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ORIGINAL ARTICLE

Development of a real-time TaqMan PCR assay for the detection of porcine and bovine Torque teno virus

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Abstract

Aims: The goal of this study was to develop and to optimize molecular tools to detect the presence of Torque teno virus (TTV) in swine and cattle. A novel real-time polymerase chain reaction (PCR) using a TaqMan probe was developed to detect both genogroups of TTV strains.

Methods and Results: Oligonucleotide primers and hybridization probes were designed based on sequence analysis of the noncoding region, a highly conserved part of the genome. The real-time PCR assay specifically detected bovine and porcine TTV DNA without cross-amplification of other common pathogens. The assay was compared with conventional PCR and nested-PCR assays for the detection of porcine genogroups 1 and 2 and bovine TTV on plasma and faecal samples, and the assay was found faster, more reliable and reduced the risk of false positive results.

Conclusions: The real-time PCR assay provided better detection results for the two TTV genogroups in both swine and cattle compared to the conventional PCR assays.

Significance and Impact of the Study: This new TaqMan PCR assay will be a useful tool for the detection of animal TTV strains, to evaluate the viral load from animal host and finally to identify the presence of these viruses in the agri-food continuum.

Introduction

The Torque teno virus (TTV) is a small, nonenveloped, single-stranded virus with a circular DNA genome of negative sense (Okamoto *et al.* 2002). The virus was first isolated in 1997 from a Japanese patient with post-transfusion hepatitis (Nishizawa *et al.* 1997). Because of its circular genome, TTV was initially classified in the family *Circoviridae*, which includes the genera *Circovirus* and *Gyrovirus*, but was recently reclassified in the 'floating' genus *Anellovirus* (Biagini *et al.* 2005). Because of the high prevalence of the virus in healthy individuals, it has not yet been possible to demonstrate any link between TTV infection and a specific disease or pathology in humans. However, TTV viral loads in patients with other diseases, including viral hepatitis, asthma, idiopathic pulmonary fibrosis and autoimmune rheumatic disorders, were found to be significantly higher compared to healthy individuals (Yzebe *et al.* 2002; Pifferi *et al.* 2005; Maggi *et al.* 2007; Bando *et al.* 2008). Five highly genetically heterogenous genogroups (G1–G5) of TTV were reported (Peng *et al.* 2002). Age-specific prevalence for the respective genogroups of TTV was observed, and a possible association between pathogenic properties and a specific genogroup was also suggested (Maggi *et al.* 2003; Naganuma *et al.* 2008).

TTV has also been detected in several domestic and wild animal species, including dogs, cats, swine, chicken, cattle, sheep, wild boars and primates (Leary *et al.* 1999; Cong *et al.* 2000; Okamoto *et al.* 2002; Martinez *et al.* 2006; Brassard *et al.* 2008). Two distinct genogroups have been identified in swine (Niel *et al.* 2005), and their prevalence varies depending on the country as well as on the genogroup, because more data are currently available on genogroup 1. Despite the fact that TTV infection in swine, as in humans, seems to be very widespread, it has

not been possible to establish a link between the presence of the virus and a disease. However, recent studies suggested the possibility of co-infection and synergy between TTV and porcine circovirus type 2 (Gagnon *et al.* 2007; Ellis *et al.* 2008).

At present, the replication of porcine TTV is not supported by any cell lines that can be cultivated in the laboratory. Molecular tools such as polymerase chain reaction (PCR) and nested PCR have therefore been developed to allow detection of the virus in various types of samples (McKeown *et al.* 2004; Kekarainen *et al.* 2007; Krakowka and Ellis 2008). However, the amplicons produced must be submitted to downstream analysis and sequenced to confirm the detection results. Technologies such as TaqMan real-time PCR allow the detection and confirmation of the presence of the targeted virus in a much shorter time than before, because a specific molecular probe is included in the assay, thereby eliminating the need for postamplification confirmatory steps. Another advantage of real-time PCR is the possibility of quantifying the number of viral genome copies in the samples based on standard curves and making quantitative comparisons, which is not possible in conventional PCR (Mackay 2004). Because the level of viremia seems to be a determining factor in the pathogenesis of TTV in a number of cases of infection, it is of paramount importance to rely on state-of-the-art technological tools for rapid detection of TTV as well as quantification of the viral particles. The methods of choice should ideally detect all strains (both genogroups) of TTV and should be sensitive enough to provide results even when there are few viral genomes in the sample.

The use of animals, particularly swine, as models to improve our understanding of the pathogenesis of TTV in both humans and animals was suggested by Kekarainen and Segales (2009). This avenue has great potential and is worthy of further exploration, because new knowledge about TTV is generally now being acquired through epidemiological study or by chance. The use of animal models requires the development of new technologies and the standardization of methods. The main objective of this study was to develop and optimize a TaqMan real-time PCR assay to detect the presence of porcine and bovine TTV genogroups 1 and 2 in plasma and faeces and to compare this TaqMan assay with the conventional PCR assays found in the literature.

Materials and methods

Sample collection

The TTV detection was assessed in 80 blood samples and 74 faecal samples from pigs of different commercial swine herds and in 81 faecal samples and 63 blood samples from cattle of various ages in several commercial herds within Quebec (kindly provided by the Faculté de médecine vétérinaire de l'Université de Montréal). The health status of the different animals was unknown.

Other micro-organisms were tested for amplification and detection cross-reactivity with the TTV real-time PCR TaqMan assay. These micro-organisms included other viral strains of adenovirus 40 and 41 as well as of Circovirus 2A and 2B kindly provided by Carl Gagnon (Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe) and bacterial DNA from *Salmonella* Typhimurium DT 104, *Campylobacter jejuni*, *Campylobacter coli* and *Escherichia coli* O157:H7 kindly provided by Sylvain Quessy and by Eric Nadeau (Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe).

Extraction of viral DNA

Faecal samples from each animal were diluted in phosphate-buffered saline, pH 7·4 (Invitrogen Canada Inc, Burlington, ON, Canada) to obtain a final 20% suspension (w/v) and stored at −80°C. Plasma was extracted from the different blood samples by sedimentation using Ficoll-Paque (GE Healthcare, Baie d'Urfé, QC, Canada) according to the manufacturer's instructions, and was stored at −80°C until use. Viral DNA was extracted from the faecal samples using the QIAamp DNA Stool Mini kit (Qiagen, Mississauga, ON, Canada) and from the plasma samples using the QIAamp DNA Blood Mini kit (Qiagen). All extractions were performed according to the manufacturer's instructions.

Primers and probe design for real-time PCR

DNA sequences from a highly conserved region were used for the development of the real-time PCR assay. The three complete swine TTV genome sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) (AB076001, AY823990 and AY823991) and aligned using the ClustalW (http://www.ebi.ac.uk/clustalw) multiple sequence alignment program. All primers and probe (IDT, Coralville, IA, USA) used are described in Table 1.

Construction of standard plasmids for real-time PCR

PCR products obtained with primers QCOM forward and QCOM reverse (Table 1) from TTV porcine and bovine strains were excised from 2% (w/v) agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen). Purified PCR products were cloned into pCR 2.1 TOPO vector using TOPO TA Cloning kit (Invitrogen) with TOP10 electrocompetent cells in accordance with the manufacturer's recommendations. Plasmid DNA was

Table 1 Sequences of PCR, nested PCR and real-time PCR oligonucleotide primers and probe used in this study

Primers	Sequences (5′–3′)	Nucleotide position*	References
PCR			
Forward-TTV1 (G1)	5′-CGGGTTCAGGAGGCTCAAT-3 ′	9–27	(Segales *et al.* 2009)
Reverse-TTV1 (G1)	5′-GCCATTCGGAACTGCACTTACT-3′	313–292	(Segales *et al.* 2009)
Forward-TTV2 (G2)	5′-TCATGACAGGGTTCACCGGA-3′	1–20	(Segales *et al.* 2009)
Reverse-TTV2 (G2)	5′-CGTCTGCGCACTTACTTATATACTCTA-3′	252–230	(Segales *et al.* 2009)
Nested PCR			
Upper 1	5′-TACACTTCCGGGTTCAGGCT-3′	1–23	(McKeown *et al.* 2004)
Lower 1	5′-ACTCAGCCATTCGGAACTGCACTC-3′	318–295	(McKeown *et al.* 2004)
Upper 2	5′-CAATTTGGCTCGCTTCGCTCGC-3′	24–45	(McKeown *et al.* 2004)
Lower 2	5′-ACTGCACTCACTTATATTCACTTTAGTGGGAACG-3′	304–270	(McKeown *et al.* 2004)
Real-time PCR			
QCOM forward	5′-CGAATGGYWGAGTTTWYGCCGC-3′†	305–326	This study
QCOM reverse	5′-GCCCGAATTGCCCCTWGACTKCG-3′†	408–382	(Bigarre *et al.* 2005)
QCOM probe	5′-FAM-CTCCGGCACCCGCCCAG-3IABlkFQ-3′‡	370–354	This study
Sequencing			
PTTV-forward	5′-TGCATAGGGTGTAACCAATC-3′	240–255	This study
PTTV-reverse	5′-TGCATAGGGTGTAACCAATC-3′	174–155	This study

TTV, Torque teno virus.

*Primer positions for TTV2 based on TTV genogroup 2 complete genome AY823991.1 (GenBank). All other primer and probe positions based on TTV genogroup 1 complete genome AB076001.1 (GenBank).

†Y = C, T; W = A, T; K = G.

‡Negative polarity and 3IABlkFQ = Iowa black Quencher 3′ end.

extracted by NucleoSpin Plasmid kit (Marcherey-Nagel, Bethlehem, PA), and sequencing was performed on recombinant plasmids in both directions using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) and a CEQ Dye Terminator Cycle sequencing kit (Beckman Coulter) with M13 forward and reverse primers, to confirm the target sequences amplified. The recombinant plasmid stocks were linearized by a digestion with Xba I restriction enzyme (Invitrogen), quantified using the NanoDrop spectrophotometer ND-1000 according to the manufacturer's instructions (NanoDrop Technologies Inc., Wilmington, DE, USA) and converted into copy number. These linear DNA plasmids were used for optimization of the TaqMan real-time PCR assay (concentration of primers, probe and $MgCl_2$), as positive control and for generation of standard curves.

TaqMan real-time PCR assay

The TaqMan PCR assays were performed in 25 μl of a reaction mixture comprising 2·5 μl of extracted DNA and 22·5 μl of master mix. Mastermix was made with the Brilliant QPCR core reagent kit (Stratagene, La Jolla, CA, USA) and contained 5·0 mmol l^{-1} of $MgCl_2$, 300 nmol l^{-1} of QCOM forward primer, 150 nmol l^{-1} of QCOM reverse primer and 300 nmol l^{-1} of TaqMan probe. The PCR amplification was performed with a Stratagene Mx 3005p system (Stratagene) in a 96-well format under the following conditions: 95°C for 10 min for initial denaturation followed by 50 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. A standard curve for each system was generated using tenfold serial dilution (10^8–10^0 genomic equivalents) of purified DNA plasmid in a 5 ng ml^{-1} salmon sperm DNA solution.

Conventional PCR and nested PCR

Porcine and bovine TTV DNA were detected by conventional PCR and nested PCR using the primers described in Table 1 in an Eppendorf Mastercycler gradient system (Brinkmann Instruments Canada, Ltd, Mississauga, ON, Canada) according to the procedures previously described elsewhere (Brassard *et al.* 2008; Segales *et al.* 2009).

Nucleotide sequencing and analysis

Arbitrarily selected amplicons were sequenced to validate the nested PCR, conventional PCR and real-time TaqMan PCR amplification results from porcine and bovine samples. Amplicons were analysed on 2% (w/v) agarose gel, excised, cloned and sequenced as described in 'Construction of standard plasmids for real-time PCR' section of this paper. The sequences obtained were deposited in GenBank (accession numbers FJ969166–FJ969191).

Determination of the entire genomic sequence of swine TTV swSTHY-TT27

Nested PCR was performed with the Taq PCR Core kit (Qiagen) using the upper/lower primers developed by McKeown *et al.* (2004) and according to the procedures previously described by Brassard *et al.* (2008). The sequence obtained with the second round of amplification (GenBank accession number EU006508) was used to design the PTTV-F and PTTV-R inverted primers used for the full-length genome amplification. This amplicon overlapped at both ends with the nested PCR sequences amplified previously and was carried out in a total volume of 50 μl using the Expand Long Template PCR System (Roche Applied Science, Laval, QC, Canada) with the buffer 1, according to the manufacturer's recommendations. PCR fragment of 2808 bp was excised from 1% (w/v) agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen). Purified PCR product was cloned into pCR-XL-TOPO vector using TOPO XL PCR Cloning kit (Invitrogen) with TOP10 electrocompetent cells in accordance with the manufacturer's recommendations. Sequencing was performed on recombinant plasmid in both directions by DNA LandMarks Inc (Saint-Jean-sur-Richelieu, QC, Canada) using the BigDye Terminator ver. 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 XL DNA Analyser (Applied Biosystems) and the obtained sequence was submitted to GenBank (accession numbers # GQ120664).

Results

TTV oligonucleotides and optimization strategies for real-time PCR

Primers and a probe were selected in the noncoding region of the genome (nt 306–408 based on the sequence AB076001) (Fig. 1). The primers and probe are located in a region that is equally highly conserved in both genogroup 1 and 2, with the result that both genogroups can be detected using the same PCR assay (Okamoto *et al.* 2002; Niel *et al.* 2005). A basic local alignment search tool (BLAST) search for the primers and probe was carried out using the National Center for Biotechnology Information (NCBI) website, and no sequence other than those of porcine and bovine TTV showed sequence identity. Both porcine and bovine TTV recombinant plasmids were used for the optimization of the TaqMan real-time PCR assay. Concentrations of primers, probe and magnesium chloride and DNA were selected based on the greatest fluorescence obtained and the lowest threshold cycle (data not given).

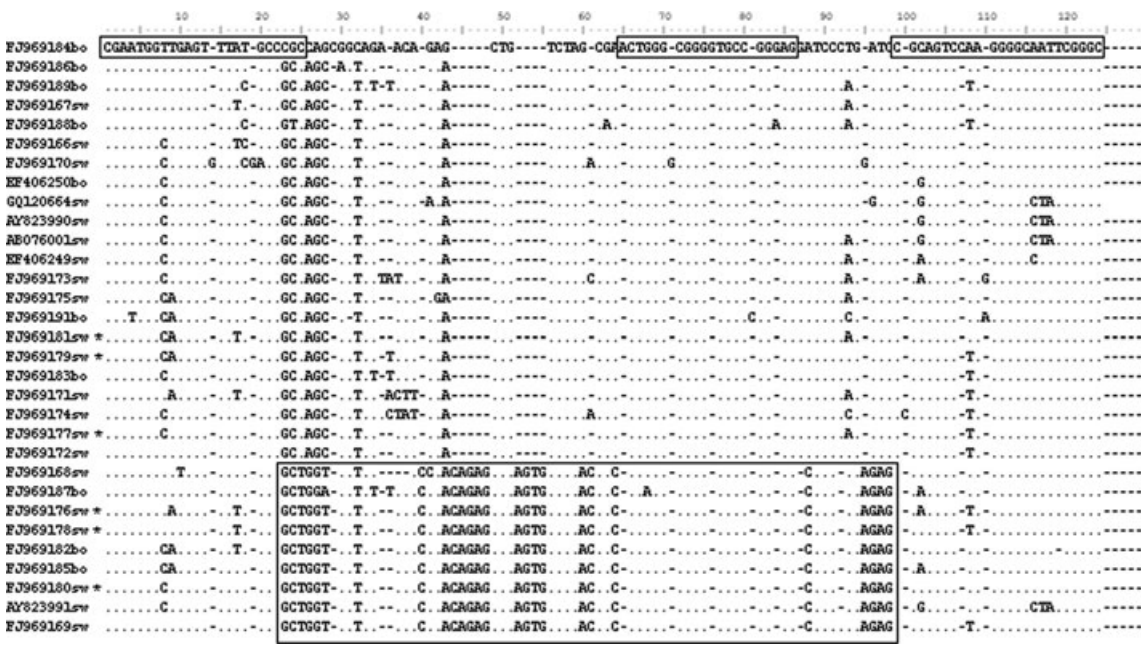

Figure 1 Sequence alignment based on a 99–115 bp nucleotides sequence of the 5′ noncoding region of known swine Torque teno virus (TTV) genogroup 1 and 2 in GenBank (AY823990, AB076001, AY82399, EF406249, EF406250) and swine (sw) and bovine (bo) TTV detected and sequenced in this study (FJ969166 to FJ969191; GQ120664). Positions 1–26 correspond to the forward primer, 99–124 to the reverse primer and 66–86 correspond to the TaqMan probe. Framed corresponds to the specific region of the genogroup 2 strains. The isolates marked with an asterisk were detected from the same animal.

Standard curves of TTV

Standard curves were established using the corresponding cloned amplicon that was serially diluted from 1×10^8 to 1×10^0 copies and amplified in triplicate. The threshold cycle number values (C_t) were plotted against genomic equivalent copies (gec). Standard curves obtained showed an efficiency of 82·4%, a slope of −3·83 and an intercept of 41·22 for porcine TTV and an efficiency of 78·9% a slope of −3·95 and an intercept of 42·67 for bovine TTV (Fig. 2a,b). The regression coefficients (R^2) were 0·99 for both standard curves (Fig. 2). The detection limits of the assay with DNA from porcine and bovine TTV clones as template were 1×10^1 and 1×10^0 gec, respectively.

Specificity of the assay

Different pathogenic bacteria and viruses were evaluated under optimum conditions using the TaqMan real-time PCR assay developed in this study. Porcine and bovine plasmid clones (10^4 copies per μl) and a porcine TTV strain were used as a positive controls and showed C_t of 27·58, 27·65 and 27·09, respectively. No cross-reactions (No C_t) were observed with Circovirus 2A, Circovirus 2B, Adenovirus 40, Adenovirus 41, *E. coli* O157:H7, *Salmonella* Typhimurium DT104, *Camp. jejuni* and *Camp. coli* DNA.

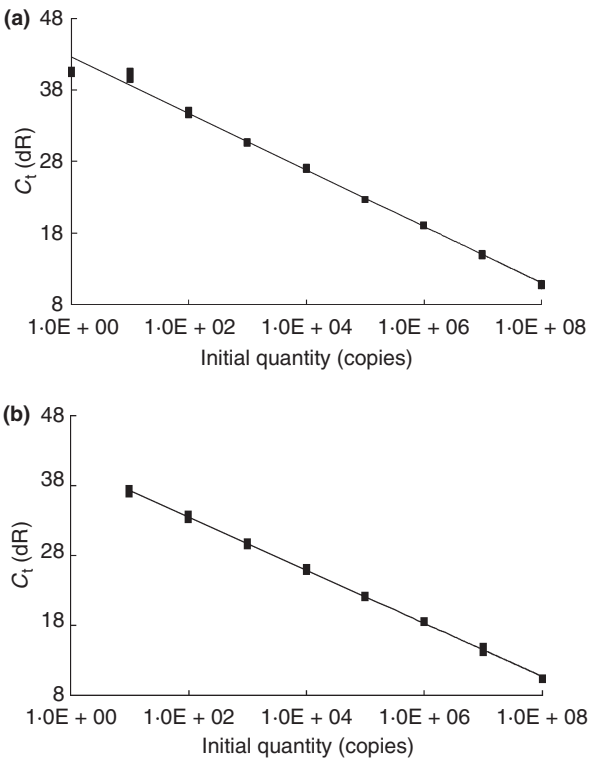

Figure 2 Standard curve generated from cloned Torque teno virus (TTV) DNA detected by TaqMan real-time PCR. Individual data points corresponding to average of triplicate wells containing 25 μl reactions are plotted. Template copy number is given on the *x*-axis and the C_t value on the *y*-axis. Graph a representing the standard curve generated using tenfold dilution of a plasmid containing DNA fragment of bovine TTV. Graph b representing the standard curve generated using tenfold dilution of a plasmid containing DNA fragment of porcine TTV.

Comparison of the results obtained by real-time PCR and conventional and nested PCR

The real-time PCR assay developed in this study, because of the design of the primers and the probe, allows the detection of both genogroups of TTV. None of the other assays tested was able to detect more than one genogroup (Table 2). In the porcine plasma samples, the real-time PCR assay provided the best TTV detection (91%) compared to the conventional PCR assays (75–90%). In the faecal samples, the conventional PCR assay for genogroup 2 detected TTV in 100% of the samples *vs* 84% for the real-time PCR assay.

In cattle, the real-time PCR was the only assay that provided detection of the presence of TTV in 42% plasma samples (Table 2). However, in faecal samples, no positive detection result was observed for the four assays tested in this study.

Sequence analysis

Sequencing of real-time PCR products confirmed that the amplification was specific to porcine and bovine TTV. A sequence alignment was constructed using those sequences as well as certain sequences found in GenBank (Fig. 1). Despite the small size of the sequenced fragment (99–115 bp), the alignment showed the formation of two distinct groups of sequences, which in fact proved to be the two TTV genogroups. A more thorough analysis of

Table 2 Detection of Torque teno virus in bovine and porcine plasma and faeces samples with four different molecular detection systems

Detection systems	Porcine		Bovine	
	Faecal	Plasma	Faecal	Plasma
	Number (%) of positive		Number (%) of positive	
Nested PCR	54/74 (73)	67/80 (84)	0/81 (0)	0/63 (0)
Genogroup 1 PCR	45/74 (61)	72/80 (90)	0/81 (0)	0/63 (0)
Genogroup 2 PCR	74/74 (100)	60/80 (75)	0/81 (0)	0/63 (0)
TaqMan real-time PCR	62/74 (84)	73/80 (91)	0/81 (0)	26/63 (42)

the sequences was conducted, and small insertions of a few base pairs were observed in a very specific region in the genogroup 2 strains. The probe used in the real-time PCR is located in the same region, and the sequence remains highly conserved between the two genogroups.

Analysis of the bovine sequences obtained showed that there are strains belonging to the two genogroups for cattle as well (Fig. 1). Certain sequences of bovine and porcine strains shared strong sequence identity as well as a high genetic heterogeneity such as those observed within each individual species.

Analysis of the sequences also showed that an animal could be infected by more than one TTV strain and by more than one genogroup (Fig. 1).

A complete genomic sequence of swine TTV isolated from plasma was determined (GQ120664). The TTV genome, a negative-sense single-stranded circular DNA, contains 2875 nucleotides with three predicted open reading frames (ORF1, nt 517–2436; ORF2, nt 428–646; ORF3, nt 428–642 + 2089–2536) potentially encoding three proteins. Nucleotide sequence identities of 86 and 73% based on the complete genome were found with two TTV genogroup 1 isolates from GenBank AY823990 and AB076001, respectively. Only 6% of sequence identity was showed with the known genogroup 2 complete genome (AY823991). It is not common to observe such genomic difference between two genogroups of the same viral species having the same origin. However, in spite of this great difference in the nucleotide sequence, the gene arrangement is strongly similar. The genomic region chosen for the detection of both genogroups of TTV is the only region which shows sufficient sequence identity to allow this detection.

Discussion

A number of challenges are involved in studying and learning more about TTV in both humans and animals. Growing TTV in cell culture provides very mixed results, and only one research group has reported a low level of replication of human TTV in cell lines (Desai *et al.* 2005). The pathogenesis of TTV remains undetermined for the time being despite the reported presence of the virus in a number of pathologies and co-infections (Davidson and Shulman 2008). Using swine as a model for studying TTV in terms of transmission, infection dynamics, distribution and, potentially, the pathologies that it causes are a promising avenue that was suggested recently (Kekarainen and Segales 2009). Despite the fact that interest in TTV has increased to some extent, much remains to be done and understood, particularly in animals. The development of new molecular tools for the detection and quantification is therefore essential for the acquisition of new knowledge about porcine and bovine TTV. The real-time PCR assay using a TaqMan probe developed in this study can detect both porcine and bovine TTV genogroups eliminating the need for confirmation by sequencing and gel analysis. Through confirmation using the probe included in the assay, this technology makes it easier to distinguish a positive result from a false positive, particularly in the case of samples presenting a lot of nonspecific amplification, such as in faecal samples. This may explain the lower detection percentage in swine faeces obtained with the real-time PCR assay when compared to conventional PCR for genogroup 2. In gel analysis, PCR amplification products for genogroup 2 revealed also lots of nonspecific bands despite optimization (data not given).

The reaction efficiencies obtained with the real-time PCR assay were only 82 and 79% for porcine and bovine TTV, respectively. Different optimization trials were conducted during the establishment of the standard curves to improve these parameters. Dilutions of viral DNA were also tested (data not given) and did not have any impact on reaction efficiencies. However, the dynamic of these systems seems to have reached its best reaction efficiencies with R^2 values of 0·99. Nevertheless, this assay can be used to obtain comparative values for the relative load of the virus present in different type of samples. In this study, real-time PCR using the TaqMan probe demonstrated greater sensitivity for detection of the virus in porcine and bovine plasma compared to the conventional PCR assays. Because virus titre during viremia appears to be a virulence marker in TTV (Pifferi *et al.* 2006; Bando *et al.* 2008) using this tool could lead to a better understanding of how TTV infection is established in animals and could also allow identification of replication sites and cell tropisms in the organism through quantitative comparisons in relation to the virus titre.

The real-time PCR assay also showed that TTV was present in considerable amounts in bovine plasma. In a previous work, a small percentage of cattle was found to have TTV in their plasma, but none of the animals was found to have the virus in its faeces (Brassard *et al.* 2008). The results obtained in the present study therefore are in agreement with the previous observations, a finding that could possibly be explained by a very short virus shedding period in the animals, the age of the animals, and/or a very low faecal concentration that was below the detection limit of the assay. More thorough studies in cattle could potentially confirm or deny these possible explanations. However, the design of new primers and a new probe made it possible to detect many more bovine strains in the plasma than were found in the previous work, as well as to compare their sequences with porcine strains. Sequence analysis showed that certain virus strains

in the two species seem to be very close genetically in this genomic region and that the bovine strains were found in the two known porcine TTV genogroups. More thorough analysis of the amplified region enabled the identification of insertions of a few specific base pairs in the genogroup 2 strains (Fig. 1), with the result that they could be effectively distinguished from the genogroup 1 strains. The advantages of this new system are the possibility of detecting the strains of the two porcine and bovine TTV genogroups using universal real-time PCR and the potential for using the genogroup 2 insertions in a second PCR run to distinguish between the two genogroups. This type of approach was already reported for human anelloviruses (Ninomiya *et al.* 2008) and contributed to better identify co-infections and reduce underestimation of the presence of certain viruses and/or genogroups.

In conclusion, the real-time PCR assay developed in this study provided better specificity based on detection results for the two TTV genogroups in both swine and cattle compared to the conventional PCR assays. This novel detection system was also faster, more reliable and reduced the risk of false positive results. TTV was detected in the plasma of up to 42% of cattle using this real-time TaqMan primer and probe system. The pathogenesis of this virus remains unknown, but this type of molecular tool could help in future studies to understand how the virus establishes infection in the host and what role the virus plays in various pathologies in both humans and animals.

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