Rolling-circle amplification of *Torque teno virus* (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup

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Multiply primed rolling-circle amplification is a novel technology that uses bacteriophage phi29 DNA polymerase to amplify circular DNA molecules, without the need for prior knowledge of their sequences. In an attempt to detect *Torque teno virus* (TTV), rolling-circle amplification was used to amplify DNA extracted from eight human and four pig serum samples. All samples gave high molecular weight (>30 kb) amplification products. By restriction endonuclease digestion, these products generated DNA fragments whose sizes were consistent with those of human TTV (3-8 kb) and swine TTV (Sd-TTV; 2.9 kb) genomes. Two TTV isolates derived from a single AIDS patient, as well as two Sd-TTV isolates derived from a single pig, were characterized by complete nucleotide sequencing. One of the Sd-TTV isolates showed very low (43-45%) nucleotide sequence similarity to the other Sd-TTV isolate and to the prototype isolate Sd-TTV31, and could be considered the prototype of a novel genogroup.

*Torque teno virus* (TTV) is a non-enveloped, single-stranded, circular DNA virus with a genomic length of 3.4-3.9 kb (Nishizawa et al., 1997; Miyata et al., 1999; Mushahwar et al., 1999) and has been recently classified into a novel, floating genus called Anellovirus (Biagini et al., 2005). TTV is found in the plasma of >80% of the human population worldwide (Prescott & Simmonds, 1998; Takahashi et al., 1998; Niel et al., 1999). Co-infection of single individuals with multiple TTV isolates is frequent (Takayama et al., 1999; Niel et al., 2000). TTV has a wide genetic diversity and virus isolates have been classified into five main phylogenetic groups (1-5) with low nucleotide sequence similarity between them (Peng et al., 2002). Anelloviruses are not restricted to human hosts and have also been detected in non-human primates, tupaias, cats, dogs and pigs (Leary et al., 1999; Verschoor et al., 2000; Okamoto et al., 2001, 2002). However, few complete nucleotide sequences from animal TTVs have been reported.

In their natural replication cycle, some DNA viruses, like circoviruses, employ a rolling-circle mechanism to propagate their circular genomes. Multiply primed rolling-circle amplification is a novel technique able to amplify circular DNA molecules such as plasmids with great efficiency (Dean et al., 2001). The method utilizes bacteriophage phi29 DNA polymerase, a high-fidelity enzyme, with a strong strand-displacing capability, high processivity and proofreading activity (Garmendia et al., 1992; Esteban et al., 1993). Unlike PCR, the primers used in the amplification reaction are random hexamers. Previous knowledge of the nucleotide sequences to be amplified therefore is not necessary. Furthermore, phi29 DNA polymerase is very stable, with linear kinetics at 30°C for over 12 h, eliminating the need for thermal cycling. The reaction products are high molecular weight, linear, double-stranded, tandem-repeat copies of the input DNA that can subsequently be digested with restriction endonucleases.

In this study, multiply primed rolling-circle amplification was used to amplify the complete genomes of human TTV and porcine (*Sus domesticus*) TTV (Sd-TTV) after direct extraction of viral DNA from serum. A novel genogroup of Sd-TTV was identified.

To evaluate the utility of the rolling-circle amplification technique for identification of TTV, eight human serum samples were used, originating from four voluntary blood donors and four patients with AIDS. All the subjects lived in Rio de Janeiro, Brazil, and were 25-35 years old. In addition, serum samples collected from four adult, randomly selected pigs belonging to a single herd from the state of Rio de Janeiro were used.

Viral DNA was extracted from 250 µl serum as described previously (Niel et al., 1994) and resuspended in 30 µl distilled water. For multiply primed rolling-circle
amplification, 5 μl DNA was denatured for 3 min at 95 °C, cooled to 30 °C and added to a mixture containing 5 U phi29 DNA polymerase (New England Biolabs), 50 μM random primers (Invitrogen), 1 mM each dNTP (Amersham Biosciences) and 200 μg BSA ml⁻¹ in a final volume of 100 μl of reaction buffer [50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 10 mM (NH₄)₂SO₄, 4 mM dithiothreitol]. Amplification was for 18 h at 30 °C, followed by 10 min at 65 °C to inactivate the phi29 DNA polymerase.

Ten microlitres of amplification product was digested with 10 U BamHI, EcoRI, HindIII, PstI or PvuII. Restriction digests were separated by 1% agarose gel electrophoresis and visualized by UV light exposure after ethidium bromide staining.

EcoRI fragments showing sizes corresponding to those of TTV (~4 kb) and Sd-TTV (3 kb) were purified using the QIAquick gel extraction kit (Qiagen). Fifty nanograms of purified DNA was ligated to 5 ng plasmid vector pUC19 linearized with EcoRI. After transformation of TOP10 Escherichia coli (Invitrogen), the bacteria were incubated for blue/white colony screening on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and ampicillin.

Bacteria carrying recombinant plasmids were grown overnight. Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen). Inserts were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing primers were universal and reverse M13 primers, as well as primers designed specifically to be used for genome walking.

Firstly, the minimal quantity of enzyme required to achieve successful multiply primed rolling-circle amplification assays was determined. Using 1 pg of plasmid pUC19 as input DNA, 1 U of enzyme (in a 20 μl reaction volume) was sufficient to generate a strong DNA band of high molecular mass (>30 kb). Secondly, the sensitivity of the method was determined with decreasing amounts of pUC19. A faint DNA band was visible on the gel using as little as 1 fg of plasmid (about 350 copies) in the amplification mixture (not shown).

To determine whether the rolling-circle amplification method could be used for detection of TTV in human serum, assays were performed with DNA extracted from serum samples collected from four blood donors and four AIDS patients. High molecular mass DNA was obtained in all cases. DNA samples were digested with five restriction endonucleases (BamHI, EcoRI, HindIII, PstI or PvuII), chosen from those having a 6 bp recognition site and, consequently, a high probability of having a unique restriction site in a 3-8 kb DNA molecule (the size of the TTV genome). The results are shown in Fig. 1. In all
samples, a band of 4 kb (or slightly shorter) was seen after digestion of rolling-circle amplification products with at least one restriction endonuclease. The eight samples under study had been previously tested by PCR for the presence of TTV isolates belonging to each of the five phylogenetic groups (Devalle & Niel, 2004). At that time, it was noticed that AIDS patients 7 and 16 were co-infected with TTV isolates belonging to four and five genogroups, respectively, whereas TTV from only one genogroup was detected in blood donors 89, 93 and 228, as well as in AIDS patients 12 and 22 (two genogroups were identified in blood donor 200). It was therefore remarkable that the rolling-circle amplification products derived from patients 7 and 16 generated a number of restriction fragments notably higher than observed in the six other individuals (Fig. 1).

In the same way, four swine serum samples were submitted to viral DNA extraction, rolling-circle amplification and restriction endonuclease digestion. For all samples, restriction fragments of approximately 3 kb, consistent with the size of the Sd-TTV genome, were observed after separate digestions with at least three restriction endonucleases (Fig. 2).

EcoRI fragments of approximately 4 and 3 kb in size, derived from AIDS patient 7 and pig 845, respectively, were cloned into the plasmid vector pUC19. Two recombinant human clones (2h and 3h) and two recombinant pig clones (1p and 2p) were selected and their nucleotide sequences determined. All four clones showed a genetic organization characteristic of TTV, with (i) a coding region covering about two-thirds of the genome and containing four (human clones) or three (swine clones) open reading frames; (ii) a GC-rich stretch in the untranslated region (UTR); and (iii) a TATA box, located upstream of the smallest open reading frame (not shown).

Human clones 2h and 3h had genomic lengths of 3816 and 3920 nt, respectively. They shared >60% sequence identity with each other and with human TTV isolate TYM9 belonging to genogroup 3, but <60% with isolates representative of the other four genogroups. Clones 2h and 3h were thus classifiable into genogroup 3.

Clones 1p and 2p, although derived from a single pig, shared a very low (43.4%) sequence identity. To date, one swine TTV isolate (Sd-TTV31), from Japan, has been fully characterized at the genome level and sequences with a length of 69–80 nt, localized in the UTR, have been determined for eight isolates (Okamoto et al., 2002). Clone 1p had a genomic length of 2872 nt, very similar to Sd-TTV31 (2878 nt) and shared a relatively high similarity (69.6%) with that prototype isolate. However, isolate 2p (2735 nt) showed very low (45.1%) sequence identity with Sd-TTV31. Actually, clone 2p was almost as distantly related to Sd-TTV31 as it was to TTVs infecting tupaias, cats, dogs and humans (Table 1). Nevertheless, within the small genome segment (69–80 nt) mentioned above, the sequence of clone 2p was identical to those of Japanese isolates Sd-TTV83 and Sd-TTV161 (not shown).

Recent studies have described the multiply primed rolling-circle amplification of complete begomovirus (Inoue-Nagata et al., 2004) and papillomavirus (Rector et al., 2004) genomes. In both cases, amplification was performed using a commercial DNA amplification kit. The amplification assays described here were performed with recombinant phi29 DNA polymerase, not included in a commercial kit. The method was inexpensive and sensitive. As little as 1 fg (about 350 copies) of input plasmid pUC19 was sufficient to produce a visible band (>5 ng of DNA) in an agarose gel.

This study is the first to report the rolling-circle amplification of a complete viral genome from a biological fluid. All serum samples tested gave a high molecular weight (>30 kb) DNA band, revealing the high efficiency of the method for amplification of viral DNA present in serum. Restriction fragments of approximately 4 kb (the size of the TTV genome) were found in all eight human samples tested (Fig. 1). Whether other viral genomes were amplified in our experiments is not known, for at least two reasons. Firstly,
the restriction fragments whose size did not correspond to the TTV genome were not analysed. Some may have resulted from digestion of TTV DNA molecules into two or more fragments, while others may have had other origins. Secondly, unlike PCR, multiply primed rolling-circle amplification is a generic method that does not favour certain DNA molecules with regard to sequence. Circular genomes of blood-borne viruses other than TTV may therefore have been amplified, although not visualized on the agarose gel, due to the low amounts in the samples tested.

A significant difference was observed between blood donors and AIDS patients, with a higher number of restriction fragments observed in the case of AIDS patients. This was particularly true for patients 7 and 16, who were co-infected with TTV isolates from different genogroups (Devalle & Niel, 2004). Further studies are necessary to confirm this correlation between co-infection and complexity of the restriction patterns of amplification products. A high TTV load in human immunodeficiency virus-positive samples (Christensen et al., 2000; Shibayama et al., 2001) could also facilitate TTV detection by rolling-circle amplification.

Due to the singularly high genetic diversity of human TTV, no truly ‘universal’ PCR system, able to detect all isolates, has been developed. Multiply primed rolling-circle amplification, as a sequence-independent strategy for detection of circular DNA viruses, should be useful for identification of novel genogroups.

Recombination between different TTV isolates co-infecting single individuals has been proposed, based on phylogenetic analysis of nucleotide sequences available from databases (Worobey, 2000; Manni et al., 2002). However, this has been questioned, due to possible artefacts (Jelic et al., 2004). Indeed, all TTV nucleotide sequences deposited in databases have been obtained through sequencing of PCR products covering part, not the whole, of the genomes. In this way, a number of ‘complete sequences’ available from the databases may result from combinations of partial sequences obtained from different DNA molecules. Nucleotide sequencing of complete TTV genomes obtained through rolling-circle amplification assays circumvents this problem.

Sd-TTV was first recognized in Japan (Okamoto et al., 2002) and, more recently, in North America, Spain, China, Korea and Thailand (McKeown et al., 2004). The present work, showing that 4/4 pigs from a single Brazilian herd were Sd-TTV-infected, confirms the global distribution of the virus. The large genetic distance existing between clone 2p on the one hand, and clone 1p and prototype strain Sd-TTV31 on the other, shows that swine TTV has a wide genetic diversity, like its human counterpart. In accordance with the criteria used to classify human TTV isolates, clone 2p (Sd-TTV2p) could be considered the prototype of a novel genogroup. Sd-TTV31 and Sd-TTV2p would thus be the prototypes of Sd-TTV genogroups 1 and 2, respectively.

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References


Table 1. Pairwise percentage nucleotide identity comparisons of full-length nucleotide sequences of TTVs infecting different mammalian species

Percentage identity > 60 % is shown in bold.

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<td>–</td>
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<td>38-9</td>
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<td>–</td>
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