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Two novel circo-like viruses detected in human feces: complete genome sequencing and electron microscopy analysis

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ABSTRACT

The application of viral metagenomic techniques and a series of PCRs in a human fecal sample enabled the detection of two novel circular unisense DNA viral genomes with 92% nucleotide similarity. The viruses were tentatively named circo-like virus-Brazil (CLV-BR) strains hs1 and hs2 and have genome lengths of 2526 and 2533 nucleotides, respectively. Four major open reading frames (ORFs) were identified in each of the genomes, and differences between the two genomes were primarily observed in ORF 2. Only ORF 3 showed significant amino acid similarities to a putative rolling circle replication initiator protein (Rep), although with low identity (36%). Our phylogenetic analysis, based on the Rep protein, demonstrated that the CLV-BRs do not cluster with members of the *Circoviridae*, *Nanoviridae* or *Geminiviridae* families and are more closely related to circo-like genomes previously identified in reclaimed water and feces of a wild rodent and of a bat. The CLV-BRs are members of a putative new family of circular Rep-encoding ssDNA viruses. Electron microscopy revealed icosahedral (~23 nm) structures, likely reflecting the novel viruses, and rod-shaped viral particles (~65–460 × 21 × 10 nm in length, diameter, and axial canal, respectively). Circo-like viruses have been detected in stool samples from humans and other mammals (bats, rodents, chimpanzees and bovines), cerebrospinal fluid and sera from humans, as well as samples from many other sources, *e.g.*, insects, meat and the environment. Further studies are needed to classify all novel circular DNA viruses and elucidate their hosts, pathogenicity and evolutionary history.

1. Introduction

Viral metagenomic techniques do not depend on viral propagation in cell culture, prior knowledge of the viral nucleic acid sequence or immunological cross-reactivity tests using panels of sera. Thus, viral metagenomic approaches have been key factors in the detection of the genomic sequences of many previously unknown viruses in human samples (Ambrose and Clewley, 2006; Barzon et al., 2011; Delwart, 2007; Mokili et al., 2012).

Despite advances in the field of etiologic diagnosis, many human clinical syndromes of suspected infectious origin have not been associated with a probable causal agent. More than 30% of cases of acute gastroenteritis (Denno et al., 2005, 2012; Finkbeiner et al., 2008), acute respiratory infection (Chiu et al., 2008) and infectious encephalitis (Glaser et al., 2003, 2006; Mailles and Stahl, 2009) do not have an identified etiologic agent, although extensive conventional diagnostic tests have been utilized. Many of these agents may be viruses. Therefore, it is becoming increasingly evident that viral metagenomic approaches, although not yet part of the routine diagnostics, might be of great assistance in public health laboratories affiliated with infectious disease control centers (Mokili et al., 2012; Svraka et al., 2010; Yang et al., 2011). Nonetheless, while the discovery of novel or unexpected viral genomes in clinical samples is a fundamental first step, the association of a virus with a disease is typically complex (Denno et al., 2012; Li and Delwart, 2011; Mokili et al., 2012).

In this study, metagenomics was utilized at a public health laboratory in Brazil to analyze a human fecal sample due to the unusual presence of a huge quantity of rigid rod-shaped viral particles detected by TEM (Fig. 1a). The viral particles resembled RNA

Abbreviations: ICTV, International Committee on Taxonomy of Viruses; ORF, open reading frame; RCR, rolling circle replication; Rep, rolling circle replication initiator (protein/gene); SISPA, sequence-independent single primer amplification; TEM, transmission electron microscopy.

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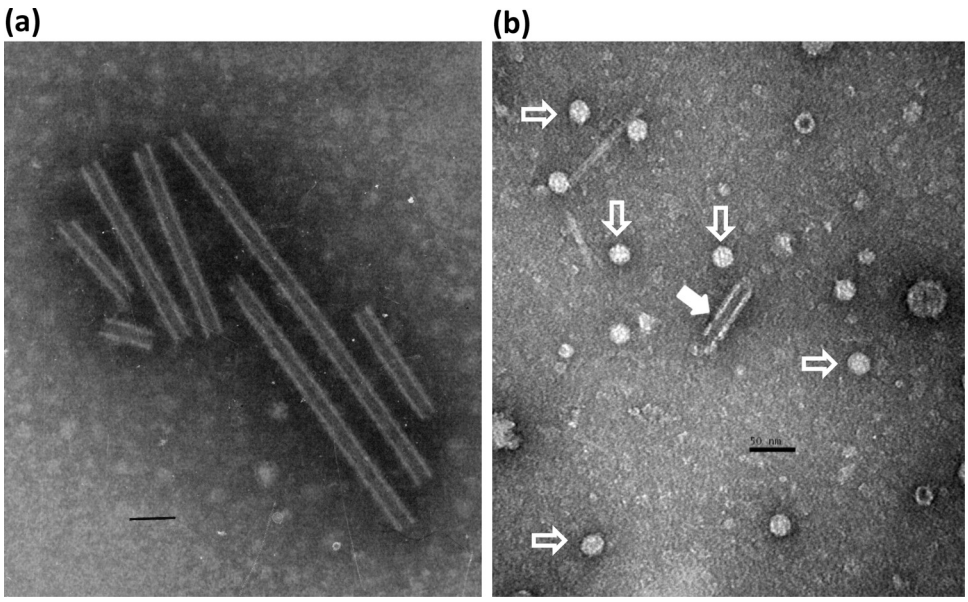

Fig. 1. Electron micrographs of the human fecal sample. (a) Rod-shaped particles negatively stained with potassium phosphotungstate in 2003. (b) Icosahedral (open arrows) and short rod-shaped particles (filled arrow) recently stained with ammonium molybdate, from the second fraction of the sucrose gradient. Scale bars = 50 nm.

plant viruses but did not match any known virus with a similar structure described by the ICTV (King et al., 2012). Instead of discovering a plant virus, this approach detected two novel genomes with circovirus-like features.

2. Materials and methods

2.1. Sample and viral genome purification

A fecal sample from a 50-year-old woman was sent to the TEM laboratory at the Adolfo Lutz Institute (São Paulo, Brazil) in March 2003 because of a clinical history of prolonged diarrhea. Negative staining revealed a large quantity of rigid rod-shaped virus particles with lengths of ~65–460 nm, a diameter of ~21 nm and a large axial canal (~10 nm) (Fig. 1a). Potential pathogenic viruses were not detected by TEM. After eight years of storage at −20 °C, the fecal sample was resuspended (1:10, w/v) in 0.9% NaCl and clarified by two rounds of centrifugation at 1000 × *g* and 4 °C for 20 min. Approximately 20 ml of supernatant was poured through 1.2- and 0.8-μm filters (Millipore) and ultracentrifuged (Beckman SW 55 Ti rotor, 40,000 rpm, 2 h, 6 °C). The pellet was resuspended in 240 μl of buffer containing 22 mM Tris–HCl, 33 mM KCl and 2 mM $MgCl_2$, totaling 600 μl due to the residual supernatant. The sample (400 μl) was treated with 133 U of DNase I (Invitrogen) and 20 μg of RNase A (Invitrogen) for 1 h at 37 °C to remove non-particle-protected nucleic acids (Allander et al., 2001; Djikeng et al., 2008; Victoria et al., 2009; Zhang et al., 2006). Viral nucleic acids were immediately extracted using the QIAamp MinElute Virus Spin Kit (Qiagen) according to the manufacturer's instructions, and 40 U of RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen) was added.

2.2. Sequence-independent single primer amplification (SISPA) and cloning

The amplification procedures used in this study were based on previously described SISPA techniques (Allander et al., 2001, 2005; Djikeng et al., 2008; Froussard, 1992; Jones et al., 2007; Klempa et al., 2009; Reyes and Kim, 1991; Stang et al., 2005). The purified viral nucleic acids were submitted to two procedures in parallel, considering the possibility that we isolated ss/dsRNA and/or ss/dsDNA.

Procedure (a). The purified nucleic acid sample (18 μl) and 1 μl of 10 μM primer FR26RV-N (Table 1) were incubated at 94 °C for 5 min and chilled on ice. RNA was reverse transcribed by the addition of 11 μl of reaction mix containing 6 μl of 5× First-Strand Buffer, 1.5 μl of 100 mM dithiothreitol, 1 μl of dNTP mix (10 mM each), 40 U of RNaseOUT Recombinant Ribonuclease Inhibitor and 300 U of SuperScript III Reverse Transcriptase (Invitrogen) with incubation at 25 °C for 10 min, 37 °C for 10 min, 50 °C for 60 min and 94 °C for 3 min. Subsequently, the product was treated with 2 U of *E. coli* RNase H (Invitrogen) at 37 °C for 20 min. After denaturation at 94 °C for 3 min and chilling on ice, 4.5 U of DNA Polymerase I Large (Klenow) Fragment, Exonuclease Minus (Promega) was added, and primer extension was performed at 37 °C for 60 min, followed by inactivation at 75 °C for 10 min.

Procedure (b). The purified nucleic acid sample (20 μl) was mixed with 2.5 μl of DNA Polymerase 10× Buffer (Promega), 1 μl of dNTP mix (10 mM each) and 1 μl of 10 μM primer FR26RV-N, and incubated at 94 °C for 2 min, immediately followed by chilling on ice; 4.5 U of DNA Polymerase I Large (Klenow) Fragment, Exonuclease Minus was then added and incubated at 37 °C for 60 min, 75 °C for 10 min and 94 °C for 2 min, followed by immediate cooling on ice and repetition of the previous Klenow step.

Thereafter, the products from (a) and (b) were processed equally. The first round of amplification was performed with the primer FR20RV (Table 1) as previously described (Allander et al., 2005). The PCR amplicons were digested with 10 U of *Csp*6 I (Fermentas) and 1× B+ Buffer (Fermentas) in a volume of 100 μl at 37 °C for 60 min. The DNA fragments were purified with the High Pure PCR Product Purification Kit (Roche). Subsequently, all vacuum-dried *Csp*6 I-digested DNA samples were ligated to 20 pmol of adaptor NCsp (Table 1) using 400 U of T4 DNA Ligase (New England Biolabs) in a volume of 10 μl for 1 h at 4 °C, 1 h at 16 °C and 10 min at 23 °C (Ferretti and Sgaramella, 1981; New England Biolabs, 2013). The ligation reaction product (3 μl) was used as a template for a second amplification reaction (Jones et al., 2007) using the primer NBam24 (Table 1) as previously described (Allander et al., 2001).

The products were analyzed on a 0.8% agarose gel. Bands at 150–400 and 400–1500 bp, originating from (a), and bands at 400–1500 bp, originating from (b), were excised, extracted using