

Rolling-circle amplification of viral DNA genomes using phi29 polymerase

Reimar Johne¹, Hermann Müller², Annabel Rector³, Marc van Ranst³ and Hans Stevens³

¹ Federal Institute for Risk Assessment, Diedersdorfer Weg 1, 12277 Berlin, Germany

² Institute for Virology, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 29, 04103 Leipzig, Germany

³ Laboratory of Clinical Virology, Rega Institute for Medical Research, University of Leuven, Minderbroederstraat 10, B3000 Leuven, Belgium

Techniques for the single-step amplification of whole genomes have been developed into powerful tools for phylogenetic analyses, epidemiological studies and studies on genome organization. Recently, the bacteriophage phi29 DNA polymerase has been used for the efficient amplification of circular DNA viral genomes without the need of specific primers by a rolling-circle amplification (RCA) mechanism. Various protocols have been applied for detection of novel viruses, for differentiation between circular and linear forms of viral genomes and for generation of infectious genomic clones directly from specimens. Here, we summarize the broad application of the RCA technique to DNA viruses infecting humans, animals and plants.

Amplification of whole genomes from microorganisms

Genome analysis of microorganisms is a scientific field of increasing importance generating data for clinical diagnostics, phylogenetic analysis, epidemiological studies and studies on genome organization and evolution. The availability of efficient tools for amplification of genome sequences is a prerequisite for successful genome analysis. The most widely used amplification method is the polymerase chain reaction (PCR), of which many specific protocols exist for various applications. Usually, however, PCR produces relatively short amplicons and is dependent on specific primer sequences. As the quality of genome analysis increases with the length of the generated sequences, techniques enabling the amplification of whole genomes are most advantageous [1,2]. These techniques are especially needed for microorganisms that cannot be cultivated *in vitro* so far, as for many of them no sequence information is available [3].

Among the new available techniques for whole genome amplification, rolling-circle amplification (RCA) using the DNA polymerase of bacteriophage phi29 is showing great value for the analysis of viral DNA genomes. This polymerase possesses several features, such as strand displacement activity, proof-reading activity and generation of very long synthesis products (see later), which make it most suitable for the efficient amplification of circular DNA molecules from complex biological samples. Although small circular DNA molecules are rare in higher organisms, they are abundant in several microorganisms

(Table 1). Therefore, phi29 polymerase-dependent RCA has been applied to the study of several virus families possessing circular DNA genomes. As specific primer sequences are not required for this technique, many novel viruses have been discovered. Moreover, one-step whole genome amplification by RCA has enabled rapid sequencing of viral genomes and generation of infectious genomic clones. Here, we describe the RCA technique and highlight recent applications to different virus families infecting humans, animals and plants.

RCA methods

RCA has been performed *in vitro* beginning in 1989 using several DNA polymerases and different circular DNA templates [4–7]. The general principle is schematically shown in Figure 1. After the annealing of a specific primer to the single-stranded template molecule, the polymerase incorporates nucleotides in 5'-to-3' direction complementary to the template strand. In case of a linear template molecule, the synthesis is completed at the end of the template (Figure 1a). For a circular template molecule, however, the polymerase reaches the primer binding site again, displaces the newly synthesized strand and goes on with DNA synthesis for several rounds dependent on the template length (Figure 1b). By this mechanism, a large DNA molecule consisting of repeated copies of the template sequence is produced, leading to a more efficient amplification of a circular target sequence as compared to a linear template. Using a single primer, this principle has been widely used in genotyping and mutational analysis for the detection of padlock probes (linear DNA molecules which, after binding to adjacent target sequences, are circularized by ligation) [8,9].

By adding an additional primer with a binding site on the complementary strand, a more efficient amplification is observed for both kinds of templates as this primer can bind to the displaced strand and initiate additional DNA synthesis. In this case, the amplification products consist of double-stranded DNA. Using a specific primer pair, efficient isothermal amplification of a given sequence is possible [10], which has been used in several techniques such as circle-to-circle amplification [11].

Multiply-primed amplification methods, in which primers with random sequences bind to many sites of the template molecule, yield a highly efficient amplification.

Corresponding author: Johne, R. (Reimar.Johne@bfr.bund.de)

Table 1. Circular DNA in microorganisms and higher eukaryotes

DNA type	Organism	DNA length
Mitochondrial	Animals	15–18 kbp
Mitochondrial	Plants	195–2,500 kbp
Chloroplast	Plants	120–160 kbp
Mitochondrial plasmid	Plants	1–2 kbp
Plasmid	Bacteria	1–200 kbp
Bacteriophage	Bacteria	5–10 kb(p)
Viral	Animals and plants	1–9 kb(p)

Several techniques for whole (linear) genome amplification of higher organisms have been developed using this principle [12]. For circular template molecules, multiply-primed amplification is even more efficient because of the aforementioned RCA effect (Figure 1c). An artificial circular DNA molecule with a length of 150 bp has been shown to be amplified 10^7 -fold using phi29 polymerase in multiply-primed RCA [13]. Circular viral DNA with a length of 8 kbp was amplified 2.4×10^4 -fold directly from infected cells, corresponding to a 2.8×10^3 -fold enrichment of circular viral DNA as compared to linear cellular DNA present in the sample [14]. Techniques for amplification of plasmid DNA [13] and cell-free cloning systems [15] have also been developed using this principle.

Phi29 DNA polymerase

The genome of bacteriophage phi29 (which infects *Bacillus subtilis*) encodes the phi29 DNA polymerase, which is used for replication of its DNA. This enzyme possesses several remarkable features, which make it suitable for efficient *in vitro* amplification of DNA [12,16]. The phi29 DNA polymerase is a member of the B-family of replicative polymerases and consists of a single protein with a molecular

mass of 68 kDa [17,18]. It catalyzes two synthetic reactions (5'-to-3' polymerization, and deoxynucleotidylation of phi29 terminal protein) and three degradative reactions (pyrophosphorolytic, 3'-to-5' ssDNA exonucleolytic and 3'-to-5' ssRNA exonucleolytic activities) [19,20] (Figure 2). In addition, it has a strand displacement activity, which enables the enzyme to displace the complementary strand in double-stranded regions of a template molecule during DNA synthesis [7,21].

In the bacteriophage phi29 replicative cycle, DNA polymerization is initiated by a terminal protein, which is first covalently linked to a nucleotide and then used as primer for replication of the linear double-stranded DNA genome [19,22]. However, *in vitro* amplification systems use (random) DNA primers bound to a single-stranded DNA template for initiation of DNA synthesis. Primers containing thiophosphate linkages are usually utilized to prevent their degradation by the exonucleolytic activity of the phi29 polymerase [16].

After DNA-primed initiation, DNA is continuously synthesized by the phi29 polymerase, resulting in products with a length higher than 70 kbp within 20 min [7]. Moreover, the long half-life of the enzyme allows prolonged reactions; under certain conditions, its average synthesis rate is ~1,500 nucleotides per minute, which lead to products with estimated lengths of 900 kb in a 10 h reaction [11,23]. If double-stranded regions are present in the template molecule, the complementary strand is displaced and DNA synthesis proceeds [7]. This strand displacement activity is used in many amplification techniques because the displaced strand is a single-stranded template suitable for further DNA synthesis, without the need of an additional denaturing step [24,25]. When using circular DNA molecules as templates, large concatemeric molecules are produced by displacement of the newly synthesized strand after one round of molecule replication and continuous synthesis of additional copies of the template sequence [7,16]. This mechanism results in a predominant amplification of small circular DNA molecules as compared to linear molecules [13,16].

The 3'-to-5' DNA exonuclease activity of the phi29 polymerase enables the enzyme to perform proofreading, which makes it suitable for genetic studies. Its error rates have been calculated to be between 10^{-5} and 10^{-6} in biochemical assays [26], or 9.5×10^{-6} by direct sequencing of 500 000 bp after phi29 polymerase-mediated amplification [27].

Application of RCA to viral genomes

In 2004, RCA using phi29 DNA polymerase was first applied to viral genomes. Rector *et al.* [14] showed that the papillomavirus genome, which is composed of circular double-stranded DNA, could be efficiently amplified from tissue samples using this technique. At the same time, Inoue-Nagata *et al.* [28] used this method for cloning of a single-stranded circular DNA genome segment of a geminivirus.

A general procedure for detecting circular DNA viruses using multiply-primed RCA is shown schematically in Figure 3. Total DNA is extracted from the sample and random hexamer primers are subsequently added. After

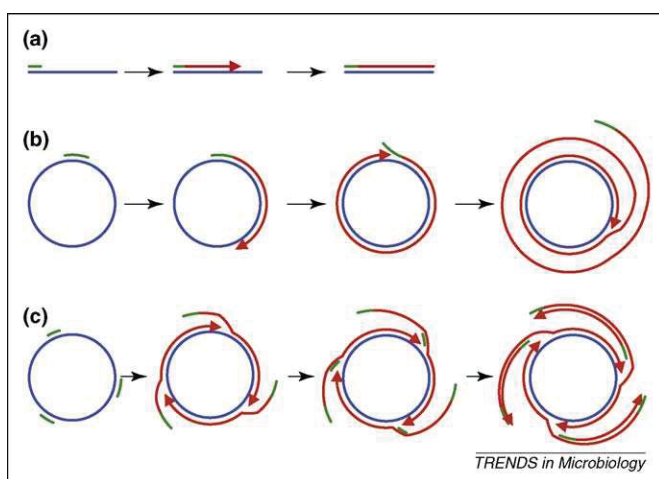


Figure 1. Principle of rolling-circle amplification. Blue lines denote target DNA sequences, green lines represent oligonucleotide primers and red lines represent new DNA synthesized by the polymerase. Arrow heads indicate 3' ends of growing DNA strands. (a) Linear template and single primer. After primer binding, the polymerase synthesizes one complementary strand. (b) Circular template and single primer. The polymerase synthesizes a complementary strand beginning at the bound primer. After one round, the primer and the synthesized strand are displaced and DNA synthesis continues for additional rounds. By this, a long concatemeric single-stranded DNA is produced. (c) Circular template and multiple random primers. The synthesis is initiated at multiple primers bound to the template. DNA synthesis using strand displacement is carried out as in (b). However, primers still present in the reaction mixture bind to the displaced strand and are used as additional initiation points for DNA synthesis. The multiple products are long concatemeric molecules of double-stranded DNA.

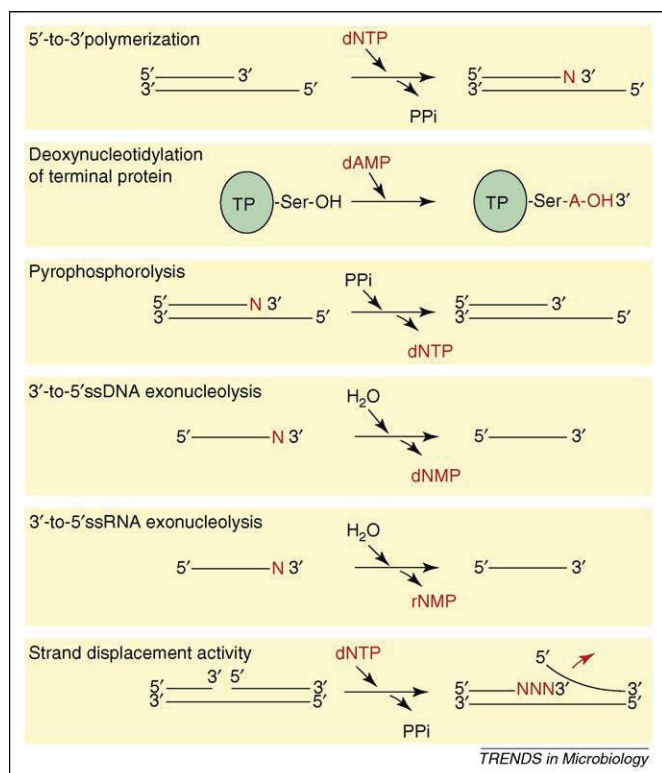


Figure 2. Enzymatic activities of phi29 DNA polymerase. The combination of 5'-to-3' polymerase activity and strand displacement activity makes this enzyme suitable for use in RCA. The 3'-to-5' ssDNA exonucleolytic activity is involved in proofreading. Abbreviations: dAMP, deoxyadenosylmonophosphate; dNMP, deoxynucleotidylmonophosphate; dNTP, deoxynucleotidyltriphosphate; H₂O, water; N, nucleotide; PPi, pyrophosphate; rNMP, ribonucleotidylmonophosphate; TP, terminal protein.

heat denaturation, the phi29 polymerase is added and an isothermal reaction (30 °C) is carried out overnight. The high molecular weight RCA products are examined by restriction enzyme analysis. Using a single cutting

Box 1. Technical hints and tips for RCA application

- **Starting material:** DNA extracted from tissue samples, swabs, cultured cells or plant tissue (even dried). Avoid repeated freezing and drying of the DNA to prevent strand breaks in the circular template DNA.
- **Amount of input DNA required:** variable, depending on the source of the template material, but only a very small amount of circular DNA (~1 ng) is required for amplification.
- **Primers:** thiophosphate linkages increase the stability of primers. However, standard primers without modification have also been used successfully.
- **Polymerase reaction:** it might be optimized for the specific application with regard to deoxynucleotidyltriphosphate (dNTP) concentration, reaction temperature and incubation time.
- **Identification of RCA products:** the easiest method consists of using restriction enzyme digestion followed by agarose gel electrophoresis.
- **Further processing of RCA products:** cloning, direct sequencing or additional PCR amplification (if RCA was used for pre-amplification of circular DNA).
- **Detailed RCA protocols:** see Refs [4,14,16,32,33].

enzyme, genome length amplification products can subsequently be cloned and sequenced. Sequence comparisons with databases are used to identify the cloned sequence. The interested reader can find a summary of technical hints and tips for RCA application in Box 1.

RCA has been applied to a large variety of DNA viruses carrying circular genomes. According to the International Committee on the Taxonomy of Viruses, a total of eight virus families with circular DNA genomes contain viruses infecting human, vertebrate animals and plants [29] (Table 2). These viral genomes are single-stranded or double-stranded, with a length between 2 to 9 kb(p), and are composed of either a single molecule or multiple circular segments. In addition, some viruses such as the adeno-associated viruses (AAVs) possess a linear genome,

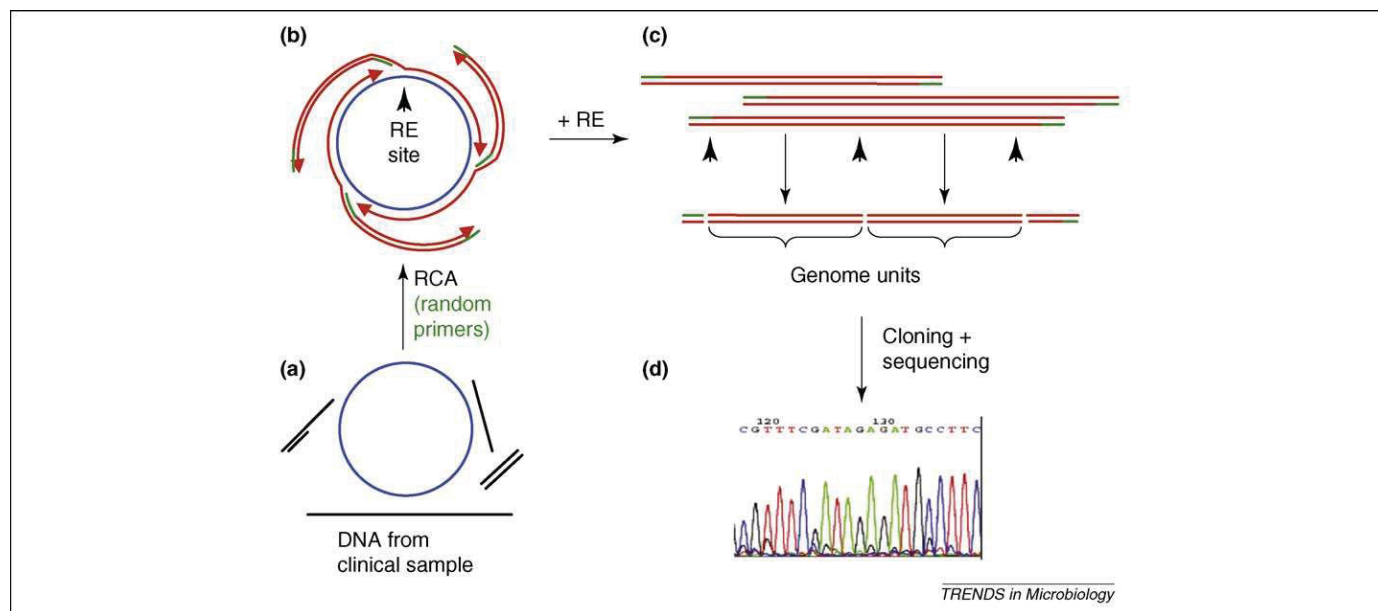


Figure 3. Application of multiply-primed RCA for detection of circular viral genomes in tissue samples. Blue lines denote native target DNA sequences, green lines represent oligonucleotide primers and red lines represent new DNA synthesized by the polymerase. Arrow heads indicate 3' ends of growing DNA strands. (a) Total DNA is isolated from the sample. (b) Multiply-primed RCA is performed. RE: a single-cutting restriction enzyme. (c) The RCA product is subjected to digestion with the single-cutting restriction enzyme resulting in fragments of whole genome length. (d) The fragments are then cloned, and their sequences are obtained for comparison with known sequences to identify the amplified viral genomes.

Table 2. Characteristics of virus families with circular DNA genomes infecting human, vertebrate animals and plants

Virus family	Host	Disease	Cultivation in tissue culture	Genome segments	Genome type	Genome length
<i>Papillomaviridae</i>	Human, animal	Skin papillomas, mucosal lesions, cervical cancer (human)	Difficult (epithelial raft culture needed)	1	ds DNA	7 to 8 kbp
<i>Polyomaviridae</i>	Human, animal	Tumours (laboratory rodents), inflammatory diseases (immunosuppressed humans, young birds)	Difficult (no culture system for several virus species)	1	ds DNA	5 kbp
<i>Anelloviridae</i>	Human, animal	Not known	Difficult (specific cell lines for selected virus species)	1	ss DNA	3 to 4 kb
<i>Hepadnaviridae</i>	Human, animal	Hepatitis B	Difficult (inefficient replication, primary hepatocytes needed)	1	ds DNA ^a	3 to 4 kb(p)
<i>Circoviridae</i>	Animal	Immunosuppression, inflammatory disease (birds)	Difficult (no culture system for most virus species)	1	ss DNA	2 kb
<i>Caulimoviridae</i>	Plant	Mosaic syndrome, chlorotic mottling	n.a.	1	ds DNA ^b	7 to 9 kbp
<i>Geminiviridae</i>	Plant (transmitted by insects)	Multiple syndromes, many economically important diseases	n.a.	1 or 2	ss DNA	3 to 6 kb ^c (3 kb per segment)
<i>Nanoviridae</i>	Plant (transmitted by insects)	Banana bunchy top syndrome (and others)	n.a.	6 to 9	ss DNA	6 to 9 kb (1 kb per segment)

Abbreviation: n.a., not applicable.

^aThe circular genome is not covalently closed and only partially composed of dsDNA. Covalently, closed circular DNA is generated during genome replication.

^bBoth strands of the circular dsDNA genome contain one or two discontinuities. Covalently closed circular DNA is generated during genome replication.

^cAdditional satellite DNAs with sizes between 0.6 kb and 1.3 kb are possible.

which is circularized at a specific time-point in the infectious cycle, enabling application of RCA. Many of the viruses mentioned later cannot be grown efficiently in tissue culture, making detection and amplification of their genome difficult. In the following paragraphs we summarize the application of RCA to viral genomes to address several questions in diagnosis of virus infections and basic research.

Detection of known viruses

The RCA technique was first applied to known papilloma-viruses and developed further using these viruses as models [14]. As proof of concept, a multiply-primed RCA protocol was tested and optimized with episomal human papillomavirus type 16 DNA from W12 cells and an amplification efficiency of 2.4×10^4 fold was obtained. Thereafter, a bovine papillomavirus type 1 variant was successfully amplified directly from extracted DNA of a tissue sample using multiply-primed RCA.

For plant viruses, multiply-primed RCA was first applied to the bipartite geminivirus DF-BR2 infecting tomatoes to amplify its DNA B component [28]. The RCA product was shown to be suitable for direct sequencing, restriction enzyme analysis and cloning of the entire genome segment. Thereafter, RCA has been applied to several different geminiviruses, including monopartite and bipartite species [30–32]. A diagnostic system using RCA combined with restriction enzyme analysis has been established for the detection and differentiation of known geminivirus species [33]. However, limitations for the use of multiply-primed RCA in diagnostic applications on plant viral diseases have also been pointed out [34]: analysis of restriction fragments generated after RCA revealed mitochondrial sequences for some of the fragments. Therefore, to exclude false positive results, it is advisable to use additional precautions such as the sequencing of RCA products, the use of standardized control fragments or a parallel analysis of seed-grown uninfected plants. Under

these conditions, however, mitochondrial plasmid DNA might be used as a positive control to assess the quality of the RCA reaction in the plant tissue.

Detection of unknown viruses

As multiply-primed RCA enables the detection of circular DNA viral genomes without the need of specific primers, the technique has become a powerful tool for the detection of unknown viruses. In fact, the majority of publications on RCA application to viruses refer to the detection and characterization of novel viruses in samples of diseased humans, animals or plants.

Until now, 13 novel papillomaviruses have been detected in tissue samples of different animals by multiply-primed RCA, and the whole viral genomes have been characterized, enabling the development of diagnostic tests for many tumour diseases of animals [35–43]. The technique was also applied to tissue samples of several bird species, in which a polyomavirus infection was suspected based on clinical signs, but which were negative by PCR for the known bird polyomaviruses [44]. Hereby, the whole genomes of two novel polyomaviruses could be cloned; one directly after RCA and the other by a combination of RCA and PCR. After application of multiply-primed RCA to several human and pig serum samples, DNA fragments with sizes of 3 to 4 kbp were repeatedly detected, which could be identified as novel anellovirus genomes [45]. In another study, a combination of RCA and sequence-independent single primer amplification was applied to four human plasma samples and one cat saliva sample, and not less than nine different anelloviruses could be detected [46]. A screening for possible etiological agents in mortality incidents of wild birds using multiply-primed RCA led to the detection of two novel circoviruses in samples derived from European starlings and Mute swans [47,48]. An investigation of plant tissues revealed three novel geminivirus species from two different genera using direct sequencing of RCA products [30,31]. Shepherd *et al.* [32]

showed that this method could also be successfully applied to dried plant tissue. They cloned 77 genomes of geminiviruses using RCA products from 102 dried plant samples, some of which had been stored for 10 years.

The application of multiply-primed RCA to clinical samples also resulted in the detection of viruses which could not be assigned to known virus families. Investigations on a tumour disease of bandicoots using multiply-primed RCA revealed the presence of a novel virus designated as 'bandicoot papillomatosis carcinomatosis virus' (BPCV) [49]. Subsequently, two different types of the virus were identified in lesions of Western barred bandicoots [49] and Southern brown bandicoots [50]. Analysis of one of the DNA strands of the amplified 7.3 kbp genome sequences revealed open reading frames coding for putative structural proteins, which showed similarities to the capsid proteins L1 and L2 of papillomaviruses. However, gene products encoded by open reading frames located at the opposite DNA strand were highly similar to polyomavirus large and small tumour antigens. It is not known so far whether a recombination event between viruses of both families led to the generation of BPCV, or if the virus represents a common ancestor of both virus families. The detection of this unexpected novel virus underlines the power of multiply-primed RCA in analysis of clinical samples derived from cases with unknown aetiology.

Whole genome analysis of non-cultivable viruses

For human and animal viruses, cultivation in tissue culture is a crucial step, enabling virus propagation, virus purification and subsequent whole genome analysis. However, many viruses could not be cultivated so far because no appropriate tissue culture system is available. In other cases, cultivation is difficult because of the need of sophisticated culture systems or due to inefficient virus growth. In those cases, whole genome analysis is hampered by the lack of a sufficient amount of the purified viral genome. As RCA allows the efficient amplification of whole viral genomes independently of virus cultivation, the sequencing of the RCA product has frequently been used for whole genome analysis of non-cultivable viruses.

For papillomaviruses, which could only be propagated in sophisticated epithelial raft cultures, RCA has become a powerful tool for phylogenetic analysis. Because these viruses are genetically very heterogeneous, PCR analysis using established consensus primers is difficult and often impossible [51,52]. Therefore, whole genome analysis using RCA contributed to the understanding of phylogenetic relationships and evolution among papillomaviruses [35–43].

For two novel avian polyomaviruses, inoculation into tissue cultures did not result in virus propagation [53,54], but the RCA-mediated whole genome analysis enabled their phylogenetic classification and revealed general features in genome organization of bird polyomaviruses [44]. New phylogenetic branches were identified within the anellovirus family using RCA-mediated genome analysis [45,46]. A detailed comparison of RCA-derived whole genome sequences of non-cultivable avian circoviruses with sequences of their hosts strengthened the hypothesis of a strict virus–host co-evolution for circoviruses [47].

Generation of infectious genomic clones

An infectious genomic clone is an isolated viral genome, usually cloned in bacteria, which is able to generate infectious virus after its transfection into a host cell. Those clones allow functional studies of viral genome sequences and can be used for specific mutagenesis of the viruses. As RCA produces repeated copies of whole viral genomes, it has been used for rapid generation of infectious genomic clones of some viruses.

Full-length genomic clones of (AAVs) have been generated directly from clinical specimens using RCA, which were shown to be infective after transfection into tissue culture [55,56]. Inoculation of plants with an RCA product from a begomovirus also resulted in typical symptoms of infection [33]. As compared to cloned begomovirus DNA, the RCA product had a higher efficiency in reproduction of symptoms independent from the inoculation method. This effect might be explained by the concatemeric structure of the RCA product, enabling efficient generation of circular genomic molecules by homologous recombination. Knierim *et al.* [31] excised genome components of another begomovirus from plasmids, which were ligated and subjected to RCA. After inoculation into plants, the RCA products were shown to be tenfold more effective in generation of infectious virus than unit-length genomic DNA. Recently, the technique for generation of infectious genomic clones has been developed further by a combination of RCA, limited restriction enzyme digestion and cloning [57,58]. By this, highly infective constructs containing dimeric genome fragments could be generated by a simplified and rapid method as demonstrated for four different geminiviruses.

Identification of persistent and replicative forms of viral genomes

The genome of (AAV) is composed of linear single-stranded DNA with a length of 4.7 kb; however, it can be circularized during persistent infection, resulting in a double-stranded circular episome [59]. To distinguish between linear and double-stranded circular forms of the AAV genome in persistently infected human tissue, Schnepf *et al.* [60] used a complex RCA approach. First, the DNA preparation was digested with a restriction enzyme which does not cut the AAV genome, resulting in small fragments of cellular DNA. Thereafter, all linear and single-stranded DNA fragments were degraded using a specific DNase. The remaining double-stranded circular DNA was amplified by RCA using two AAV-specific primers. By this, specific RCA products were detected in most of the persistently infected tissues. This indicated, for the first time, that persistence of human AAV genomes is mainly mediated by circular double-stranded forms of their genome, but not by integrated forms of viral DNA. In addition, a combination of this technique with Southern dot blot analysis enabled quantification of circular DNA present in the samples.

Circoviruses have a single-stranded circular DNA genome, which is converted into double-stranded DNA during genome replication. For the detection of the porcine circovirus-2 genome in infected tissue cultures, Navidad *et al.* [61] used in a first step a multiply-primed RCA. In a second step, the authors used a primer specific for the second strand of the circular DNA genome in an RCA to demon-

strate the replicative form of the circovirus genome. This allowed differentiation between active virus replication and passive presence of the circovirus genome in tissue culture.

Conclusion and future perspectives

Following the first application to viruses in 2004, phi29 polymerase-mediated RCA techniques have been developed into important tools for many applications in virology. These include (i) virus diagnostics with differentiation of known virus species; (ii) detection of unknown virus variants, species and families; (iii) whole genome analysis of viruses which could not be efficiently propagated in tissue culture; (iv) generation of infectious genomic clones directly from clinical specimens and; (v) differentiation between linear and circular forms, in addition to identification of replicative forms of viral genomes. Despite this success, there are still several open questions for future research (Box 2).

Until now, the technique has been successfully applied to viruses originating from six known virus families. It could be expected, however, that RCA is also suitable for other virus families with circular DNA genomes (e.g. nanoviruses) (Table 2). In some virus families, such as hepadnaviruses and caulimoviruses, the genome is covalently closed to circular DNA at a specific stage of replication, which can represent a suitable substrate for phi29 polymerase. It might also be speculated that a treatment of viral linear DNA genomes with ligating enzymes, or a reverse transcription of viral RNA genomes, could transform their genomes into suitable substrates for RCA, thereby expanding the applicability of the technique to a larger number of virus families.

In summary, RCA has proven to be an efficient amplification technique, relatively fast and easy to perform. However, some limitations of the method are evident. First, as amplification efficiency decreases with the length of the circular DNA template, RCA is less suitable for larger genomes. This effect can be explained by the longer time needed to synthesize a complete copy of a large circular genome as compared to a small DNA molecule. For very long circular templates, the amplification efficiency of RCA can be expected to be similar to linear molecules. In addition, the probability of strand breaks increases with the length of the DNA molecule, resulting in a termination of the RCA. Second, products generated by multiply-primed RCA from complex samples have to be

analysed further to exclude possible unspecific amplification of bacteriophage genomes, bacterial plasmids or DNA of cellular organelles (Table 1). Until now, restriction analysis, cloning and sequencing of the RCA products are the favoured methods for their identification. The development of reliable and fast methods for easier identification of viral RCA products might be one important issue to be addressed in future studies.

Acknowledgements

This work was supported by the Flemish Fund for Scientific Research (Fonds voor Wetenschappelijk Onderzoek, FWO) grant G.0513.06 and by the Deutsche Forschungsgesellschaft grant JO 369/3-2.

References

- 1 Leebens-Mack, J. *et al.* (2006) Taking the first steps towards a standard for reporting on phylogenies: minimum information about a phylogenetic analysis (MIAPA). *OMICS* 10, 231–237
- 2 Gresham, D. *et al.* (2008) Comparing whole genomes using DNA microarrays. *Nat. Rev. Genet.* 9, 291–302
- 3 Ambrose, H.E. and Clewley, J.P. (2006) Virus discovery by sequence-independent genome amplification. *Rev. Med. Virol.* 16, 365–383
- 4 Fire, A. and Xu, S.Q. (1995) Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. U. S. A.* 92, 4641–4645
- 5 Shavitt, O. and Livneh, Z. (1989) Rolling-circle replication of UV-irradiated duplex DNA in the ϕ X174 replicative-form→single-strand replication system *in vitro*. *J. Bacteriol.* 171, 3530–3538
- 6 Liu, D. *et al.* (1996) Rolling circle DNA synthesis: small circular oligonucleotides as efficient templates for DNA polymerases. *J. Am. Chem. Soc.* 118, 1587–1594
- 7 Blanco, L. *et al.* (1989) Highly efficient DNA synthesis by the phage phi29 DNA polymerase. *J. Biol. Chem.* 264, 8935–8940
- 8 Nilsson, M. *et al.* (2006) Analyzing genes using closing and replicating circles. *Trends Biotechnol.* 24, 83–88
- 9 Nilsson, M. (2006) Lock and roll: single-molecule genotyping *in situ* using padlock probes and rolling-circle amplification. *Histochem. Cell Biol.* 126, 159–164
- 10 Lizardi, P.M. *et al.* (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* 19, 225–232
- 11 Dahl, F. *et al.* (2004) Circle-to-circle amplification for precise and sensitive DNA analysis. *Proc. Natl. Acad. Sci. U. S. A.* 101, 4548–4553
- 12 Silander, K. and Saarela, J. (2008) Whole genome amplification with phi29 DNA polymerase to enable genetic or genomic analysis of samples of low DNA yield. *Methods Mol. Biol.* 439, 1–18
- 13 Nelson, J.R. *et al.* (2002) TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. *Biotechniques* (Suppl), 44–47
- 14 Rector, A. *et al.* (2004) A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. *J. Virol.* 78, 4993–4998
- 15 Hutchison, C.A. *et al.* (2005) Cell-free cloning using phi29 DNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* 102, 17332–17336
- 16 Dean, F.B. *et al.* (2001) Rapid amplification of plasmid and phage DNA using phi29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Res.* 11, 1095–1099
- 17 Blanco, L. and Salas, M. (1984) Characterization and purification of phage phi29-encoded DNA polymerase required for the initiation of replication. *Proc. Natl. Acad. Sci. U. S. A.* 81, 5325–5329
- 18 Berman, A.J. *et al.* (2007) Structures of phi29 DNA polymerase complexed with substrate: the mechanism of translocation in B-family polymerases. *EMBO J.* 26, 3494–3505
- 19 Blanco, L. and Salas, M. (1996) Relating structure to function in phi29 DNA polymerase. *J. Biol. Chem.* 271, 8509–8512
- 20 Lagunavicius, A. *et al.* (2008) Duality of polynucleotide substrates for phi29 DNA polymerase: 3'→5' RNase activity of the enzyme. *RNA* 14, 503–513
- 21 Kamtekar, S. *et al.* (2004) Insights into strand displacement and processivity from the crystal structure of the protein-primed DNA polymerase of bacteriophage phi29. *Mol. Cell* 16, 609–618

Box 2. Questions for future research

- Can RCA be applied to all virus families with circular DNA genomes (e.g. nanoviruses, caulimoviruses and hepadnaviruses)?
- Is an efficient amplification of large circular viral DNA possible (e.g. baculovirus genome, replicative form of herpesvirus genome)?
- Could RCA be used for efficient amplification of linear viral genomes or genomes of RNA viruses after treatment with ligating or reverse transcribing enzymes, respectively?
- Will novel techniques be developed for quick identification of viral RCA products, maybe in a 'real time' fashion?
- Could the RCA reaction conditions be standardized to enable reliable quantitation of circular viral DNA in a sample?

- 22 Watabe, K. *et al.* (1984) Replication of bacteriophage phi29 DNA *in vitro*: the role of terminal protein and DNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* 81, 5374–5378
- 23 Baner, J. *et al.* (1998) Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res.* 26, 5073–5078
- 24 Spits, C. *et al.* (2006) Whole-genome multiple displacement amplification from single cells. *Nat. Protoc.* 1, 1965–1970
- 25 Luthra, R. and Medeiros, L.J. (2004) Isothermal multiple displacement amplification: a highly reliable approach for generating unlimited high molecular weight genomic DNA from clinical specimens. *J. Mol. Diagn.* 6, 236–242
- 26 Esteban, J.A. *et al.* (1993) Fidelity of phi29 DNA polymerase. *J. Biol. Chem.* 268, 2719–2726
- 27 Paez, J.G. *et al.* (2004) Genome coverage and sequence fidelity of phi29 polymerase-based multiple strand displacement whole genome amplification. *Nucleic Acids Res.* 32, e71
- 28 Inoue-Nagata, A.K. *et al.* (2004) A simple method for cloning the complete begomovirus genome using the bacteriophage phi29 DNA polymerase. *J. Virol. Methods* 116, 209–211
- 29 Fauquet, C.M. *et al.* (2005) *Virus Taxonomy. Eighth Report of the ICTV.* Elsevier Academic Press
- 30 Schubert, J. *et al.* (2007) Surveying cereal-infecting geminiviruses in Germany – diagnostics and direct sequencing using rolling circle amplification. *Virus Res.* 127, 61–70
- 31 Knierim, D. and Maiss, E. (2007) Application of phi29 DNA polymerase in identification and full-length clone inoculation of tomato yellow leaf curl Thailand virus and tobacco leaf curl Thailand virus. *Arch. Virol.* 152, 941–954
- 32 Shepherd, D.N. *et al.* (2008) A protocol for the rapid isolation of full geminivirus genomes from dried plant tissue. *J. Virol. Methods* 149, 97–102
- 33 Haible, D. *et al.* (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *J. Virol. Methods* 135, 9–16
- 34 Homs, M. *et al.* (2008) Mitochondrial plasmids of sugar beet amplified via rolling circle method detected during curtovirus screening. *Virus Res.* 136, 124–129
- 35 Ogawa, T. *et al.* (2007) Complete genome and phylogenetic position of bovine papillomavirus type 7. *J. Gen. Virol.* 88, 1934–1938
- 36 Tobler, K. *et al.* (2006) Detection of the prototype of a potential novel genus in the family *Papillomaviridae* in association with canine epidermodysplasia verruciformis. *J. Gen. Virol.* 87, 3551–3557
- 37 Van Doorslaer, K. *et al.* (2006) The genetic characterization of the *Capra hircus* papillomavirus; a novel close to root Artiodactyl papillomavirus. *Virus Res.* 118, 164–169
- 38 Rector, A. *et al.* (2005) Isolation and cloning of a papillomavirus from a North American porcupine by using multiply primed rolling-circle amplification: the *Erethizon dorsatum* papillomavirus type 1. *Virology* 331, 449–456
- 39 Rector, A. *et al.* (2007) Ancient papillomavirus-host co-speciation in Felidae. *Genome Biol.* 8, R57
- 40 Rector, A. *et al.* (2004) Characterization of a novel close-to-root papillomavirus from a Florida manatee by using multiply primed rolling-circle amplification: *Trichechus manatus latirostris* papillomavirus type 1. *J. Virol.* 78, 12698–12702
- 41 Rector, A. *et al.* (2008) Genomic characterization of novel dolphin papillomaviruses provides indications for recombination within the *Papillomaviridae*. *Virology* 378, 151–161
- 42 Rehtanz, M. *et al.* (2006) Isolation and characterization of the first American bottlenose dolphin papillomavirus: *Tursiops truncatus* papillomavirus type 2. *J. Gen. Virol.* 87, 3559–3565
- 43 Stevens, H. *et al.* (2008) Novel papillomavirus isolated from the oral mucosa of a polar bear does not cluster with other papillomaviruses of carnivores. *Vet. Microbiol.* 129, 108–116
- 44 John, R. *et al.* (2006) Characterization of two novel polyomaviruses of birds by using multiply primed rolling-circle amplification of their genomes. *J. Virol.* 80, 3523–3531
- 45 Niel, C. *et al.* (2005) Rolling-circle amplification of *Torque teno virus* (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. *J. Gen. Virol.* 86, 1343–1347
- 46 Biagini, P. *et al.* (2007) Circular genomes related to anelloviruses identified in human and animal samples by using a combined rolling-circle amplification/sequence-independent single primer amplification approach. *J. Gen. Virol.* 88, 2696–2701
- 47 John, R. *et al.* (2006) Genome of a novel circovirus of starlings, amplified by multiply primed rolling-circle amplification. *J. Gen. Virol.* 87, 1189–1195
- 48 Halami, M.Y. *et al.* (2008) Detection of a novel circovirus in mute swans (*Cygnus olor*) by using nested broad-spectrum PCR. *Virus Res.* 132, 208–212
- 49 Woolford, L. *et al.* (2007) A novel virus detected in papillomas and carcinomas of the endangered western barred bandicoot (*Perameles bougainville*) exhibits genomic features of both the *Papillomaviridae* and *Polyomaviridae*. *J. Virol.* 81, 13280–13290
- 50 Bennett, M.D. *et al.* (2008) Genomic characterization of a novel virus found in papillomatous lesions from a southern brown bandicoot (*Isodon obesulus*) in Western Australia. *Virology* 376, 173–182
- 51 Van Ranst, M. *et al.* (1992) Phylogenetic classification of human papillomaviruses: correlation with clinical manifestations. *J. Gen. Virol.* 73, 2653–2660
- 52 Sundberg, J.P. *et al.* (1997) The nonhuman (animal) papillomaviruses: host range, epitope conservation, and molecular diversity. In *Human Papillomavirus Infections in Dermatovenereology* (Gross, G. and von Krogh, G., eds), pp. 47–68, CRC Press
- 53 John, R. and Müller, H. (2007) Polyomaviruses of birds: etiologic agents of inflammatory diseases in a tumorvirus family. *J. Virol.* 81, 11554–11559
- 54 Wittig, W. *et al.* (2007) Detection of DNA of the finch polyomavirus in diseased birds of the order *Passeriformes*. *Berl. Munch. Tierarztl. Wochenschr.* 120, 113–119
- 55 Chen, C.L. *et al.* (2005) Molecular characterization of adeno-associated viruses infecting children. *J. Virol.* 79, 14781–14792
- 56 Schnepf, B.C. *et al.* (2008) Infectious molecular clones of adeno-associated virus isolated directly from human tissues. *J. Virol.* 83, 1456–1464
- 57 Wu, C.Y. *et al.* (2008) A simplified method of constructing infectious clones of begomovirus employing limited restriction enzyme digestion of products of rolling circle amplification. *J. Virol. Methods* 147, 355–359
- 58 Ferreira, T. *et al.* (2008) One-step cloning approach for construction of agroinfectious begomovirus clones. *J. Virol. Methods* 147, 351–354
- 59 Duan, D. *et al.* (1998) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol.* 72, 8568–8577
- 60 Schnepf, B.C. *et al.* (2005) Characterization of adeno-associated virus genomes isolated from human tissues. *J. Virol.* 79, 14793–14803
- 61 Navidad, P.D. *et al.* (2008) Rolling-circle amplification for the detection of active porcine circovirus type 2 DNA replication *in vitro*. *J. Virol. Methods* 152, 112–116

Reproduction of material from Elsevier articles

Interested in reproducing part or all of an article published by Elsevier, or one of our article figures? If so, please contact our *Global Rights Department* with details of how and where the requested material will be used. To submit a permission request online, please visit:

www.elsevier.com/locate/permissions