the host for months and as such is extremely persistent (Mengeling and Paul, 1986). Furthermore, although PPV has little if any effect on mature boars they may act as non-infected carriers. Eradication is impossible due to this persistence but vaccination programs are used successfully worldwide.

Molecular beacon assays (Tyagi and Kramer, 1996) are based on fluorescent resonance energy transfer (FRET). As with conventional PCR, the assays require amplification of a target nucleic acid sequence facilitated by the binding and extension of two oligonucleotide primers. A molecular beacon is a 15–40 mer single-stranded DNA or PNA probe complementary to a section of the target sequence amplified by the primers. This probe sequence is flanked by two complementary 5–7 mer arms sequences, one with a fluorophore attached, the other with a quencher. The arms form a stem/loop structure when the molecular beacon is in an unbound state. In this state the fluorophore and quencher are in close proximity and fluorescence is quenched. When the molecular beacon anneals to a target molecule the complementary arms separate. As such the fluorophore and quencher are separated and an increase in fluorescence occurs. During PCR, presence of target nucleic acid will result in an accumulation of amplicons with complementary sequences to the molecular beacon probe sequence. Accordingly a concomitant increase in fluorescence will result. Absence of target nucleic acid will result in no accumulation of PCR amplicons, the molecular beacons will remain in the stem/loop configuration and no increase in fluorescence will occur.

The development of molecular beacon-based real-time PCR assays is described, providing novel powerful means for the

improved, high throughput detection of ADV, ASFV, PCV2 and PPV in the pig populations.

2. Materials and methods

2.1. Virus isolates

The development of the molecular beacon assays used nucleic acids from a virulent Northern Ireland ADV field isolate, designated NIA 3, ASFV isolates L60 and Spain75, a Canadian PCV2 isolate Stoon 1010 (Genbank accession no. AF055392) and PPV isolates 1005 (GenBank accession no. NC_001718) and Northern Ireland isolate 59e. Specificity testing also used the PCV1 isolate Weybridge and a Northern Ireland field isolate of porcine adenovirus. The optimised assays were also run on a selection of other target isolates. Table 1 shows all the isolates used in this study.

2.2. Clinical material

Fourteen porcine tissue samples from Northern Ireland ADV field cases were tested. Spleen, mesenteric lymph node, tonsil and brain tissues were collected and approximate 10% (w/v) homogenates were produced in Minimum Essential Medium

Table 1
Virus isolates used in this study

| Virus | Isolate | Country | Details |
|--------------------|-----------|------------------|---------------------|
| ADV | Imp 76 | England | Epidemic diarrhea |
| | Imp 95 | Holland | Bovine |
| | Imp 156 | Denmark | Bovine |
| | Imp 186 | Belgium | Acute death |
| | Imp 187 | Belgium | Virulent |
| | Imp 188 | Belgium | Mild virulent |
| | Imp 209 | USA | Bovine |
| | Bartha | Hungary | Vaccine |
| | NIA3 | Northern Ireland | Virulent |
| | Van Doorn | Holland | 14 day old piglet |
| ASFV | Lisbon60 | Portugal | Virulent |
| | Malawi | Malawi | Virulent |
| | Malta | Malta | Moderately virulent |
| | Spain75 | Spain | Virulent |
| PCV1 | Weybridge | England | |
| PCV2 | 999 | USA | Field case |
| | 1010 | Canada | Field case |
| | 1017 | Denmark | Field case |
| | 1019 | Spain | Field case |
| | 1206 | Belgium | Field case |
| | 1306 | England | Field case |
| | 1247 | Sweden | Field case |
| | 5549 | Northern Ireland | Field case |
| | 9367 | Italy | Field case |
| | 48285 | France | PMWS field case |
| H2755 | Hungary | Field case | |
| Porcine adenovirus | | Northern Ireland | Field case |
| PPV | 59e | Northern Ireland | Field case |
| | 1005 | | Reference strain |
| | 1008 | France | Field case |

with Earle's salts (MEME) (Invitrogen Ltd., Paisley, U.K.). Homogenisation was carried out in a Stomacher80 (Seward Ltd., Worthing, U.K.). These were then clarified by centrifugation at 3000 rpm for 30 min and stored as 5% homogenates in SPGA buffer (0.22 M sucrose, 3.8 mM KH_2PO_4 , 5.44 mM L-Glutamic acid-mono sodium salt, and 0.8% irradiated foetal calf serum), pH 6.0.

Whole blood was taken from one ASFV experimentally infected pig and five sentinel pigs at -1, 7, 9 and 14 days post-infection.

A total of 16 field sera from PMWS affected and non-affected English farms were selected for testing along with 10 tissues from two PCV2 and PPV experimentally infected animals. The tissue samples consisted of liver, mesenteric lymph node, small intestine, inguinal lymph node and kidney. All were 10% homogenates, produced by placing 100 mg of tissue and 1 ml of MEME in Lysing Matrix A tubes (Q-BIOgene, Cambridge, U.K.) and shaking in a Hybaid Ribolyser (now called the FastPrep Homogenizer and Isolation System, Thermo Electron Corporation, Basingstoke, U.K.) for 20 s at speed 4. Homogenates were then clarified by centrifugation for 30 min at 3000 rpm. An additional five sera were selected from two pigs infected experimentally. Sampling was at days 9, 18 and 24 days post-infection for pig Tag No. 6 and at 9 and 18 days post-infection for pig Tag No. 21. These pigs were infected with French isolate 48285.

2.3. Design of primers and probes

Alignments were created from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) sequences using the Genedoc program (<http://www.psc.edu/biomed/genedoc/>).

To design each assay a primer set was first generated using Oligo 5.0 (Molecular Biology Insights). Design of the probes was carried out by selecting conserved 25 mer sequences within the primer regions. The melting temperatures (T_m) of these sequences were then analysed using Hyther software (<http://ozone2.chem.wayne.edu/Hyther/hythermenu.html>).

A set of two 6 mer arms was added to suitable sequences and the resulting molecular beacons were checked in mFold (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>) to ensure that they assumed the correct stem loop configuration. Those that did were analysed along with their primer set for dimer formation using Oligo 5.0.

Sequences were aligned for ADV from regions coding for glycoproteins gB, gC, gD, gE, gH, latency associated transcription unit, UL42 polymerase accessory protein and the 28k membrane protein. The ADV beacon was designed using an alignment of eleven sequences from the glycoprotein gD region, as evaluation of all the ADV alignments showed this region to be suitably conserved. A set of primers producing a 110 bp amplicon and a 25 mer molecular beacon were designed. Complete conservation was observed in all strains in this area for both primers and probe.

The ASFV molecular beacon was designed using an alignment of 23 sequences from the 9GL region. A set of primers producing a 264 bp amplicon and a 25 mer molecular beacon

were designed. However, the alignment was incomplete at the 5' end and only nine sequences were available for the forward primer region. No mismatches were present in this area. In the reverse primer region two sequences had a single mismatch, but these were not near the 3' end of the primer. There were no mismatches in the molecular beacon region.

A circovirus alignment was produced containing 193 PCV2 and 21 PCV1 sequences. A conserved primer set producing a 263 bp amplicon and a 25 mer molecular beacon were chosen in an area spanning the 5' end of the replicase gene. The forward primer was not PCV2 specific as it matched to the PCV1 and PCV2 sequences. The reverse primer had a single mismatch in 14 out of the 193 PCV2 sequences, none of which were near the 3' end. This primer mismatched to all of the PCV1 sequences in five to seven positions including a double mismatch and a treble mismatch at the 3' end. The PCV2 molecular beacon mismatched in one position to 14 of the PCV2 sequences. These 14 sequences represented 6 mismatched positions. In addition one sequence had three mismatches. Compared to the PCV1 sequences the beacon probe region mismatched in at least six positions with a further four deletions. As such the region was an ideal choice to confer PCV2 specificity.

Two PPV alignments were made, one with complete genome sequences and one with VP2 sequences. A set of primers producing a 130 bp amplicon and a 25 mer molecular beacon were designed in the VP2 region against 23 sequences. The forward primer mismatched to just one sequence in a single position at the 5' end of the primer. The reverse primers matched perfectly to all sequences on the alignment. The beacon probe region showed one mismatch to three sequences in the same position.

Primers and probes were purchased from MWG Biotech (90 Long Acre, Covent Garden London, U.K.).

2.4. Extraction of DNA and routine conventional PCR

DNA was extracted from 200 μl of virus pool, cell culture material, lysed tissue homogenates, whole blood or serum using the Qiagen DNA Blood Mini Kit (Qiagen Ltd., Crawley, U.K.) according to the manufacturer's instructions. Conventional PCR was carried out using HotStarTaq Master Mix (Qiagen Ltd., Crawley, U.K.) according to the manufacturers instructions with 0.5 μM each primer. The primers used for our routine conventional PCR assays are shown with their annealing temperatures (T_a) and amplicon sizes in Table 2. 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, and extension at 72 °C. This was followed by a final extension step of 5 min at 72 °C before holding at 8 °C. Reaction volumes were 25 μl .

2.5. Real-time PCR

Molecular beacon assays used JumpStart Taq (Sigma-Aldrich, Gillingham, U.K.). Table 3 shows the reaction

Table 2
Primers and T_a used in routine conventional PCR assays

| Name | Sequence | T_a (°C) |
|------------------------------|------------------------|------------|
| ADV F375–394 ^a | TTTATCGAGTACGCCGACTG | 52 |
| ADV R604–622 ^a | CGGGCGAACGGGCACTCTT | |
| ASFV OIE F ^b | ATGGATACCGAGGGAATAGC | 59 |
| ASFV OIE R ^b | CTTACCGATGAAAATGATAC | |
| PCV2 OUARDANI F ^c | CACGGATATTGTAGTCCTGGT | 50 |
| PCV2 OUARDANI R ^c | CCGCACCTTCGGATATACTGTC | |
| PPV 414 bp F ^a | AAGAGCCTGCTTTGGTGAAA | 55 |
| PPV 414 bp R ^a | AGAGTTTTGGAGCAAAGGCA | |

^a Primers designed in-house.

^b Office International des Epizooties, http://www.oie.int/eng/en_index.htm.

^c Ouardani et al. (1999).

conditions and amplicon sizes for all the molecular beacon assays. All real-time assays were carried out on MJ Research Opticon2 real-time thermal cycler (Bio-Rad Laboratories Ltd., Herts, U.K.). Cycling for all molecular beacon assays was initiated with a hot start denaturation of 2 min. Thermal cycling consisted of 40 cycles consisting of 30 s each of denaturation at 94 °C, annealing and extension at 72 °C. This was followed by an 8 °C hold. Reaction volumes were 25 µl. All molecular beacons were labelled with FAM, except for PPV, which was labelled with HEX. Black Hole Quenchers were used in all cases. Table 4 shows the details of all oligonucleotides used in the molecular beacon assays.

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

Bulk amounts of PCR product were used to produce dilution series of quantified standard DNA for each assay. The PCR products were run on a 1.5% agarose gel, the bands were excised, purified from the gel using Qiaex Gel Extraction Kit (Qiagen Ltd., Crawley, U.K.) and quantified using a spectrophotometer. Based on the size of the amplicon and on the average molecular weight of a base pair the number of copies of the amplicon/µl was calculated. From this a dilution series of known concentration of amplicon copy number was produced, down to 10⁰ copies/µl. This process was carried out on one isolate for each virus. For ASFV and PPV standard material the real-time assay primers were used to generate the PCR product from isolates L60 and 1005, respectively. For ADV our conventional PCR primers were used and these generated a 248 bp product from isolate NIA3 that spanned the real-time assay amplicon. For PCV2 a 689 bp spanning product from isolate Stoon 1010 was produced using primers pair PCV2 SG 181F AAGATGCCATTTTTCCTT

Table 3
Reaction conditions for molecular beacon assays

| | T_a (°C) | Mg ²⁺ (mM) | For primer conc. (µM) | Rev primer conc. (µM) | Beacon conc. (µM) |
|------|------------|-----------------------|-----------------------|-----------------------|-------------------|
| ADV | 50 | 4 | 0.75 | 0.75 | 0.5 |
| ASFV | 51 | 5.5 | 0.5 | 0.5 | 1.0 |
| PCV2 | 50 | 4 | 0.5 | 0.5 | 0.5 |
| PPV | 52 | 4 | 0.5 | 0.5 | 0.5 |

and PCV GROUP 852–871R CCCGCTCACTTTCAAAGTT, used with a T_a of 50 °C.

2.7. Melting curve analysis of molecular beacons to determine T_m , signal to background ratio and tolerance to mismatches

Prior to optimisation of molecular beacon assays, the beacons were tested for correct functionality by means of a melting curve. 0.5 µM of each beacon was melted with 1.0 µM of target and 4 mM Mg²⁺ in 25 µl of 10 mM Tris pH 8.0. The target oligonucleotides were reverse complements of the molecular beacon probe sequence with the addition of two thymines at each end. In addition replicates were melted containing no target. Melting was carried out on the MJ Research Opticon2 real-time thermal cycler (Bio-Rad Laboratories, Herts, U.K.) and involved slowly raising the temperature from 40 to 95 °C taking a fluorescent reading every 0.1 °C with a dwell time of 0.1 s at each temperature.

The tolerance of the PCV2 and PPV molecular beacons to mismatched targets was analysed by carrying out the melting curve analysis on target oligonucleotides containing the mismatches seen in the Genbank alignments. As such one mismatched target was tested for PPV containing a single mismatch. Seven mismatched targets were tested for PCV2, six with a single mismatch and one with a single and a double mismatch. The sequences of these mismatched targets are shown in Table 5. The T_m s of the molecular beacons binding to the mismatched and perfectly matched targets were calculated by the Opticon2 software as the negative derivative of fluorescence over temperature versus temperature. As there were no mismatches in the ADV and ASFV alignments in the beacon probe regions these were not included in the analysis.

2.8. Sequencing

Sequencing of standard DNA was carried out using BigDye Terminator Kit Version 3.1 (Applied Biosystems, Warrington, U.K.). Forward primers from the pairs used to produce the standard material were used. One microliter of 3.2 pmol/µl primer was added to 8 µl of a 1 in 4 dilution of Terminator Ready Reaction Mix in BigDye sequencing buffer and 1 µl of target, made up to a 20 µl volume with DEPC treated water (Ambion, Cambridgeshire, U.K.). This reaction mix was subject to an initial denaturation of 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Excess dye-terminator was removed using DyeEx 2.0 spin columns (Qiagen Ltd., Crawley, U.K.). The sequencing reactions were analysed

Table 4

Molecular beacon nucleotide sequences, region of design and fluorophore used for the molecular beacon assays

| Name and sequence ^a | Gene | Fluor |
|---|----------|-------|
| ADVgd MB462-25: cgctcgGACTACATGTTCCCCACGGAGGACGcgagcg ADVgd F429-445: CGCACCACGCCGATGTG ADVgd R519-538: CGGTACTGGCCCTCGTTGAA | GD | FAM |
| ASFV_9GL_MB162-25: cgctcgGGCTCCAATAAAGTCGGTTTTTCCCAcgagcg ASFV_9GL_F77: CGGGAGACGTTGTTTTAT ASFV_9GL_R340: CGCCTTTTCGTATCTTAC | 9GL | FAM |
| PCV2 MB25 394-418: gcgagcCACCTCAGCAGCAACATGCCCAGCAgctcgc PCV GrFor 283-300: CTTCTGCGGTAACGCCTC PCVILR529Match_GFor: TTACCCTCCTCGCCAACA | Rep gene | FAM |
| PPV VP2 MB156-25: cgctcgTCAATACTTGGGGGAGGGCTTGGTTcgagcg PPV VP2 F112-132: GGGTTGGTGTGTCTACAGGT PPV VP2 R190-210: GTATGAGTCTTGATGCGTGTGC | VP2 | HEX |

^a Lower case sequence letters denote molecular beacon arms.

Table 5

Melting curve analysis of PCV2 and PPV molecular beacons against perfectly matched and mismatched targets

| Virus | Probe sequence | No. of mismatches | No. of isolates in alignment | T_m of beacon against target |
|-------|---------------------------|-------------------|------------------------------|--------------------------------|
| PCV2 | CACCTCAGCAGCAACATGCCCAGCA | 0 | 193 | 74.5 |
| | CACCTCAGCAGCAACATGCCCAcCA | 1 | 1 | 72 |
| | CACtTCAGCAGCAACATGCCCAGCA | 1 | 1 | 70 |
| | CACCTCAGCAGCAACATGCCtAGCA | 1 | 6 | 68.5 |
| | CACCTCAGCAGCAACATGCCgAGCA | 1 | 1 | 67 |
| | tACCTCAGCAGCAACATGCCCAGCA | 1 | 1 | 73.5 |
| | CACCTCgGCAGCAACATGCCCAGCA | 1 | 3 | 69.5 |
| | CACCTCAaCAGCTGCAtgCCCAGCA | 3 | 1 | 61 |
| PPV | TCAATACTTGGGGGAGGGCTTGGTT | 0 | 15 | 68.5 |
| | TCAATACTTGGGGGAGGGgTTGGTT | 1 | 3 | 59 |

Mismatches are shown in lower case.

on an ABI 3100 Genetic Analyser (Applied Biosystems). The other target isolates for each assay were also sequenced.

2.9. Optimisation of molecular beacon assays

The first stage of optimisation involved performing a gradient PCR with T_a and Mg^{2+} concentrations between 50 and 60 °C, and 3 and 7 mM, respectively. On determining the T_a/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 μ M concentrations. Finally a variety of beacon concentrations from 0.1 to 1.0 μ M were tested with the optimal annealing temperature, Mg^{2+} and primer concentrations.

2.10. Comparison of assay sensitivity to conventional PCR, specificity testing and testing of assays on a range of isolates

In order to compare sensitivity of the ADV, PCV2 and PPV molecular beacon assays to conventional PCR, DNA was extracted from neat virus pool and was diluted 10-fold in 10 mM Tris pH 8.0. Viral pool NIA3 was used for the ADV assay, Stoon 1010 for the PCV2 assay and 59e for the PPV assay. Dilution of DNA extracted from whole blood of a pig infected with the

Spain 75 was used for ASFV. Dilution series were then run in duplicate with their respective molecular beacon assays and with our conventional PCR assays.

Each assay was tested using ADV, ASFV, PCV1, PCV2, porcine adenovirus and PPV nucleic acids as target. Nucleic acids from the same isolates used for sensitivity comparison were used as well as PCV1 from the Weybridge isolate and porcine adenovirus nucleic acid from a field isolate. In addition each assay was further tested using more assay specific isolates as shown in Table 1. The ASFV and PPV assays were tested on an extra two isolates in addition to those used for assay optimisation. The ADV assay was tested on an additional 9 isolates and the PCV2 assay on an additional 10 isolates.

3. Results

3.1. Sequencing

As it is known that molecular beacons are sensitive to mismatches, sequencing was carried out in order to establish that the standard material was suitable for evaluation of the molecular beacon assays.

Sequencing of gel purified standard material showed that all four molecular beacon sequences matched perfectly to the

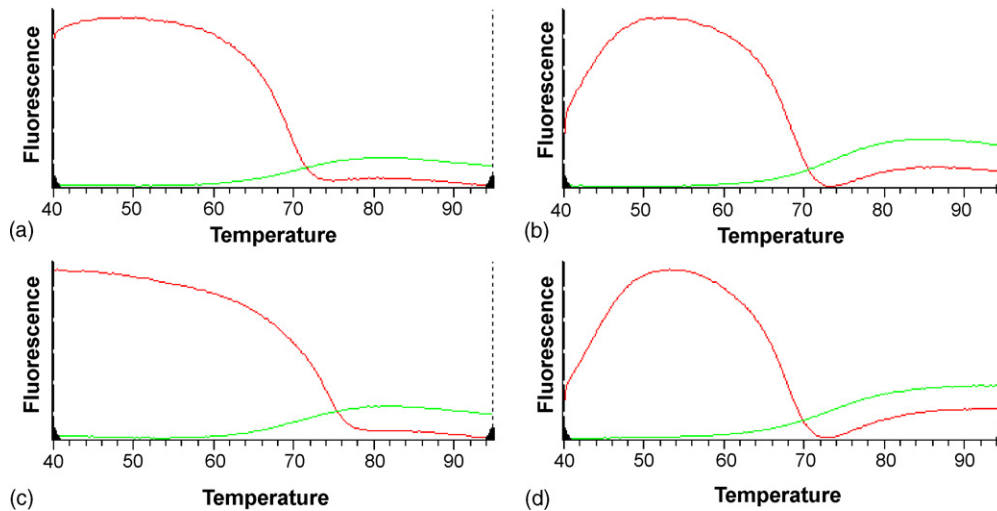


Fig. 1. Melting profile of the molecular beacons. Red traces are beacon and target; green traces are beacon without target. Fluorescence vs. temperature data shows typical melting profile for a functional molecular beacon. Part (a) is the ADV molecular beacon melting profile, (b) is ASFV, (c) is PCV2 and (d) is PPV.

probe sequences for all four assays. The other target isolates for each assay were sequenced and these also all showed perfect homology to the beacon probe sequences.

3.2. Melting curve analysis

Melting curve analysis of all four probes showed the typical melting profile associated with functional molecular beacons, as exemplified by Fig. 1.

The red traces represent a sample with probe and target, whereby the initial fluorescence is high due to binding of the probe and the concomitant separation of the fluorophore and quencher. The fluorescence begins to fall as the T_m of the probe approaches until it reaches baseline level where no probe is bound. The green traces show the behaviour of the beacons with no target present. From the melting curves it can be seen that the assay T_a s are at temperatures of optimal binding for all beacons signal to background ratios for the beacons, as determined by the difference in fluorescent signal between the bound and unbound probes, was between 4 and 11-fold.

Melting curve analysis of the PCV2 and PPV molecular beacons against their mismatched targets is summarised in Table 5. For the PPV assay the single mismatch caused a drop in T_m of 9.5 °C. For PCV2 the single mismatches caused drops in T_m of 1–7.5 °C. The target with three mismatches showed a drop in T_m of 13.5 °C. Fig. 2 shows the variation in melting curve profile when the PCV2 molecular beacon is melted with some of the mismatched targets.

3.3. Optimisation of molecular beacon assays, determination of sensitivity and linear dynamic range

Optimisation of each assay involved determining the best T_a , Mg^{2+} concentration, primer concentrations and probe concentration. Mg^{2+} concentration and T_a were optimised together

using the Opticon2 gradient facility. In a real-time PCR assay the cycle number at which the fluorescent trace for a particular reaction crosses the threshold of background fluorescence is known as the Ct value. Conditions for the molecular beacon assays were chosen that gave the lowest Ct values for a particular known starting concentration of template. In this way assays of the greatest sensitivity were developed. Table 3 shows the optimised conditions for each assay.

Dynamic range and sensitivity of the optimised assays were determined using templates of known starting quantity ranging from 2×10^0 to 2×10^9 copies per microliter. All of the assays were linear from 2×10^9 to 2×10^1 . Fig. 3 shows the standard curves for all four assays.

3.4. Comparison of assay sensitivity to conventional PCR, specificity testing and testing of assays on a range of isolates

Sensitivity of the molecular beacon assays compared well to that of the conventional PCR. The molecular beacon assay

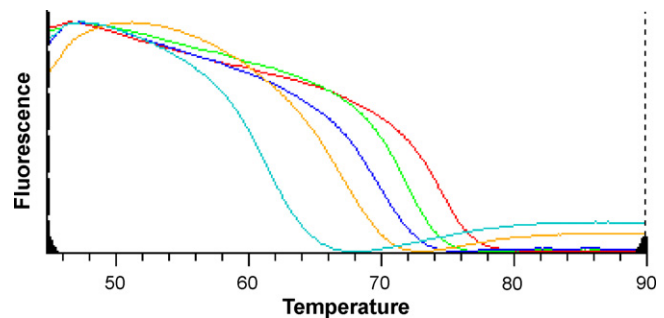


Fig. 2. Tolerance of PCV2 molecular beacon to mismatched targets. The right-hand most trace is the beacon melted with perfectly matched target, with a T_m of 74.5 °C. The next trace is the beacon melted with single mismatched target with a T_m of 72 °C. The next trace is the beacon melted with single mismatched target with a T_m of 70 °C. The next trace is the beacon melted with single mismatched target with a T_m of 67 °C. The left-hand most trace is the beacon melted with target containing three mismatches with a T_m of 61 °C.

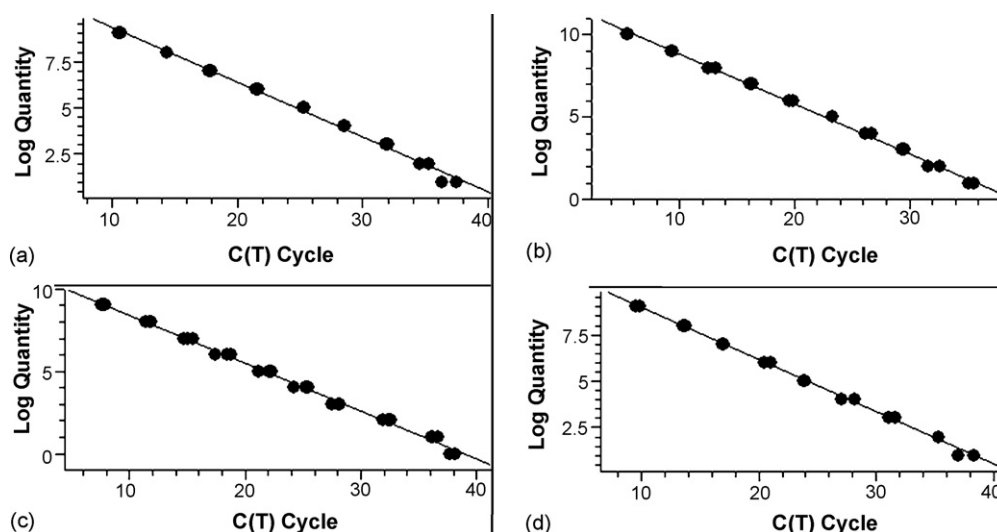


Fig. 3. Standard curves for molecular beacon assays. Graph shows log of initial copy number against threshold cycle number. Duplicate samples are shown for each dilution. (a) ADV assay: $y = -0.30x + 12.31$, $r^2 = 0.995$. Range is 2×10^9 to 2×10^1 . (b) ASFV assay: $y = -0.30x + 11.84$, $r^2 = 0.997$. Range is 2×10^{10} to 2×10^1 . (c) PCV2 assay: $y = -0.30x + 11.32$, $r^2 = 0.996$. Range is 2×10^{10} to 2×10^1 . (d) PPV assay: $y = -0.28x + 11.78$, $r^2 = 0.997$. Range is 2×10^{10} to 2×10^1 .

for ADV detected target to a 10^{-3} dilution of NIA3 virus pool, as did the conventional PCR assay. The PCV2 molecular beacon assay detected target to a 10^{-8} dilution of Stoon 1010 virus pool, also showing identical detection to the conventional assay. The PPV molecular beacon assay detected target to a 10^{-8} dilution of 59e virus pool, one 10-fold dilution more sensitive than the conventional assay. Comparative testing of the ASFV assays was carried out using a 10-fold dilution series of DNA extracted from porcine whole blood taken from a Spain75 experimentally infected animal. The molecular beacon assay detected this target to a dilution of 10^{-5} , one dilution more sensitive than our conventional assay.

Each assay was tested using ADV, ASFV, PCV2, PCV1, PPV and porcine adenovirus type 4 as target. All assays only produced a fluorescent signal with their own specific target.

Each assay also amplified all the additional target isolates specific to that assay.

3.5. Testing of clinical samples

3.5.1. ADV clinical samples

A total of 14 tissue samples (brain, mesenteric lymph node, spleen and tonsil) from nine ADV naturally infected pigs from Northern Ireland were tested using the ADV molecular beacon and conventional assays. Both assays detected 10 of these as positive and were capable of detecting virus in all four tissue types. Results are shown in Table 6.

3.5.2. ASFV clinical samples

DNA was extracted from whole blood taken from one experimentally infected pig (Tag No. 29) and five sentinel pigs (Tag Nos. 31–35) at -1, 7, 9 and 14 days post-infection. These DNA samples were tested by conventional PCR using the OIE recommended primers. This PCR was carried out as described in the OIE manual for diagnostic tests (Office International des Epizooties, http://www.oie.int/eng/en_index.htm).

Table 6
Results of molecular beacon and conventional PCR assay runs for ADV tissue samples of naturally infected pigs

| No. | Sample | Tissue type ^a | Conventional PCR results | Molecular beacon results |
|-----|-----------|--------------------------|--------------------------|--------------------------|
| 1 | 01-10444 | Brain | + | + |
| 2 | 01-10444 | Tonsil | + | + |
| 3 | 01-12322 | Brain | + | + |
| 4 | 02-404 | Brain | - | - |
| 5 | 02-404 | Tonsil | - | - |
| 6 | VP97-5319 | Brain | + | + |
| 7 | VP97-5382 | Brain | + | + |
| 8 | 03-2082 | Tonsil | - | - |
| 9 | 03-2301 | Brain | + | + |
| 10 | 05-3536 | Brain | + | + |
| 11 | 05-3536 | Tonsil | + | + |
| 12 | 05-3536 | MLN | + | + |
| 13 | 05-3743 | MLN | - | - |
| 14 | 05-3743 | Spleen | + | + |

^a MLN: mesenteric lymph node.

Table 7
Results of ASFV molecular beacon assay run on whole blood samples obtained from experimental infected and sentinel animals

| Days post-infection | Pig no. | ASFV OIE PCR | ASFV beacon assay |
|---------------------|---------|--------------|-------------------|
| -1 | 29 | - | - |
| | 31 | - | - |
| | 32 | - | - |
| | 33 | - | - |
| | 34 | - | - |
| | 35 | - | - |
| 7 | 29 | + | + |
| | 31 | - | - |
| | 32 | - | - |
| | 33 | - | - |
| | 34 | - | - |
| | 35 | - | - |
| 9 | 29 | + | + |
| 14 | 31 | + | + |
| | 32 | + | + |
| | 33 | + | + |
| | 34 | + | + |
| | 35 | + | + |

In addition they were tested using the ASFV molecular beacon assay. DNA from the experimentally infected pig was positive using both assays at 7 and 9 days post-infection. At day 14 post-infection DNA samples from the experimentally infected pig and all five sentinel pigs were positive with both assays. Results are shown in Table 7.

3.5.3. PCV2 and PPV clinical samples

A total of 16 field sera from PMWS affected and non-affected English farms were selected for testing with PCV2 and PPV molecular beacon assays. The sera were also tested with PCV2 (Ouardani et al., 1999) and PPV conventional PCR. Four of these field sera were found to be positive for PCV2 with both the molecular beacon assay and the standard PCR assay. In addition, the molecular beacon and conventional PCV2 assays both found two field sera to be positive which the other did not. None of these field sera were found to be positive for PPV by conventional PCR but four were weakly positive using the molecular beacon assay.

Sera from two pigs experimentally infected with PCV2 in another study were also tested. Sera had been taken at days 9, 18 and 24 days post-infection for pig Tag No. 6 and at 9 and 18 days post-infection for pig Tag No. 21. Both the PCV2 molecular beacon assay and the conventional PCV2 PCR found all seven sera positive for PCV2. Both pigs showed the highest levels of PCV2 at 18 days post-infection as shown by the lowest Ct value at this time point and by a stronger electrophoresis band. Conventional PCR also confirmed the drop in PCV2 levels in pig Tag No. 6 at 24 days post-infection as shown by a weaker electrophoresis band. No PPV was detected in any of these sera by either the PPV molecular beacon assay or by conventional PCR.

Ten tissues from two pigs infected experimentally with both PCV2 and PPV in another study were tested with molecular beacon and conventional assays. Tissues used were liver, mesenteric

lymph node, small intestine, inguinal lymph node and kidney. All tissues samples were strongly positive with all assays.

The results of testing these clinical samples are shown in Table 8.

4. Discussion

PCR has been shown to be a rapid and sensitive method for the detection of virus by the specific amplification of viral nucleic acid sequences. Conventional and real-time PCR assays are widely used for detection of viruses in swine (Belak, 2005). Several real-time PCR chemistries are now used widely and they allow a closed-tube assay format whereby accumulation of PCR product can be measured by a concomitant increase in fluorescence. Accumulating fluorescence can be measured in the tube after every cycle of PCR by a variety of commercially available instruments. Generation of a fluorescent signal can be via non-specific intercalation of SYBR Green into the DNA helix or by the binding of fluorescent probes to an area within a PCR amplicon. Such probe based systems include molecular beacons (Tyagi and Kramer, 1996), TaqMan[®] probes (Jebbink et al., 2003) and PriProET (Rasmussen et al., 2003). All these real-time PCR assays have several important improvements over conventional PCR. The closed-tube format means that no agarose gel electrophoresis is necessary. Accordingly, future PCR amplifications are not exposed to potentially cross-contaminating amplicon, run time is reduced and potential exposure to ethidium bromide is eliminated. The increased specificity of probe-based systems is a further improvement over SYBR Green assays as a fluorescent signal is only generated when probes are bound to a specific matching amplicon.

Molecular beacons now have many uses including single nucleotide polymorphism (SNP) detection (Marras et al., 2003), biosensors and arrays (Liu et al., 2000) and nucleic acid sequence based amplification (NASBA) (Leone et al., 1998). To date molecular beacon assays have been developed for detection of many pathogens including viruses such as Epstein Barr virus and cytomegalovirus (Jebbink et al., 2003), human immunodeficiency virus (Vet et al., 1999), adenovirus (Poddar, 2000) and hepatitis C virus (Yang et al., 2002).

Molecular beacons are characterised by high levels of sensitivity and specificity. The molecular beacon assays in this study proved to be highly sensitive, detecting at least 2×10^1 copies of gel purified PCR product and exhibited levels of sensitivity equal to or better than those shown by the conventional PCR assays used in our laboratories. Molecular beacon assays are not only inherently more specific than SYBR Green assays due to the presence of the probe, but due to their stem/loop configuration they display a very low tolerance to target point mutations (Bonnet et al., 1999) conferring an extra degree of specificity on diagnostic assays that use this chemistry. As such this technology is well suited to the diagnosis of DNA viruses with high levels of conservation between isolates, but development of assays for RNA viruses with more variable genomes could be difficult. The PCV2 and PPV proved tolerant to all the single mismatched targets as determined by melting curve analysis against mismatched oligonucleotides based on available

Table 8

Results of molecular beacon and conventional PCR assay runs for PCV2 and PPV on sera and tissue samples of experimentally and naturally infected pigs

| | PCV2 molecular beacon assay ^b | Conventional PCV2 PCR | PPV molecular beacon assay ^b | Conventional PPV PCR |
|------------------------------------|--|-----------------------|---|----------------------|
| Field sera | | | | |
| Field sera 1 | + | + | +w | – |
| Field sera 2 | + | + | – | – |
| Field sera 3 | + | + | – | – |
| Field sera 4 | – | – | – | – |
| Field sera 5 | – | – | – | – |
| Field sera 6 | – | – | – | – |
| Field sera 7 | – | – | – | – |
| Field sera 8 | – | – | – | – |
| Field sera 9 | – | – | – | – |
| Field sera 10 | +w | – | +w | – |
| Field sera 11 | +w | + | – | – |
| Field sera 12 | – | – | – | – |
| Field sera 13 | +w | – | +w | – |
| Field sera 14 | – | + | +w | – |
| Field sera 15 | – | + | – | – |
| Field sera 16 | – | – | – | – |
| PCV2 experimentally infected sera | | | | |
| Tag 6 9 dpi | +(Ct 24) | + | – | – |
| Tag 6 18 dpi | +(Ct 19) | + | – | – |
| Tag 6 24 dpi | +(Ct 23) | + | – | – |
| Tag 21 9 dpi | +(Ct 25) | + | – | – |
| Tag 21 18 dpi | +(Ct 19) | + | – | – |
| Dual infected tissues ^a | | | | |
| 14774 liver | + | + | + | + |
| 14774 MLN | + | + | + | + |
| 14774 SI | + | + | + | + |
| 14774 ILN | + | + | + | + |
| 14774 kidney | + | + | + | + |
| 14783 liver | + | + | + | + |
| 14783 MLN | + | + | + | + |
| 14783 SI | + | + | + | + |
| 14783 ILN | + | + | + | + |
| 14783 kidney | + | + | + | + |

^a MLN: mesenteric lymph node, SI: small intestine, ILN: inguinal lymph node.^b +w: weak positive.

Genbank sequences. Melting curve analysis of the PCV2 beacon also showed that it would bind to a target with three mismatches at the assay annealing temperature, albeit with a 13.5 °C drop in T_m . Using molecular beacon assays, as in this study, with T_a s considerably below the beacon T_m s allow some tolerance to mismatches while maintaining a high degree of specificity. The assays in this study proved highly specific, only generating a fluorescent signal in the presence of their specific target DNA.

The presence of ADV limits the competitiveness of the pig industry in countries affected by it due to export restrictions. Reliable diagnostics are a vital element in any eradication programme. Usually the detection of ADV is performed by immunofluorescence, virus isolation (Allan et al., 1984), enzyme linked immunofluorescent assay (ELISA) (Arias et al., 1992), PCR (Cao et al., 2005) and real-time PCR (van Rijn et al., 2004). Our ADV molecular beacon assay represents an improvement over the conventional PCR assays in terms of speed, reliability and safety and was found to be as sensitive as our conventional PCR assay. In animals other than pigs detection of ADV by serological methods is often impossible due to the fact that they

may not live long enough to produce a serological response. In such cases methods of diagnosis such as PCR could provide a useful tool, particularly if infections of animals other than pigs contribute to the persistence of the virus.

The OIE prescribed test for ASFV is ELISA but diagnosis can also be by a range of serological tests, by virus isolation (http://www.oie.int/eng/en_index.htm), by PCR (Aguero et al., 2004) or TaqMan[®] real-time PCR (King et al., 2003). A novel Invader assay for ASFV has been described recently (Hjertner et al., 2005), which involves isothermal amplification of DNA using a cleavase enzyme. As the virus 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. The use of our molecular beacon assay will facilitate such rapid diagnosis, due to the short assay run time and increased sensitivity compared to the conventional PCR assay. In addition, the use of real-time PCR will reduce the possibility of cross contamination associated with traditional PCR, the consequences of false positive results being

potentially serious. In an outbreak situation the added reliability of real-time PCR assays in combination with increased precautions when carrying out the testing should reduce the possibility of false positive results. The highly variable nature of the clinical signs of African swine fever (ASF) can make diagnosis difficult. Diagnosis can be confused with a range of bacterial and viral diseases, including classical swine fever (CSF) (Kleiboeker, 2002). Again, delays in accurate diagnosis could have severe implications in the case of a disease outbreak. This molecular beacon assay represents a rapid and reliable method for definitively confirming the presence of ASFV. The most serious case of misdiagnosis would be the confusion of CSF with ASF. To this end traditional PCR has been applied to the simultaneous detection of, and differentiation between CSFV and ASFV (Aguero et al., 2004). Further work could involve the development of a real-time multiplex assay for these two viruses.

The development of the molecular beacon assays for PCV2 and PPV has provided a useful tool in the ongoing research efforts into the nature of the role of PCV2 in PMWS. Diagnosis of this disease complex requires the presence of PCV2 virus associated with lesions, in addition to clinical signs. Despite the fact that it is now known that PCV2 alone can cause lesions consistent with PMWS (Ladekjaer-Mikkelsen et al., 2002), the exact pathogenesis of PCV2 is not fully known. Several studies have shown that co-infection of PCV2 and PPV can cause the development of severe clinical symptoms (Allan et al., 1999). The most common methods of detecting PCV2 and PPV are virus isolation, ELISA, conventional and real-time PCR, in situ hybridisation and immunohistochemistry (Brunborg et al., 2004; Cao et al., 2005; Jenkins, 1992; Kim and Chae, 2004; McNeilly et al., 2002; Ouardani et al., 1999). PCR has been used extensively in research efforts into the combined roles of PCV2 and PPV. This PCV2 molecular beacon assay was as sensitive as its conventional counterpart whereas the PPV molecular beacon assay was one log more sensitive than its counterpart. As such these assays, providing all the benefits associated with a rapid real-time probe-based closed-tube assay, represent an improved tool to aid the research efforts into the role of these two viruses in PMWS pathogenesis. Molecular beacon assays lend themselves well to multiplexing (Vet et al., 1999). These allow more than one target to be detected in a single reaction, by means of several probes in a single tube, each labelled with a unique fluorophore. Multiplex assays will be developed for the simultaneous detection of PCV2 and PPV.

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 format 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. These molecular beacon assays could be adapted readily for such automated processes.

This study has shown that molecular beacon assays are powerful, specific diagnostic tools for the detection of viral DNA in clinical samples with improved speed, reliability, sensitivity and ease of use. As such they represent a powerful tool for virus research and the monitoring of endemic and exotic diseases.

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