MARCIA HELENA BRAGA CATROXO

PESQUISA DO VÍRUS TT (TORQUE TENO VÍRUS) EM PRIMATAS NÃO HUMANOS E EM FRANGOS DE CORTE (*Gallus g. domesticus*), PELA TÉCNICA DE REAÇÃO EM CADEIA PELA POLIMERASE (PCR) E CARACTERIZAÇÃO DO GENOMA VIRAL

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do Título de Doutor em Ciências.

SÃO PAULO
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Catroxo, Márcia Helena Braga

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Research of TT virus (Torque Teno Virus) in non-human primates and in chickens of cut (*Gallus g. domesticus*) by the polymerase chain reaction (PCR) technique and characterization of viral genome.

1.torque teno virus 2.non-human primates 3.chickens of cut 4.coding region 5.non coding region

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“A civilização de um povo se avalia pela forma que seus animais são tratados”

Humboldt

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RESUMO

O Torque Teno Vírus (TTV) foi primeiramente identificado em 1997, no Japão, em um paciente com hepatite aguda pós-transfusão, de etiologia desconhecida, sendo após, caracterizado como um pequeno vírus DNA circular de fita simples e não envelopado. Recentemente, o Torque Teno Vírus foi classificado em um novo gênero chamado *Anellovírus,* que compreende também, o Torque Teno Mini Vírus (TTMV), Torque Teno Midi Vírus (TTMDV) e pequenos anelovírus (SAV). O TTV tem sido detectado em uma ampla gama de primatas não humanos, bem como em animais domésticos. Este trabalho teve por objetivo pesquisar o TTV no soro e sangue total de primatas não humanos e em plasma de frangos domésticos de corte (*Gallus g. domesticus)*, pela aplicação da Nested-PCR da região não codificadora (UTR) e pela Semi-Nested-PCR da região codificadora N22, seguido de seqüenciamento genômico e de análise filogenética. Através da Nested-PCR da região não codificadora (UTR) o DNA do TTV foi detectado no soro de 4 (5,3%) de 75 *Cebus apella*, 2 (40%) de 5 *Alouata fusca*, 1 (20%) de 5 *Alouata caraya*, 1 (5.2%) de 19 *Callithrix penicilata*, 1 (4%) de 25 *Callithrix jacchus*, 1 (20%) de 5 *Saimiri sciureus* e 1 (25%) de 4 *Leontopithecus chrysomelas*. Não se obteve amplificação por PCR-UTR em nenhuma amostra de sangue total. A análise filogenética revelou que as seqüências detectadas em 8 amostras apresentaram maior identidade com as seqüências de TTV isoladas de macacos japoneses do novo mundo: So-TTV2 (*Sagüínus oedipus)* e At-TTV3 (*Aotes Trivirgatus*). Três seqüências (uma de *Callithrix penicilata*, uma de *Leontopithecus crysomelas* e uma de *Cebus apella*) mostraram similaridade com uma seqüência de Torque Teno Mini Vírus (TTMV) de humanos. Não se obteve amplificação por PCR-UTR *em nenhuma amostra de plasma de frangos domésticos de corte (Gallus g. domesticus*). Três amostras foram positivas pela amplificação da região ORF2. O DNA do TTV da região ORF2 foi detectado em uma amostra de soro de *Cebus apella* e em uma amostra de sangue total de *Callithrix jacchus*, e em uma de plasma de frango doméstico de corte (*Gallus g. domesticus).* As seqüências amplificadas pela região ORF2 não mostraram diferenças entre as de humano, primatas não humanos e de frango doméstico.

Pela Semi-Nested-PCR da região codificadora (N22), o DNA do TTV foi detectado no sangue total de 3 (4%) de 75 *Cebus apella* e de 1 (25%) de 4 *Leontopithecus chrysomelas*. Não se obteve amplificação por PCR em nenhuma amostra de soro. A análise filogenética revelou que uma amostra de *Cebus apella* agrupou-se com seqüências de macacos japoneses do novo mundo: *Saguinus oedipus* (So-TTV2) e *Aotes trivirgatus* (At-TTV3); duas amostras de *Cebus apella* mostraram similaridade com uma seqüência de Torque Teno Mini Vírus (TTMV) de humanos e uma (*Cebus apella*) mostrando similaridade com uma seqüência de chimpanzé (Pt-TTV6) e com uma seqüência de TTV de humano, cepa protótipo denominada TA278. Não se obteve amplificação por PCR-N22 em nenhuma amostra de plasma de frangos domésticos de corte (*Gallus g. domesticus*). Os resultados apresentados mostraram que este é o primeiro relato da ocorrência de Torque Teno Vírus e Torque Teno Mini Vírus em primatas não humanos do novo mundo e em frangos de corte (*Gallus g. domesticus*) no Brasil.

SUMMARY

Torque Teno Virus (TTV) was first identified in the serum of a patient with acute post-transfusion hepatitis of unknown etiology in 1997 and was characterized as a small nonenveloped virus with a circular, single-stranded DNA. Recently, Torque Teno Virus has been classified to the newly genus called *Anellovirus*, which also comprises Torque Teno Mini Virus (TTMV), Torque Teno Midi Virus (TTMDV) and small *Anellovirus.* TTV has been detected in a range of non-human primates as well as domestic animals. The purpose of this study was to search TTV in the serum and total blood of non-human primates and in plasma of domestic chickens of cut (*Gallus g. domesticus*) by application the nested-PCR of the non-coding region (UTR) and by semi-nested-PCR of the coding region (N22), followed by a genomic sequence and phylogenetic analysis. By nested-PCR of non-coding region (UTR), the TTV DNA was detected in sera from 4 (5.3%) of 75 *Cebus apella*, 2 (40%) of 5 *Alouata fusca*, 1 (20%) of 5 *Alouata caraya*, 1 (5.2%) of 19 *Callithrix penicilata*, 1 (4%) of 25 *Callithrix jacchus*, 1 (20%) of 5 *Saimiri sciureus* e 1 (25%) of 4 *Leontopithecus chrysomelas*. No PCR-UTR amplification in any total blood sample was obtained. Phylogenetic analysis revealed that sequences detected in 8 samples had presented greater identity with TTV sequences isolated of Japanese new world non-human primates (So-TTV2 - *Sagüínus oedipus* and At-TTV3 - *Aotes Trivirgatus*). Three sequences (1 of *Callithrix penicilata*, 1 of *Leontopithecus crysomelas* and 1 of *Cebus apella*) showed similarity with a human Torque Teno Mini Virus (TTMV) sequence. No PCR-UTR amplification in any domestic chicken plasma sample was obtained. Three additional samples were positive by the amplification of the ORF-2 region. TTV ORF2 DNA was detected in one sera sample of *Cebus apella* and one whole blood sample of *Callithrix jacchus* and in one plasma sample of domestic chicken of cut (*Gallus g. domesticus*). The sequences amplified by the ORF2 region showed no differences between human, non-human primates and domestic chicken. By semi-nested-PCR of coding region (N22), the TTV DNA was detected in total blood from 3 (4%) out of 75 *Cebus apella* and from 1 (25%) out of 4 *Leontopithecus chrysomelas*. No PCR amplification in any serum sample was obtained. Phylogenetic analysis revealed that one sample of *Cebus*

apella clustered with sequences isolated of Japanese new world non-human primates (*Saguinus oedipus* - So-TTV2 and *Aotes trivirgatus* - At-TTV3); two samples of *Cebus apella* showed similarity with a human Torque Teno Mini Virus (TLMV) sequence. The other sample of *Cebus apella* showed similarity with one sequence of the chimpanzee (Pt-TTV6) and with the human TTV strain called TA278. No PCR amplification any domestic chicken plasma sample was obtained. The presented results showed that this is the first occurrence of Torque Teno Virus and Torque Teno Mini Virus in new world non-human primates and domestic chicken of cut (*Gallus g. domesticus*) in Brazil.

1. Introdução

Nas últimas décadas, o aparecimento de diversas viroses emergentes pouco conhecidas tem aumentado a incidência de doenças infecciosas em nosso meio. As hepatites virais fazem parte das chamadas enfermidades emergentes, sendo uma das causas mais comuns de doenças hepáticas, agudas e crônicas que afetam tanto o homem como muitas espécies de animais silvestres e domésticos.

Em 1997, uma equipe japonesa realizou a clonagem de um novo vírus DNA através da técnica de "análise de diferença representacional" a partir de uma amostra de soro de um paciente com hepatite pós-transfusão de etiologia desconhecida (Nishizawa et al., 1997). O vírus foi denominado TT (TTV) de acordo com o nome do paciente do qual foi isolado. Coincidentemente TTV são as iniciais de vírus transmitido por transfusão (Abe et al., 1999; Okamoto et al., 1998a, b).

Posteriormente foi demonstrado que o TTV apresenta um genoma DNA circular, de fita simples, de polaridade negativa, que compreende cerca de 3,6 a 3,8 kb (Okamoto et al., 1998a,b; Okamoto et al., 1999 a,b,c; Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 2000a,b). Através de estudos por microscopia eletrônica de transmissão, comprovou-se que a pequena e esférica partícula do TTV não possui envelope e o seu diâmetro mede aproximadamente 30 a 32 nm (Itoh et al., 2000).

Devido ao seu genoma circular, o TTV foi inicialmente classificado na família *Circoviridae*, na qual estão incluídos, o circovírus porcino (PCV 1 e 2) e o vírus da doença das penas e bicos dos psitacídeos (BFDV), no gênero *Circovírus* e o vírus da anemia das galinhas (CAV), no gênero *Gyrovírus* (Pringle, 1999; Todd et al., 2000). Devido à ausência de significante similaridade entre as seqüências e determinantes antigênicos do TTV e os dos membros da família *Circoviridae,* foi proposto que o TTV fosse enquadrado em uma nova família, tentativamente designada por *Circinoviridae* (Mushahwar et al., 1999); *Paracircoviridae*

(Takahashi et al., 2000b) ou por família TTV (Tanaka et al., 2001). Recentemente, o Torque Teno Vírus (TTV) foi classificado em um novo e flutuante gênero chamado *Anellovírus*, que compreende também, o Torque Teno Mini Vírus (TTMV; 2,8 – 2,0 kb), Torque Teno Midi Vírus (TTMDV; 3,2 kb) e pequenos *Anellovírus* (SAV; 2,2 – 2,6 kb) (Takahashi et al., 2000a; Niel et al., 2001; Biagini et al., 2005; Ninoniya et al., 2007).

O genoma do TTV é dividido em duas regiões: uma região codificadora (N22) de aproximadamente 2,6 kb, extremamente variável e uma não codificadora, mais conservada de 1,2 kb, possuindo entre 3808 nucleotídeos (isolado SANBAN) e 3853 (isolados TA278 e JA 20) (Higikata et al., 1999).

A região codificadora consiste de 6 ORFs com fases de leitura aberta (ORF1 a ORF6), sendo ORF1 e ORF2 os principais genes codificadores de proteínas (Yokoyama et al., 2002). À região ORF1 tem sido atribuída a função de codificar a proteína do capsídeo viral. Na porção central da ORF1 foram identificadas três regiões genômicas hipervariáveis (HVRs), designadas HVR1, HVR2 e HVR3, que contribuem decisivamente para a elevada diversidade genética viral, resultando na forma circulante do TTV em “quasispécies” (Nishizawa et al., 1999).

A N22 é constituída de 500 nt, localiza-se logo após a HVR3 e representa aproximadamente 220nt do genoma do TTV (Okamoto et al, 1998 a, b; Miyata et al., 1999; Mushahwar, et al., 1999; Nishizawa et al., 1999). A maioria das análises filogenéticas do TTV é dirigida a essa região genômica (Lemey et al., 2002).

A ORF2 às vezes apresenta-se dividida em ORFs 2a e 2b, provavelmente codificando uma proteína não estrutural (Kamahora et al., 2000; Okamoto et al., 2000b) com função direta na replicação viral (Okamoto et al., 1999c).

Estudos *in vitro* têm demonstrado que o gene da ORF3 gera dois tipos de proteínas (P38–migração de forma rápida e P41 – migração de forma lenta), com diferentes estados de fosforilação, o que sugere que as proteínas da ORF3 têm função similar à de outras proteínas virais fosforiladas, como a proteína não estrutural NS5A do vírus da hepatite C. A similaridade entre as seqüências da NS5A do HCV e da ORF3 do TTV sugere a possibilidade de que a ORF3 do TTV

possa atuar como moduladora das proteínas celulares do hospedeiro, participando na manutenção da infecção persistente (Asabe et al., 2001).

A Região ORF4 codifica uma proteína de junção que somente é observada nos TTVs e TTMVs isolados de humanos e de primatas não humanos. Em mamíferos de ordem inferior, entretanto, essa região ainda não foi reconhecida (Okamoto et al., 2001a, b, 2002).

As funções de outras ORFs até o momento ainda não foram estabelecidas Yokoyama et al. (2002).

A região não codificadora do TTV (UTR) ocupa cerca de 30% do genoma do TTV e contém uma seqüência de aproximadamente 120 nt enriquecidos com guanina (G) e citosina (C). Esta seqüência forma uma estrutura secundária em alça (Okamoto et al., 1999c; Ukita et al., 2000) exercendo uma importante função na replicação viral (Okamoto & Mayumi, 2001).

Os isolados do vírus TT, em contraste com a maioria dos vírus DNA, apresentam um alto nível de heterogeneidade genética, sendo que as seqüências nucleotídicas da região codificadora (N22) diferem mais que 30% umas das outras (Okamoto et al., 1999, a, b, c, Okamoto & Mayumi, 2001).

Baseados em sua análise filogenética, 39 genótipos foram classificados em 5 genogrupos principais (grupos 1 a 5) (Muljono et al., 2001; Tanaka et al., 2001; Peng et al., 2002; Devalle & Niel, 2004).

Os genótipos 1, 2 e 3 são os mais comumente encontrados em todo o mundo, exceto na áfrica, onde o genótipo 3 ainda não foi encontrado (Gallian et al., 2000).

Dois diferentes métodos de PCR são utilizados para a detecção do DNA do TTV: PCR da região N22 (codificadora) e PCR da região UTR (não codificadora) (Okamoto et al, 1998 b; Okamoto et al., 2000 a, b). A PCR N22 pode detectar os genótipos de TTV de 1-6 que são classificados no grupo 1, enquanto que a PCR da UTR pode detectar essencialmente todos os 39 genótipos incluídos nos grupos 1 – 5, inclusive aqueles detectáveis pela PCR N22 (Okamoto et al., 2004; Muljono et al., 2001; Peng et al., 2002).

O TTV é transmitido parenteralmente por transfusão (Okamoto et al., 1999a,b,c), por transmissão entérica demonstrada experimentalmente em chimpanzés (Luo & Zhang, 2001; Tawara et al., 2000) e por transmissão vertical (Saback et al., 1999). Também tem sido detectado em saliva, secreção nasal, cordão umbilical, sêmen, lágrima, leite materno, cabelo e pele de humanos (Saback et al., 1999; Goto et al., 2000; Inami et al., 2000; Osiowy et al., 2000). Em animais, foi identificado em leite e pele de camelo (Al-Moshih et al., 2007). A presença viral em sêmen de suínos sugere a ocorrência de transmissão venérea, bem como em recém nascidos, a transplacentária (Kekarainen & Segalés, 2008).

Após o descobrimento do TTV, muitos estudos têm sido publicados descrevendo a prevalência da infecção em pacientes com hepatite aguda ou crônica e entre indivíduos de alto risco, como hemofílicos, hemodializados e usuários de drogas intravenosas (Biagini et al., 1998; Mac Donald et al., 1999).

Estudos revelam que 60 a 100% de indivíduos saudáveis são infectados mundialmente com TTV ou TTMV (Peng et al., 2002).

A freqüente infecção com múltiplos genótipos tem sido reportada em adultos e crianças (Peng et al., 2002; Ninomiya et al., 2008).

A presença do TTV tem sido atribuída a alguns tipos de patologias, como, rinite, asma, doenças hepáticas e pulmonares, câncer, desordens hematológicas e miopatias idiopáticas inflamatórias (Miyamoto et al., 2000; Bando et al., 2001; Tajiri et al., 2001; Gergely et al., 2005; Bando et al., 2008).

A infecção por TTV tem sido detectada em muitas espécies de animais domésticos, como cães, gatos, bovinos, suínos, ovinos, aves, tupaias, javalis e camelos (Leary et al., 1999; Okamoto et al., 2001b; 2002; Martinez et al., 2006; Al-Moslih et al., 2007).

Recentemente, uma alta prevalência do TTV foi reportada em granjas de suínos, no Canadá, China, Korea, Espanha, Tailândia, EUA, França e Brasil (Thom et al., 2003; Barnett et al., 2004; Mc Keown et al., 2004; Bigarré et al., 2005; Niel et al., 2005; Martelli et al., 2006; Kekarainen & Segalés, 2008; Brassard et al., 2008).

Os chimpanzés são susceptíveis à infecção por TTV (Mushahwar et al., 1999) e um estudo de transmissão experimental nessas espécies, mostrou que os animais podem ser infectados cruzadamente com TTV humano (Okamoto et al; 2000 a, b).

A transmissão com espécies específicas de TTV é relatada em alta freqüência entre chimpanzés e outros primatas não humanos. Prescott & Simmonds (1998) foram os primeiros a identificar a presença do vírus em soro de chimpanzés silvestres, na África, obtendo 3% de positividade (1 de 31) através da PCR da região N22. Utilizando a PCR da UTR, Leary et al. (1999) encontraram 23,5% de positividade de DNA do TTV no soro de *Saguinus labiatus* e em 20% de *Aotus trivirgatus.*

Em outro estudo realizado por Okamoto et al. (2000a) foi obtido positividade de 100% em 4 amostras de soro de *Saguinus labiatus,* 83% em 6 amostras de *Saguinus aedipus* e 100% de 5 *Aotes trivirgatus.* Pela PCR da N22, outros estudos realizados não obtiveram positividade em amostras de macacos do novo mundo (Abe et al., 2000; Pujol et al., 2005).

Em macacos do velho mundo, entretanto, utilizando tanto a PCR da UTR quanto da N22, a taxa de positividade encontrada variava de 9 a 100% (Leary et al., 1999; Abe et al., 2000; Romeo et al., 2000; Thom et al., 2000; Noppornpanth et al., 2001; Okamoto et al., 2000 a,b; Barnett et al., 2004; Pujol et al., 2005).

Com relação à análise filogenética, Okamoto et al. (2000b) afirmaram que o agrupamento entre algumas cepas de macacos do novo mundo (*Saguinus oedipus, Aotes trivirgatus* e *Macaca fuscata)*, reflete haver uma estreita relação entre os primatas de ordem inferior. De acordo com Verschoor et al. (1999), o TTV de chimpanzé pigmeu e de Chimpanzé comum agrupava-se com o TTV de humanos e de chimpanzés comuns subespécie *Pan troglodytes verus* e *Pan troglodytes schewein furthii*, sugerindo que a existência de um antigo parentesco hospedeiro-patógeno antes da sub-especialização há 1,6 milhões de anos atrás, possa ter ocorrido e que chimpanzés comuns e pigmeus possam ter adquirido o TTV através dessas espécies, em diferentes eventos zoonóticos há mais de 2,5 milhões de anos atrás.

Com relação aos genótipos, Okamoto et al. (2000 a, b) e Nopporpanthy et al. (2001) reportaram que as seqüências de TTV de gibons e de chimpanzés pigmeus que estudaram, agrupavam-se no genótipo 1. Verschoor et al. (1999), entretanto, relataram que as seqüências de chimpanzés pigmeus estudadas em seu trabalho eram relacionadas ao TTV de humanos dos genótipos 2 e 3, enquanto que as de chimpanzés comuns ao de genótipos 5 e 6. De acordo com o trabalho de Romeo et al. (2000), entretanto, a análise filogenética das seqüências consenso da região ORF1 do TTV obtida de 2 chimpanzés (CH1304 e CH1545) representam 2 novos genótipos.

O TTV de símian (s-TTV) foi dividido em 3 genótipos, sendo o tipo 1 o mais encontrado (Abe et al., 2000).

Somente dois relatos fazem menção à pesquisa do TTV em amostras de frangos domésticos. Um estudo investigou a presença do vírus em 29 amostras de frangos domésticos, bem como, em 20 de bovinos, 20 de ovinos e 20 de caprinos, porém todas eram PCR-UTR negativas (Thom et al., 2003). Leary et al. (1999) detectou o vírus em amostras de várias espécies de animais de criação, como galinhas, suínos, bovinos e ovinos, verificando que 19% das aves eram positivas na região UTR, cujas seqüências eram similares às encontradas em humanos.

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Alguns estudos têm mostrado que a infecção com ambos Torque Teno Vírus (TTV) e com Torque Teno Mini Vírus (TTMV), tem sido detectada em um grande número de primatas não humanos (Verschoor et al., 1999; Abe et al., 2000; Okamoto et al., 2000 a,b; Romeo et al., 2000; Thom et al., 2003). TTMV é o único vírus de pequeno genoma que pode constituir uma ligação genética evolucionária entre o vírus da anemia das galinhas (chicken anemia virus) e o TTV (Takahashi et al., 2000a).

Os primatas não humanos são hospedeiros de patógenos que podem infectar o homem por transmissão zoonótica. Muitos deles estão em vias de extinção e, por sua vez em risco de adquirir diversas doenças de humanos (Pujol et al., 2006). Assim neste estudo, propôs-se isolar o DNA do TTV de primatas não humanos e de frangos domésticos de corte, através da padronização da técnica de PCR, bem como, caracterizar o Torque Teno Vírus através de seqüenciamento

e análise filogenética das amostras positivas nessas espécies animais, encontradas em abundância no Brasil.

O estudo das infecções virais em primatas não humanos se reveste de extrema importância tanto para a preservação da biodiversidade quanto para a prevenção de epidemias de grande impacto em saúde humana (Pujol et al., 2006). Os resultados de nosso estudo poderão ser de grande valia e contribuir em futuras pesquisas para elucidar diversos aspectos relativos ao ciclo de disseminação e patogenicidade do Torque Teno Vírus, tanto na espécie humana quanto em outras espécies animais.

2. Objetivos

Os trabalhos aqui relatados tiveram por objetivo:

1. Determinar a freqüência do Torque Teno Vírus (TTV) através da aplicação da técnica de PCR, direcionada para a região codificadora (N22) e para a não codificadora (UTR) em amostras de soro e sangue total de primatas não humanos do novo mundo, provenientes de diversos Parques Ecológicos e Zoológicos do Estado de São Paulo, SP.

2. Determinar a freqüência do Torque Teno Vírus (TTV) através da aplicação da técnica de PCR, direcionada para a região codificadora (N22) e para a não codificadora (UTR) em amostras de plasma de frangos domésticos de corte (*Gallus g. domesticus*), colhidas em diversas granjas do Estado de São Paulo, SP.

3. Caracterizar o Torque Teno Vírus através de seqüenciamento genômico e análise filogenética.

Torque Teno Virus (TTV) is prevalent in Brazilian non-human primates and chickens(*Gallus g. domesticus*).

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SUMMARY

O Torque Teno Vírus (TTV) is an infectious agent of worldwide distribution isolated by the first time as the agent of an acute post-transfusion hepatitis in a patient in Japan. It has been classified into a new floating genus called *Anellovirus*. Recent studies showed that TTV can also be identified in serum specimens obtained from domesticated farm animals and from non-human primates. To better understand the relationship between TTV and their hosts, a study to detect virus in the serum and whole blood of Brazilian non-human primates and in the plasm of chickens was performed by applying the PCR-UTR-A technique, followed by a genomic sequence and phylogenetic analysis. By nested-PCR-UTR, the DNA of TTV was detected in sera from 4 (5.3%) of 75 *Cebus apella*, 2 (40%) of 5 *Alouata fusca*, 1 (20%) of 5 *Alouata caraya*, 1 (5.2%) of 19 *Callithrix penicilata*, 1 (4%) of 25 *Callithrix jacchus*, 1 (20%) of 5 *Saimiri sciureus* e 1 (25%) of 4 *Leontopithecus chrysomelas*. Phylogenetic analysis revealed that sequences detected in 8 samples clustered with TTV sequences So-TTV2 (*Sagüínus oedipus)* and At-TTV3 (*Aotes Trivirgatus*). Three sequences showed similarity with a human Torque Teno Minivirus (TLMV). TTV ORF2 DNA was detected in one sera sample and one whole blood sample of non-human primates and in one plasm sample of chicken. Phylogenetic analysis revealed that the sequences amplified by the ORF2 region show no difference between human, non-human primates and chicken. This is the first report of TTV in Brazilian new world non-human primates and chicken.

KEY WORDS: Torque Teno Virus; Torque Teno Minivirus; Non-humans primates; Untranslated region; Nucleotide sequencing.

INTRODUCTION

Torque teno virus (TTV) is an infectious agent of worldwide distribution (Prescott & Simmonds, 1998; Abe et al., 1999), which was first isolated in Japan in the serum of a patient hospitalized with acute post-transfusion hepatitis of unknown etiology (Nishizawa et al., 1997; Okamoto et al., 1998 a, b). Since then it has been detected in non A-G hepatitis patients, being highly prevalent among individuals at risk for blood borne pathogens, such as haemophilics, hemodialysis patients, and intravenous drug users (Okamoto et al., 1998a).

TTV and Torque Teno Minivirus (TTMV) have been recently classified into a new floating genus called *Anellovirus* (Biagini et al., 2005).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). TTV associated particles with a diameter of 30-32nm recovered from the sera of infected humans are observed as aggregates of various sizes on electron microscopy (Itoh et al., 2000).

Transmission of this agent is caused not only by parenteral exposure but also by fecal exposure, which have been recently demonstred in a chimpanzee transmission study (Tawara et al., 2000). In addition, TTV has been detected in saliva, throat swabs, semen, tears, breast milk, hair skin (Saback et al., 1999; Goto et al., 2000; Inami et al., 2000; Osiowy, 2000), and raw or pasteurized milk (Al-Moslih et al., 2007).

TVV DNA genome is divided into two regions: one extremely variable coding sequence, of 2.6 kb, and one more conserved non-coding (UTR) of 1.2 kb which varies in length between 3808 nt (SANBAN) and 3853 nt (TA 278 and JA 20) (Erker et al., 1999; Higikata et al., 1999). The coding region consists of 6 open reading frames (ORF1 to ORF6) (Yokoyama et al., 2002). The two major genes that codify the proteins are the ORF1 and ORF2 (Okamoto et al., 1998c; Miyata et al., 1999). The non coding region is located between the end of ORF3 (Romeo et al., 2000). PCR with primers deduced from noncoding region (UTR) can detect TTV DNA of different genotypes (Okamoto et al., 1999c).

TTV isolates present highly divergent sequences, classified into 39 genotypes inserted into five major groups (1-5) (Peng et al., 2002; Devalle & Niel, 2004). Studies recently pointed the existence of new members of the family TTV, the SANBAM, YONBAN, TTMV, SEN virus (SENV) and PM virus (PMV) considered new viral species based on the genomic divergences present in these strains (Cong et al., 2000; Hallet et al., 2000; Khudyakov et al., 2000; Takahashi et al., 2000; Diniz-Mendes et al., 2004).

Chimpanzees are susceptible to TTV infection (Mushahwar et al., 1999) and one study of experimental transmission in these species showed that the animals can be cross infected by human species (Okamoto et al., 2000a,b). Species-specific TTV transmissions are reported in high frequencies among chimpanzees and other non-human primates (Leary et al., 1999; Verschoor et al., 1999; Abe et al., 2000; Cong et al., 2000; Okamoto et al., 2000a,b; Noppornpanth et al., 2001; THOM et al., 2003; Barnet et al., 2004; Pujol et al., 2005) as well as between other domestic animals, such as dogs, cats, pigs, bovines, chickens, ovines, tupaias (thee shrews), wild boar (*Sus scrofa*) and camels (Leary et al., 1999; Okamoto et al., 2001b; 2002; Martinez et al., 2006; Al-Moslih et al., 2007). Recently high prevalence of TTV have been reported in swine herds in Canada, China, Korea, Spain, Thailand, USA, France, and in Brazil (Thom et al., 2003; Barnet et al., 2004; Mc Keown et al., 2004; Bigarré et al., 2005; Niel et al., 2005; Martelli et al., 2006).

To better understand the relationship between TTV and their hosts, we conducted a study to detect and characterize virus in Brazilian monkeys and domestic chickens (Ga*llus g. domesticus*).

MATERIAL AND METHODS

Animals:

A total of 90 serum samples and 70 whole blood samples were obtained from non-human primates of both genders, including 26 *Callithrix jacchus*, 3 *Callithrix kulii*, 4 *Leontopitecus crysomelas*, 75 *Cebus apella*, 19 *Calllithrix penicilata*, 2 *Callithrix sp*, 5 *Alouata caraya*, 5 *Alouata fusca*, 1 *Saguinus m. niger*, 1 *Callithrix humeralifer*, 3 *Callithrix geoffroyi*, 1 *Aotus trivirgatus*, 1 *Calimico goeldi,* 3 *Saguinus m. midas*, 1 *Lagothrix lagotricha*, 1 *Erytrocebus pata*, 2 *Ateles b. marginatus*, 1 *Ateles peniscus*, 4 *Saimiri sciureus,* 1 *Saimiri fuscicollis*, 1 *Leontopitecus chrysopygus*. These species are usually found in the Amazon Forest and Atlantic Rain Forest, in Brazil. These animals came from donnations or rescues and were apprehended and sent to several ecological and Zoological Parks in São Paulo, Brazil where they were maintained in captivity. The samples were collected from August 2000 to February 2001. Almost 117 samples of chicken plasm (*Gallus g. domesticus*) of the following lineages: white, brown leghorn chickens and hens of yard, of both genders, from different poultry farms in the state of São Paulo, Brazil, were collected from May to September 2001. Samples were kept undisturbed at -80ºC freezer until testing.

Extraction of nucleic acids and amplification by PCR:

Blood DNA was extracted using a DNA Extraction Kit (Qiagen) from 100 µl whole blood. DNA was ressuspended in 100 µl buffer. Serum DNA (50 µl) was mixed with 100 µl Dnazol and 3 µl Dextran T500 (1µg/µl). The DNA was precipited with isopropanol and ressuspended in 25 µl distilled water.

Nested UTR PCR:

The first round of PCR was performed with primers NG133 (sense) and NG 147 (antisense) and the second round was carried out with primers NG 134

(sense) and NG 132 (antisense). PCR was carried out in 50 µl volume using 5 µl of DNA, 10 pmol of NG133 and NG 147 primers, 1.5 mM $MgCl_2$ and 5U Taq polymerase. The PCR conditions were 94ºC 30s, 55ºC 30s, 72ºC 30s, 35 cycles, with a final extension at 72ºC 7 min. The sequences of the primers utilized were NG 133 5'- GTA AGT GCA CTT CCG AAT GGC TGA G- 3' and NG 134 5'-AGT TTT CCA CGC CCG TCC GCA GC–3' and NG 132 5'-AGC CGG AAT TGC CCC TTG AC– 3' and NG 147 5'-GCC AGT CCC GAG CCC GAA TTG CC-3' according to Okamoto et al. (1998a).

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Five µl of the first PCR product and 10 pmoles/µl of NG 134 and NG 132 primers were used for the second PCR under the same conditions. The amplification products of the first round of PCR were a 143 pb fragment, and the product of the second round was a 110 bp fragment. It was considered positive control for the TTV, a human serum from a TTV positive patient. Amplified products were separated by electrophoresis through a 2% agarose gel containing ethidium bromide, and visualized under UV light.

Sequencing

Sequence reactions were performed with the ABI Prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems). Cycle sequencing was carried out with an automatic DNA Sequencer (ABI Prism 377 Sequencer – Applied Biosystem). The primers used were NG 134 and NG 132 of the UTR region. Sequence edition was performed using the Sequencher program 4.0.5 (Gene Code Corporation). Sequence alignments were generated by the Bioedit Program (Hall, 1999). The phylogenetic analysis was performed using Philip version 3.5 c (Felsentein, 1993).

Alignment with 14 edited sequences (11 from the UTR region and 3 from ORF-2 region) and series of standard sequences from different TTV genotypes from Gen Bank and from the literature (16 from UTR and 15 from ORF-2) were performed.

The sequence alignments were assessed using 1,000 bootstrap replicates using SEQBOOT Program (Felsentein, 1993). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1997). The consense tree was calculed using the Consense Program and visualized in the Treview Program version 1.6.6 (win 32) (Roderic, 2001). The genotypes of the TTV had been determined by comparing the lined sequences (GenBank accession numbers EU518620-EU518633).

RESULTS

Non humans Primates.

90 serum samples and 70 whole blood samples obtained from different species of the new world non human primates were analysed using the nested PCR of the UTR-A region.

Among these samples, 11 serum samples showed positive results. 2 aditional serum samples were positive by the amplification of the ORF-2 region. All DNAs from whole blood samples were negative.

Of these 11 samples, TTV UTR-A positive PCR detected in serum, 4 (5.3%) out of 75 were from *Cebus apella*, 2 (40%) of 5 were from *Alouata fusca*, 1 (20%) of 5 from *Alouata caraya*, 1 (5.2%) of 19 from *Callithrix penicilata*, 1 (4%) of 25 from *Callithrix jacchus*, 1 (20%) of 5 from *Saimiri sciureus* e 1 (25%) of 4 from *Leontopithecus chrysomelas*. Regarding gender, 5 (45.5%) were from female primates and 6 (54.5%) from males. Among these animals 10 positive samples were from adults (91%) and 1 (9.0%) was from a younger animal.

Regarding the 2 samples positives for the ORF-2 region, the DNA TTV was detected in sera from 1 (1.3%) out of 75 *Cebus apella* and in whole blood of 1 (4%) of 25 *Callithrix jaccus*, of which 1 (50%) was a female and the other (50%) a male, all adults (table 1).

Phylogenetic analysis of the UTR-A region (Figure1) of the 11 serum samples, showed that among these TTV sequences obtained from non-human primates analysed in this study, eight of them clustered with one sequence of the cotton-top tamarin (*Saguinus oedipus*) (So-TTV2) and with one sequence of the *Aotes trivirgatus* (At-TTV3), from the primates Park in Japan. The other three sequences showed similarity with a human TLMV sequence (Torque teno Minivirus).

The two sequences amplified by ORF-2 region clustered with the human TTV strain TA278.

***Gallus g. domesticus*.**

No positive PCR was obtained from 117 plasma samples obtained from domestic chicken (*Gallus g. domesticus*) white, and brown leghorn lineages and also leghorn var. (chicken from poultry farm) by the nested-PCR for the UTR-A, and a positive samples was detected in a female adult white leghorn by the ORF-2 PCR**.** Phylogenetic analysis revealed that the amplified sequence did not differ from strains isolated in human or non-human primates. (Figure 2).

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DISCUSSION

Two distinct methods are widely used for the detection of TTV DNA: the N22 PCR, using primers based on the sequence of the coding region and the UTR PCR, using primers based on the sequence of the conserved UTR (Okamoto et al., 2000a,b). The UTR PCR can detect essentially all genotypes, and it has been called "universal" or "general" PCR for the detection of TTV (Okamoto & Mayumi, 2001).

In our study, approximately 160 samples, of which 90 of serum and 70 of whole blood obtained from different non-human primate species from Brazil were tested by the nested PCR for the UTR-A region to detect TTV DNA. This was detected in 11 out of 90 non-human-primates of this study (6.9%).

Similarly, one study found 23.5% of TTV DNA in serum of *Saguinus labiatus* and in 20% in *Aotus trivirgatus*, whereas other studies did not detect TTV DNA in any other serum of new world primates (Leary et al, 1999, Verschoor et al., 1999; Romeo at al., 2000; Barnett et al., 2004; Pujol et al., 2005). Additionally, other study (Okamoto et al. 2000a) obtained 100% of TTV DNA positivity in 4 *Saguinus labiatus* serum´s, in 83% out of 6 *Saguinus aedipus*, and 100% of 5 *Aotes trivirgatus* samples. On the other hand, some authors obtained positive from 14 to 100% investigate among samples from old world primates, (Abe et al., 2000; Romeo et al., 2000; Thom et al., 2000; Pujol et al., 2005; Barnett et al., 2004; Verschoor et al., 1999; Okamoto et al., 2000a; Leary et al., 1999; Noppornpanth et al., 2001).

In our study the most prevalent non-human primate specie was the *Cebus apella*, which is the specie with the wildly geographical distribution in the Americas (Auricchio, 1995). However, another study did not detect TTV DNA in any of 9 samples of *Cebus apella* (Abe et al. 2000).

Of the 11 positive samples detected in our study, 5 (45.5%) were from female monkeys and 6 from males (54.5%), confirming that there is probably no bias of TTV infection among males and females, as showed by other study that detected 48.3% (29 of 60) TTV infection among male chimpanzee and 49.2% among females (31 of 63) (Verschoor et al.,1999).

It is interesting to note that one study observed a higher incidence of TTV infection among adult animals (30 of 46, 65.2%) as compared to new born and juvenile chimpanzees (8 of 37, 21.6%) (Verschoor et al., 1999). Therefore, older animals may play an important role in the transmission pattern in a monkey breeding place and further studies should be conducted to specifically address the causes for these findings.

Interestingly, 8 out of 11 samples characterized in our study clustered in the phylogenetic analysis with two previously described strains isolated from non-human primates (Okamoto et al. 2000a), thus suggesting that, although there are some strains from non-human primates similar to strains found in humans (Leary et al, 1999), some strains may be specific for non-human primates. It is important to point out that usually TTV sequences isolated from chimpanzees will be distinct from strains isolated in humans. (Abe et al., 2000; Romeo et al., 2000; Barnett et al., 2004).

The results of our research showed that three sequences had similarity with a human TLMV sequences. Indeed, several studies showed that the infection with both TTV and TLMV have been detected in a range of non-human primates (Verschoor et al., 1999; Abe et al., 2000; Okamoto et al., 2000ab; Romeo et al., 2000; Thom et al., 2002). There are some evidences that corroborate frequent infection by TTV and TLMV related virus in a wide range of old world apes (chimpanzees, gorillas, orangutans, gibbons, mangabeys, drills and mandrills), raising the hypothesis that the TTV-like viruses co-evolved with their hosts over the period of primate speciation (Thom et al. 2003).

Considering that the inespecifity of primers which amplify the UTR, and that for some animals species the UTR region is located close to the ORF2 region, two samples (one of *Cebus apella* and one of *Callithrix jacchus*) were amplified only with ORF primers. When comparing the 2 sequences from the ORF2 region samples a clustering was observed with the TA278 human prototype sequence, which to our knowledge, is a unique finding in the literature. This sort of similarity was found among strains isolated from one chimpanzee (Pt-TTV6) and the TA278 from humans,

by the phylogenetic tree constructed on the amino acid sequences of ORF2 (Okamoto et al., 2002).

When chickens' samples were analyzed (*Gallus g. domesticus*), it was observed that none among the 117 plasm samples studied in our researches were amplified by the nested-PCR UTR-A reaction. These data are in accordance with the findings of other study Thom et al. (2003) which observed that all the 29 chicken samples, as well as the 20 samples from cows, 20 from sheep and 20 from goats, were PCR negative.

On the other hand, it was observed in one chicken sample that the amplification of the ORF2 region was obtained.

In our study, the sequence of chicken clustered with the prototype TTV human TA 278, which is in accordance with other study (Leary et al. 1999) that found similarity between sequences amplified in humans and other animals, including birds, thus confirming TTV is able to infect a wide range of mammals as well as avian species.

Our date indicate that TTV is present in Brazilian new world monkeys in captivity, although in low prevalence, and these viruses can be hardly ever encountered in chickens. Considering that there is a great deal of contact between the Brazilian population and the animal life and captivity, virus might be transmitted through feeding processes or by the treatment applied to animals.

The enteric transmission demonstrated experimentally in non-human primates (Luo & Zhang 2001), indicates that the fecal-oral route, associate to sanitary and also the precarious conditions of hygiene can be of importance in the dissemination of the TTV. Additional explanations can be considered regarding TTV presence in the animals used in this study. These animals when submitted to stress situations such as changes of temperatures or feeding, transport, intercourse, etc. could develop the disease resulting in great threat mainly to those species included in the official list of the Brazilian fauna at risk of extinction, such as the golden faced lion tamarin (*Leontopithecus crysomelas*).

We acknowledge that the small sample size and the bias of the analysis of animals in captivity preclude more conclusive considerations. However,

we believe that the results of our study can be of importance for the design of future studies to address questions related to the dissemination cycle and pathogenicity of TTV in animal species and humans.

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Figure Legends:

TABLE 1: Description of TTV positive samples by PCR of UTR-A and ORF-2 regions according to the species, gender, age and captivity origin. M= male, F=female, A= adults, Y=young.

FIGURE 1: Comparison between TTV-UTR-A nucleotide alignment from sequences obtained from Brazilian non-human primates and sequences isolated from humans: Sequences TA278 (AB17610), JA1 (AF122916), T3PB (AF247138), TGP96 (AB041962), SANBAN (AB025946), TLMV-CDB203 (AB026929), TLMV-CDB231 (AB026930), TUS01 (AB017613), TJN01 (AB028668) are from human sequences MfTTV3 (AB041958), MfTTV9 (AB041959), PtTTV6 (AB041957), Pt-TTV8II (AB041963), SoTTV2 (AB041960), AtTTV3 (AB041961) are from non-human primates, and sequence is from CAV/U65414 (U65414) chicken. Identification of isolates characterized in this study is presented in bold. Dots indicate nucleotide identities, whereas dashes indicate gaps.

FIGURE 2: Comparison between TTV-ORF-2 nucleotide sequences obtained from Brazilian non-human primates and sequences isolated from humans: TA278 (AB17610), PMV (AF261761), TUSO1 (AB017613), KC009-YONBAN (AB028668), JT33F (AB064606), TLMV-CDB231 (AB026930), TGP96 (AB041962) non-human primates: PtTTV6 (AB041957), Pt-TTV8II (AB041963), MfTTV3 (AB041958), MfTTV9 (AB041959), SoTTV2 (AB041960), AtTTV3 (AB041961), tupaia: Tbc-TTV14 (AB057358), swine: Sd-TTV31 (AB076001) and from chickens: CAV (M55918). Isolates determined in this study are presented in bold.

FIGURE 3: Phylogenetic relationship between TTV UTR-A nucleotide sequences obtained from Brazilian non-human primates and sequences isolated from humans, non-humans primates and chickens. The phylogenetic trees were constructed by the

neighbour-joining method (Saitou & Nei, 1987). Isolates determined in this study are presented in bold. The database-derived isolates and their accession numbers is given in the legend to fig. 1.

FIGURE 4: Phylogenetic relationship between TTV ORF2 nucleotide sequences obtained from Brazilian non-human primates and from chicken (*Gallus g. domesticus*) and sequences isolated from humans, non-humans primates, tupaia, swine and chicken. The phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1997). Bootstraps above 700 are presented. Isolates determined in this study are presented in bold. The database-derived isolates and their accession numbers is given in the legend to fig. 2.

Table 1:

№ sample	Specie	Gender	Age	Origin	Positive PCR
07	*Cebus apella*	M	A	Zoo Sorocaba, SP	UTR
13	*Callittrix penicilata*	M	A	Zoo Sorocaba, SP	UTR
15	*Cebus apella*	M	A	Zoo Sorocaba, SP	UTR
18	*Cebus apella*	F	A	Parque Tietê, SP	ORF-2
21	*Cebus apella*	M	A	Zoo Campinas, SP	UTR
27	*Callithrix jacchus*	F	A	Zoo Sorocaba, SP	UTR
32	*Callithrix jacchus*	M	A	Zoo Sorocaba, SP	ORF-2
65	*Alouatta caraya*	F	A	Zoo Mogi-Mirim, SP	UTR
66	*Cebus apella*	F	A	Zoo Guarulhos, SP	UTR
71	*Allouata fusca*	F	Y	Zoo Taboão da Serra, SP	UTR
79	*Allouata fusca*	F	A	Zoo São Paulo, SP	UTR
85	*Saimiri sciureus*	M	A	Zoo São Paulo, SP	UTR
87	*Leontopithecus chrysomelas*	M	A	Zoo São Paulo, SP	UTR
92	*Gallus g. domesticus* (white leghorn)	F	A	Itapevi, SP	ORF-2

Figure 1:

```
                  10        20        30        40        50        60
              ....|....|....|....|....|....|....|....|....|....|....|....|
1.TA278     1 GAGTTTTCCACGCCCGTCCGC-AGC--GGTGAAGC----CACG----GAGGGAGATCTCC 49
2.JA1       1 .....................-G.A--.AG.G...----....----....TGATC.CGA 49
3.T3PB      1 .....................-...--........----....----.......C..A.. 49
11.TUS01    1 .....................-...--.AGA.C..----....----.....GAG..CG. 49
12.TJN01    1 .....................-...--.AG..GCA----GC..----A.........CG. 49
13.SANBAN   1 .....................-...--.AG.G...----G...----....AGCTC.CGA 49
Pt-TTV6     1 ......ATGC...........-..ACG.AG..G..----GCT.----CC.CCGACCGCG. 51
Mf-TTV3     1 ......ATACT.T...G....AG.TCG.TG..G.A----GCGACA--.TAACGACC.CAG 54
Mf-TTV9     1 .......T.C.-..GAGA...-..GCGA.GACGAACCGCGCA.CCTG.T.A.T..C...G 58
TLMV-CBD231 1 ......AT--------CA...C..ACG.AGACG..----AT.AGA--ACACTGAC.GC.G 46
TLMV-CBD203 1 ......AT--------G....T..ACG.AGAG.AG----ACA.----.TTACT.....T. 44
TGP96       1 ......AT--------G....C..ACG.AGACG.G----AT.ACT--TCA.TGAC..CAG 46
So-TTV2     1 ......A.--------.....CG.GTGTAG..GA.GCG-AC..GAGG...A.GC...CG- 50
At-TTV3     1 ......A.--------CGT..CG.GGA.CA.CGT.T---GC..AGCG...C...GCGCGA 49
Pt-TTV8-II  1 ......AT--------G....T..ACG.AGAG.AG----ATA.TC--.CA.ACT.A..T. 46
Ac-TTV65    1 ----------------CA...CC.TCC.CA.CG.A----GC..GC--TC..CGA.CACT. 38
Af-TTV71    1 -.......--------CA...CC.TCC.CA.CG.A----G-------------A.CGA.. 34
Af-TTV79    1 T.......--------CA...CC.TCC.CA.CG.A----G-------------A.CGA.. 35
Ss-TTV85    1 T.......--------CA...CC.TCC.CA.CG.A----G-------------A.CGA.. 35
Ca-TTV21    1 T.......--------CA...CC.TCC.CA.CGA-----G----------.--..CGCGA 35
Lc-TTV87    1 T.......--------CA...CC.TCC.CA.CGAA------GC---------GA.A..A. 37
Ca-TTV13    1 ------------------------------------------------------------ 1
Ca-TTV07    1 ---.....--------CA...CC.TCC.CA.C---------------------------- 21
Cj-TTV27    1 T.......--------CA...CC.TCC.CA.CGA.----G----------A--A.CTCGA 36
Gg-TTV15    1 T.......--------CA...CC.TCC.CA.CGA-------------.G.ACGCGA.CGA 39
Ca-TTV66    1 T.......--------CA...CC.TCC.CA.CGT.T---GC..AGCG...C...GCGCGA 49
CAV/U65414  1 .TAGGTAT---------A...A..GCG.TCCGG.--------------T..AT.CA.GGG 37

                    70        80        90       100       110       120
              ....|....|....|....|....|....|....|....|....|....|....|....|
1.TA278     50 G-CGTCCCGAGGGCGGGTGCCGAAG------GTGAGTTTACACACCGAAGTCAAGGGGCA 102
2.JA1       50 A-....................G..------................G............ 102
3.T3PB      50 .-....................T..------............................. 102
11.TUS01    50 .-....................G..------................C............ 102
12.TJN01    50 .-....................G..------................C............ 102
13.SANBAN   50 .-....................G..------.....C..........C............ 102
Pt-TTV6     52 .T................A..CG..------.................G..T........ 105
Mf-TTV3     55 AC.AG...T-............G..------................C............ 107
Mf-TTV9     59 .C..C..GT-...............CG----.GAG......C.C.GCG............ 113
TLMV-CBD231 47 .CT.AT.TT-.......A.......------......GA.AC..........T....... 99
TLMV-CBD203 45 .CTAA.T.A-.......A.......------......GA.AC..........T....... 97
TGP96       47 .CT.AA.TT-.......A.......------......GA.AC.....T....T....... 99
So-TTV2     50 --.AG...T........AC...C..AGCATTCC..AGG..TG.GG..C-..GT....... 107
At-TTV3     50 CCGAG.GACT.......A....GGAACA--TCC..AGG..TGTG--..C...T....... 105
Pt-TTV8-II  47 .CT.....T-...............------......GA.AC.................. 99
Ac-TTV65    39 .CG.AT.-CT.......A...ACG.AGCATTCC..AGG..TG.GGA.T-........... 96
Af-TTV71    35 .CG.AT.-CT.......A...TCG.AGCATTCC..AGG..TG.GGA..-........... 92
Af-TTV79    36 .CG.AT.-CT.......A...TCG.AGCATTCC..AGG..TG.GGA..-........... 93
Ss-TTV85    36 .CG.AT.-CT.......A...TCG.AGCATTCC..AGG..TG.GGA..-........... 93
Ca-TTV21    36 CCGAG.GACT.......A....CGAAGCATTCC..AGG..TG.GGA.C-........... 94
Lc-TTV87    38 .C.AG...T........A....G..------......GA.AC.................. 91
Ca-TTV13    1  --.AG............A....G..------......GA.AC.................. 52
Ca-TTV07    21 --.----..........A.......------......GA.AC.................. 69
Cj-TTV27    37 CCG.C.GACT............CG.AACATTCC..AGG..TGTG-A.C-........... 94
Ca-TTV15    40 .G..A.T---.......A....CG.AGCATTCC.GAGG..TG.GGA.C-........... 95
Ca-TTV66    50 CCGAG.GACT.......A....GGAACA--TCC..AGG..TGTG--..C........... 105
CAV/U65414  38 AA..G.GGACAAC...CC..T.GG.-GCA--....A.CGG.G.TTA.CC.-AG....... 93

               ....|....
1.TA278     103 ATTCGGGCT 111
2.JA1       103 ......... 111
3.T3PB      103 ......... 111
11.TUS01    103 ......... 111
12.TJN01    103 ......... 111
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13.SANBAN   103 ......... 111
Pt-TTV6     106 ......... 114
Mf-TTV3     108 ........A 116
Mf-TTV9     114 ........A 122
TLMV-CBD231 100 ......... 108
TLMV-CBD203 98  ......... 106
TGP96       100 ......... 108
So-TTV2     108 ........A 116
At-TTV3     106 ATTCGGGCA 114
Pt-TTV8-II  100 ........T 108
Ac-TTV65    97  ........T 105
Af-TTV71    93  ........- 100
Af-TTV79    94  ........T 102
Ss-TTV85    94  ........T 102
Ca-TTV21    95  ........T 103
Lc-TTV87    92  ........T 100
Ca-TTV13    53  ....----- 56
Ca-TTV07    70  ........T 78
Cj-TTV27    95  ........T 103
Ca-TTV15    96  ........T 104
Ca-TTV66    106 ........T 114
CAV/U65414  94  .CCT....C 102
```

Figure 2:

```
                  10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
1.TA278      1  GAATTTAGCACCCCGGTGCGTAGCGGCGAAGCGACCGAAGGCGATCTGCGTGTGCCGCGT 60
17.PMV       1  -TG.GGC.TC.G......TA..A..TGCCG.GCCAG...C.TC.GT.TTA..AAG..T.C 59
11.TUS01     1  --T.ACCA.C--...ACCAT..A..CGGGCATTCGT...CAGC.GTG.TT..AAAGCACC 56
KC009        1  --G.GGC.TC.G....GC.A..A..TGCCG.GCCGTG..AA.C.GTG.TT..C.G..GTG 58
JT33F        1  -TT.GGC.TC.G.......A..A..CGACC.GC.TTC.GC.TCTGTG.TA..AAAGCTT. 59
Pt-TTV6      1  ACC.GGT..C.G.......A..A..TGCCG.GCCTG...C.TA.CTG.TA..AAAGCT.C 60
CBD231       1  TTTC.G.C.C.GAGC...TA....AAAA.C.GCCTG...AA.C.GTG.ATGAACA.CAT. 60
TGP96        1  TGC.A..AAC.GA.CTATTA..A.AC.A...GC.AAC.GAC.C.GTG.AT.AACGATAT. 60
Pt-TTV8-II1     A.C....AAC.GA.CT.TTA..C.CCGA..AGC.AA.GCAAA.CG....TGAACAGCGTG 60
Mf-TTV3      1  --G.GGGAAC.G...CA..AGG...TG.GCC..CGT...CTG...TG.T.GCGTGGCACC 58
Mf-TTV9      1  C.G.GGGAAC.GA.CACC.TG.A..CGTGG.GCCGT....AA.CGTG..TGCGTAGCGTG 60
So-TTV2      1  --------------ATGCA.CGT..TACC----CAG...CTGAT.TG.AAAGAA.T.GTG 42
At-TTV3      1  TT..GC..GG.AG.CCGC..G.TTTTGCGGTG-CAG....ATCG.TG..TGAAAG..GTG 59
Tbc-TTV14 1     ---------------------------ATG.ATC.G.CGAAAAT.TG.T.GCATAGCTGC 33
CAV          1  .TG...C.TC.G...ACCA.C...C.TCCG---CTG...AC.CCG.-ATTGCC.TGAAA. 56
CaTTV18      1  ---........................AGCA.C.............C............. 57
GgTTV92      1  T.............................................AT............ 60
CjTTV32      1  T........................AAA.CC...........CCG.-.T.C.A....T.G 59

                          70        80        90        100       110
                ....|....|....|....|....|....|....|....|....|....|..
1.TA278      61 GCGGGCGCGGAAGGCGAATTTACCCATCGTAGCCAGGGCGCGATTCGTGCGC 112
17.PMV       60 CT.CAT...C.T.AT.CG...TG.GGCT.CG..G.TTTT.T.GCG.A.ATTA 111
11.TUS01     57 CT.CGTAGCC.TCATAGC...TG.GGCT.CG..G.TCCG.T.C.G.A.TTTA 108
KC009        59 TTTCATAGCC.T.CGAGC.GGTG.GGCT.CG..G.TTTT.T.GGC.A.CT.A 110
JT33F        60 CATC.T.GCC.T.CG.CG...TG.GGCT.CG..G.TCCGATTC.G.A.ATTA 111
Pt-TTV6      61 CT.C.TAGCC.T.CG.CG...TG.GGCT.CG..G.TTTT.T..GC.A.CT.A 112
CBD231       61 TTTAA.A.CC.T.ATCTGA.GTG.GGCT.C.A.G.TAC.ATT.AA.A.CT.T 112
TGP96        61 CATT..A.CC.T.ATCTG.GGTG.AGCT.CGAT..T.TGCT..AA.A.CT.. 112
Pt-TTV8-II61    ...CATAGCC.T.ATCTGC.GTG....T.CGAT..TCCGCT..AA.A.CT.- 111
Mf-TTV3      59 TG.T.GAACC.T.CG.CG...TG.GGCT.CG..G.TCCGAGCT...A.CT.G 110
Mf-TTV9      61 .T..ATAGCC.TCAGAGC...TG.GGCT.C.A.G.TCCG.GCT...A.CT.G 112
So-TTV2      43 .ATAA.AGCC.TAAACTG...TG.A.CT.C.TGG.TCCGCA..AC.A.TAT. 94
At-TTV3      60 .AAAG.TGCC.TCAGCTG...TG.AGCT.C...AGC.CGTG.GA..A.CT.. 111
Tbc-TTV14 34    CT.CTGAGCC.TAAAAGC.GGTG.A.CT.C.C.G.ACCGCGT.AC.A.CT.. 85
CAV          57 T..TATTG.C.TT...GGCA.TA....TACCCTG..CCT.T.---..GCT.. 105
CaTTV18      58 ...................................................- 108
GgTTV92      61 ..........GC.......................................- 111
CjTTV32      60 C..T.T..C.G.A.T..GCC.G.AT.C..CG.TG.AA.GC.AG..T.GC.TG 111
```

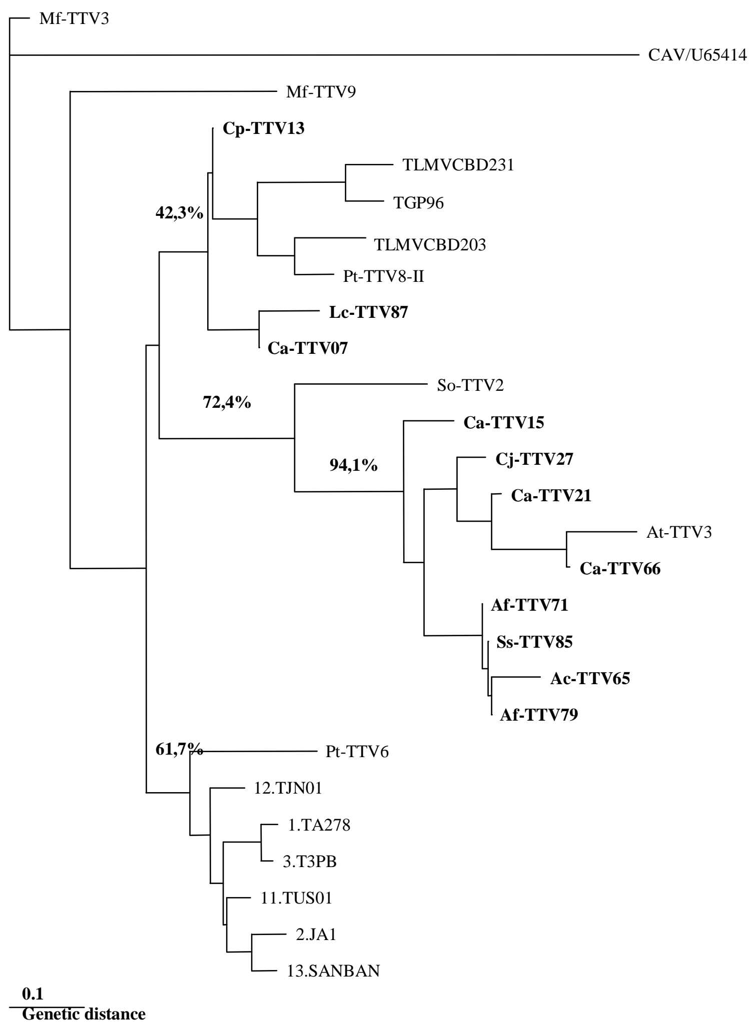

Fig. 3- Phylogenetic relationship between TTV UTR-A nucleotide sequences obtained from Brazilian non-human primates and sequences isolated from humans, non-humans primates and chickens. The phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). Isolates determined in this study are presented in bold. The database-derived isolates and their accession numbers is given in the legend to fig. 1.

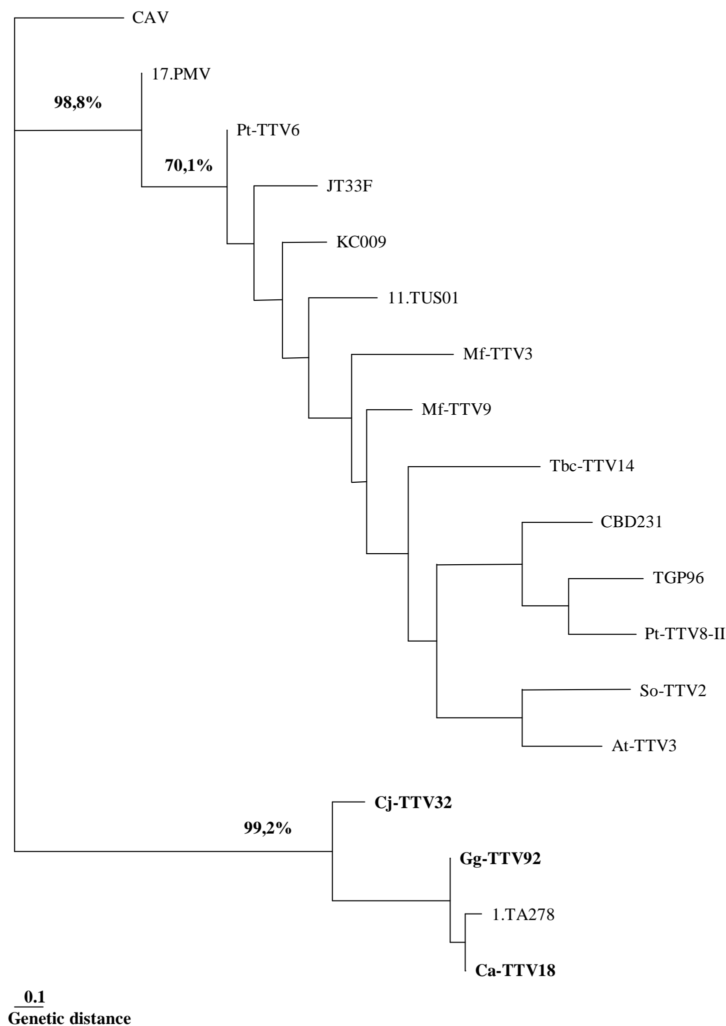

Fig. 4- Phylogenetic relationship between TTV ORF2 nucleotide sequences obtained from Brazilian non-human primates and from chicken (*Gallus g. domesticus*) and sequences isolated from humans, non-humans primates, tupaia, swine and chicken. The phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1997). Bootstraps above 700 are presented. Isolates determined in this study are presented in bold. The database-derived isolates and their accession numbers is given in the legend to fig. 2.

Research of Torque Teno Virus (TTV) in serum and total blood of Brazilian non-human primates and in chicken plasma (*Gallus g. domesticus*) by the PCR N22 region.

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CATROXO, M.H.B.; NISHIYA, A.; SABINO, E. ; TEIXEIRA, P.S.; PETRELLA, S.; MILANELO, L.; DIAZ, R.S. Research of Torque Teno Virus (TTV) in serum and total blood of non-human primates and in chicken plasma (*Gallus g. domesticus*) by the PCR-N22 region. ***Int. J. Morphol., 26(2):***377-384, 2008.

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SUMMARY:

Torque teno virus (TTV) is a recently discovered DNA virus that was originally isolated from a Japanese patient (initials, TT) with post-transfusion hepatitis of unknown aetiology. TTV is an circular DNA virus classified recently together with related Torque teno minivirus, into a new genus called *Anellovirus.* Infection TTV has been detected in a range of non-human primates as well as domestic animals. The purpose of this study was to search TTV in the serum and total blood of Brazilian monkeys and in plasma of domestic chickens by seminested PCR of coding region (N22), followed by a genomic sequence and phylogenetic analysis. No serum sample was amplified. TTV DNA was detected in total blood from 3 (4%) out of 75 brown-capuchin (*Cebus apella*) and from 1 (25%) out of 4 golden-headed lion-tamarin (*Leontopithecus chrysomelas*). Phylogenetic analysis revealed that one sample showed similarity with one sequence of the cotton top tamarin (*Saguinus oedipus)* (So-TTV2) and with one of the douroucoulis (*Aotes trivirgatus)* (At-TTV3). Two samples showed similarity with a human Torque Teno Mini Virus (TLMV). The other sample clustered with one sequence of the chimpanzee (Pt-TTV6) and with the human TTV strain TA278. The plasma chicken samples tested were all negative. The amino acid sequences reported in this study are the first obtained in Brazil from total blood of non-human primates naturally infected by TTV.

KEY WORDS: Torque teno virus; Non-humans primates; Coding region; Amino acid sequencing.

INTRODUCTION

Torque Teno virus (TTV) is a recently discovered DNA virus that was originally isolated from a Japanese patient (initials, TT) with post-transfusion hepatitis of unknown aetiology (Nishizawa et al., 1997). Since discovery of TTV, studies have been published describing the prevalence of TTV infection in people with acute or chronic hepatitis as well as in blood donors and drug users and also in health persons (Biagini et al., 1998; Mac Donald et al., 1999). TTV is an unenveloped, single-stranded, and circular DNA virus with 30-32 nm of diameter (Miyata et al., 1999; Mushahwar et al., 1999; Itoh et al., 2000), classified recently together with the related Torque Teno Minivirus, into a new genus called *Anellovirus* (Biagini et al., 2005).

TTV is transmitted parenterally by transfusion (Okamoto et al., 1999), by enteric transmission demonstrated experimentally in chimpanzees (Luo & Zhang, 2001; Tawara et al., 2000) and by vertical transmission (Saback et al.,1999). Also has been detected in saliva, on throat swabs, semen, tears, breast milk, hair, skin and raw (Saback et al., 1999; Goto et al., 2000; Inami et al., 2000; Osiowy, 2000; Al-Moshih et al., 2007).

Infection TTV has been detected in a range of non-human primates (Leary et al., 1999; Verschoor et al., 1999; Abe et al., 2000; Cong et al., 2000; Okamoto et al., 2000a,b; Noppornpanth et al., 2001; Thom et al., 2003; Barnet et al., 2004; Pujol et al., 2005) as well as domestic animals, such as dogs, cats, swines, bovines, chickens, ovines, tupaias (thee shrews), wild boar (*Sus scrofa*) and camels (Leary et al., 1999; Okamoto et al., 2001a; 2002; Mc keown et al., 2004; Bigarré et al., 2005; Niel et al., 2005; Martelli et al., 2006; Martinez et al., 2006; Al-Moslih et al., 2007).

Study of experimental transmission points out that the chimpanzees are susceptible to TTV infection and that can be cross infected with human (Mushahwar et al., 1999; Okamoto et al., 2000a,b).

TTV genome includes two regions: a coding region (N22) and an untranslated region (UTR). The UTR is located at nt 3075-3853 and nt 1-352

occupying approximately 30% of the genome (Okamoto et al., 1999; Ukita et al., 2000). Coding region consist of 6 ORFs with open reading frames (ORF1-ORF6) (Yokoyama et al., 2002). The N22 amplifies a fragment in open reading frame 1 (ORF1) and represents approximately 220 nts of a genome that usually encompasses more than 3800 nts (Miyata et al., 1999; Mushahwar et al., 1999). Consequently, almost every phylogenetic analysis of TTV is focused on this genomic region (Lemey et al., 2002).

The significant diversity of the TTV genome allows for its classification into more than 40 genotypes which cluster in five clearly distinct phylogenetic groups that are designated 1 to 5 (Okamoto & Mayumi, 2001; Hino, 2002; Peng et al., 2002; Biagini et al., 2005).

Other members of this virus family (SANBAM, YONBAN, TLMV (Torque teno Minivirus), SENV (SEN virus) and PMV (PM virus), presenting some genomic divergences have been related (Cong et al., 2000; Hallet et al., 2000; Khudyakov et al., 2000; Takahashi et al., 2000b; Diniz-Mendes et al., 2004).

The purpose of this study was to search TTV in the serum and blood of Brazilian monkeys and plasma of domestic chickens (Ga*llus g. domesticus*) by PCR of coding region (N22), followed by a genomic sequence and phylogenetic analysis.

MATERIAL AND METHODS

Animals:

Were collected from August, 2000 to February, 2001, 90 serum samples and 70 total blood samples from various non-human primates, both sexes, including 26 *Callithrix jacchus*, 3 *Callithrix kulii*, 4 *Leontopitecus crysomelas*, 75 *Cebus apella*, 19 *Calllithrix penicilata*, 2 *Callithrix sp*, 5 *Alouata caraya*, 5 *Alouata fusca*, 1 *Saguinus m. niger*, 1 *Callithrix humeralifer*, 3 *Callithrix geoffroyi*, 1 *Aotus trivirgatus*, 1 *Calimico goeldi,* 3 *Saguinus m. midas*, 1 *Lagothrix lagotricha*, 1 *Erytrocebus pata*, 2 *Ateles b. marginatus*, 1 *Ateles peniscus*, 4 *Saimiri sciureus,* 1 *Saimiri fuscicollis*, 1 *Leontopitecus chrysopygus*. These species are usually found in the Amazon Forest and Atlantic Rainforest (*Mata Atlântica*), in Brazil. These animals came from donations or rescues and were apprehended and sent to several ecological and Zoological Parks in São Paulo, Brazil where they were maintained in captivity. A total of 117 samples of chicken plasma (*Gallus g. domesticus*) of the following lineages: white, brown leghorn and hens of yard, of both sexes from different poultry farms in the state of São Paulo, SP, Brazil, were collected from May to September 2001. Samples were kept at -80ºC until testing.

Extraction of nucleic acids and amplification by PCR:

Blood DNA was extracted using a DNA Extraction Kit (Qiagen) from 100 µl total blood. DNA was resuspended in 100 µl buffer. Serum DNA (50 µl) was mixed with 100 µl Dnazol and 3 µl Dextran T500 (1µg/µl). The DNA was precipitated with isopropanol and resuspended in 25 µl distilled water.

Heminested N22 PCR:

In the first round, outer primers NG059 and NG063 (sequences 5’ ACA GAC AGA GGA GGG AAC ATC – 3’ and 5’ – CTG GCA TTT TAG CAT TTC CAA AGTT – 3’) were used.

PCR was carried out in 50 µl volumes, using 5 µl of DNA, 10 pmol of NG059 and NG063 primers, 1.5 mM $MgCl_2$ and 5U Taq polymerase.

In the second round, semi-inner primer NG061 (sequence 5' – GGC AAG ATG YTR TGG ATA GAC TGG – 3') and the outer primer NG063 were used.

Five µl of the first PCR product and 10 pmoles/µl of NG061 and NG063 primers were used for the second PCR. Amplifications conditions were 94ºC 30s, 55ºC 30s, 72ºC 30s, 35 cycles, with a final extension at 72ºC 7 min.

The amplification products of the first PCR round were 286 pb, and those of the second round were 271bp. It was considered positive for the TTV control, human serum from a TTV positive patient, and negative, ultra pure sterile water, free from inhibitors or contaminants, which could avoid amplification of PCR products. Amplified products were separated by electrophoresis through a 2% agarose gel containing ethidium bromide, and visualized under UV light.

Sequencing

Sequence reactions were performed with the ABI Prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems). Cycle sequencing was carried out with an automatic DNA Sequencer (ABI Prism 377 Sequencer – Applied Biosystem). The primers used were NG061 and NG063 of the N22 region. Sequence edition was performed using the Sequencher program 4.0.5 (Gene Code Corporation). Sequence alignments were generated by the Bioedit Program (Hall, 1999). The phylogenetic analysis was performed using Philip version 3.5 c (Felsentein, 1993).

Alignment with 3 edited sequences from the N22 region and series of standard sequences from different TTV genotypes from Gen Bank and from the literature were performed.

The sequence alignments were assessed using 1000 bootstrap replicates using SEQBOOT Program (Felsentein, 1993). Phylogenetic trees were constructed by the neighbor-joining method (Saitou & Nei, 1997). The consense tree was calculed using the Consense Program and visualized in the Treview Program version 1.6.6 (win 32) (Roderic, 2001). The genotypes of the TTV had been determined by comparing the lined sequences (GenBank accession numbers EU919749-EU919752).

RESULTS

Non-human primates.

Of 160 samples obtained from different species of new world non-human primates (90 serum and 70 whole blood) analyzed by the semi-nested PCR reaction for the N22 region only 4 samples of total blood were amplified, generating a band with a 271 pb fragment in the second round. No serum sample was amplified.

TTV N22 DNA was detected in total blood from 3 (4%) of 75 brown capuchin (*Cebus apella*) and from 1 (25%) of 4 golden-headed-lion-tamarin (*Leontopithecus chrysomelas)*. Among these 2 (50%) were males and 2 (50%) female, all adults (table 1).

Phylogenetic analysis revealed that one sample showed similarity with one sequence of the cotton top tamarin (*Saguinus oedipus)* (So-TTV2) and with one of the douroucoulis (*Aotes trivirgatus)* (At-TTV3). Two samples showed similarity with a human Torque Teno Mini Virus (TLMV). The other sample clustered with one sequence of the chimpanzee (Pt-TTV6) and with the human TTV strain TA278 (figure 2).

Gallus g. domesticus.

The 117 plasm samples tested by the semi-nested PCR reaction for the **N22** region were all negative.

DISCUSSION

The diagnostic of TTV infection is frequently realized by DNA amplification by PCR utilizing primers designed or by coding region of the ORF 1 (N22) or by non-coding region (UTR) of the viral genome (Ott et al., 2000).

The phylogenetic analysis of the TTV has been based on a small genome region that exhibits extensive sequence heterogeneity (Lemey et al., 2002).

Our results obtained by the semi-nested PCR reaction for the N22 region revealed amplification in 4 (5.7%) out of the 70 samples of total blood of new world non-human primates. However, no amplification was observed in 117 serum of the animals submitted to the same reaction.

On the contrary, other studies did not obtain positivity by the PCR of the N22 region, in samples of non-human primates of the old and new world (Abe et al., 2000; Pujol et al., 2005), not even in pig, cat and dog samples (Okamoto et al., 2002).

Studies in the literature have demonstrated variations in positivity percentage in samples of the old world non-human primates. Nopporpanth et al. (2001) detected 9 (13.4%) of 67 gibbons, Tom et al. (2003) 1 (20%) of 5 chimpanzees, Romeo et al. (2000) 2 (25%) out of 8 naive chimpanzees, Verschoor et al. (1999) 60 (48,8%) out of 123 chimpanzees e 4 (66,7%) out of 6 pygmy chimpanzees, Barnett et al (2004) 4 (80%) of 5 chimpanzees of the Congo community and 3 (100%) of 3 chimpanzees of the Sonso community of the Budongo Forest Reserve of Uganda.

Using specific primers for each species of chimpanzee, Okamoto et al (2000a) detected 57% in 49 out of 104 chimpanzees, but they did not obtain positivity in any sample that they have tested with new world non-human primates.

No other study in the literature has detected the presence of DNA TTV in new world non-human primates by the N22-PCR.

Okamoto et al. (2000a) e Leary et al. (1999), however, detected DNA TTV in new world monkeys by PCR of the non-coding region (UTR), since the primers used in the N22-PCR only allow the amplification of a restricted number of TTV genotypes (Bendinelli et al., 2001; Vasconcelos et al., 2002).

In this study DNA TTV was detected in 3 samples of *Cebus apella* and in 1 sample of L*eonthopithecus crysomelas*. Abe et al. (2000) however, had detected DNA TTV in none of the 9 *Cebus apella* samples submitted to N22-PCR.

Cebus apella is the greatest specie, largely distributed in South America, present in all Amazon Forest, swamp, woodsy pasture, upland caatinga and Atlantic Rain Forest a (Silva Júnior, 2002).

In our study one sequence of the *Cebus apella* clustered with one strain isolated from *Saguinus oedipus* (So-TTV2) and other with from *Aotes trivirgatus* (At-TTV3) of the Okamoto et al. (2000b).

Okamoto et al. (2000b) performed by cloned from UTR-PCR products representing the full-length TTV genomes obtained from sera of humans and non-human primates that showed common genomic organization with two open reading frame (ORFs), designated ORF1 and ORF2 and their sequences were determined. In accordance with the author, these sequences bifurcated from the same branch harboring Japanese macaque TTV, reflecting a close relationship of these lower classes non-human primates TTVs.

Our results showed that one sequence clustered with a human TLMV (Torque Teno Mini Virus) sequence.

Furthemore, TTV and TLMV DNA sequences have been found in non-human primates and farm animals (Verschoor et al., 1999; Abe et al., 2000; Leary et al., 1999; Okamoto et al., 2000 a, b; Romeo et al., 2000).

TLMV is a unique virus with a smaller genome that may constitute a genetic evolutionary link between chicken anemia virus and TTV (Takahashi et al., 2000a).

In this study, 1 amplified sample (*Cebus apella*) was grouped with one strain of chimpanzee (Pt-TTV6) (Okamoto et al., 2000b) and with the TA278 human prototype strain.

A close proximity was observed, between the gibbon virus and those detected in Thai individuals, whereas that, chimpanzee strains were phylogenetically more remote (Noppornpanth et al., 2001).

According to Verschoor et al. (1999) TTV from pygmy chimpanzees and the common chimpanzees closely to viruses from human TTV and from the common

chimpanzees subspecies *Pan troglodytes verus* and *Pan troglodytes schwein furthii* cluster together, suggesting an ancient host-pathogen relationship before sub speciation 1.6 million years ago and TTV of common and pygmy chimpanzees may have been acquired by these animals in different zoonotic events not longer than 2.5 million years ago.

Abe et al. (2000) reported one study that all TTV isolates obtained from simians (s-TTV) (old world non-human primates) were clearly distinct from TTV found in humans.

Inami et al (2000) stated that a strain CH65-1 in ORF-1 region showed only 35% identity to the prototype TA278 human isolate at the amino acid level.

The results of our research showed that the sequence of DNA TTV obtained of the *Cebus apella* (Ca-TTV17) were close to human TTV of the genotype1.

In the same manner, gibbons sequences (Nopporpanthy et al., 2001) and in pygmy chimpanzees Okamoto et al. (2000ab) were grouped in genotype 1.

Verschoor et al. (1999) however, reported that TTV sequences from pigmy chimpanzees are closely related to viruses from human genotypes 2 and 3, while that sequences obtained from common chimpanzees were genotypes 5 and 6, the latter only at the protein level.

The phylogenetic analyses of the ORF1 nucleotide consensus sequences suggested that TTV recovered from two chimpanzees (CH1304 and CH 1545) represented two new genotypes (Romeo et al., 2000).

Simian TTV (s-TTV) was further divided into three genotypes and showed type 1 as the major genotype (Abe et al., 2000). In this study, it was not possible to detect DNA TTV by N22-PCR in 117 samples chicken plasma.

Only two reports refer to TTV research in chicken by PCR of the non-coding region (UTR). Thom et al. (2003) had investigated the presence of virus in 29 chicken samples, as well as the 20 samples from cows, 20 from sheep and 20 from goats, but all were PCR-UTR negative. Also, Leary et al (1999) detected the virus in various farm animal species (chickens, pigs, cows and sheep) and verified that 19% of chickens were positive by PCR-UTR, whose sequences clustered with human TTV.

This different result is due to the fact of using primers deduced from a coding region sequence (N22).

The primers of the N22 region only allow the amplification of a restricted number of TTV genotypes (Bendinelli et al., 2001; Vasconcelos et al., 2002).

Our results indicate that in spite of the TTV has been detected worldwide in wide scale in various animal species, in Brazil, however, their presence in new world non-human primates, in captivity and in chickens occurs in low number.

All the animals were clinically healthy, but very little is known, however, about the TTV infection of new world primates. The virus might have been transmitted from human handler to animals in the process of feeding or the wise handling the monkeys.

According Luo & Zhang (2001) TTV is transmitted both by blood and enteric routs and perhaps in areas with inadequate hygienic standards, the virus is predominantly spread via the fecal-oral route.

The amino acid sequences reported in this study are the first obtained in Brazil from total blood of non-human primates naturally infected by TTV. The amino acid identity between human and non-human primate sequence in genotype 1 observed in this study supports the evidence that TTV is a zoonotic agent.

Further studies to evaluate the pathology, genetic diversity and host range, transmission, and persistence of TTV infection, in human and animal species will be conduced in order to outline considerable conclusions.

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Figure Legends

TABLE 1: Description of TTV positive samples by PCR of N22 region according to the species, gender, age and captivity origin. M= male, F= female.

FIGURE 1: Comparison between TTV N22 aminoacid sequences obtained from Brazilian non-human primates and sequences isolated from humans: TA278 (ABO17610) (Okamoto et al., 1999), TGP96 (AB041962) (Okamoto et al., 2000b), SANBAN (AB025946) (Hijikata et al., 1999), TLMV-CDB203 (AB026929) (Takahashi et al., 2000a), TLMV-CDB231 (AB026930) (Takahashi et al., 2000a), TUS01 (AB017613) (Okamoto et al., 1999), non-human primates: MfTTV3 (AB041958) (Okamoto et al., 2000b), MfTTV9 (AB041959) (Okamoto et al., 2000b), PtTTV6 (AB041957) (Okamoto et al., 2000b), Pt-TTV8II (AB041963) (Okamoto et al., 2000b), SoTTV2 (AB041960) (Okamoto et al., 2000b), AtTTV3 (AB041961) (Okamoto et al., 2000b) and chickens: CAV/U65414 (U65414) (Hamooleh et al., 1996), M55918 (M55918) (Noteborn et al., 1991). Isolates determined in this study are presented in bold. Dots indicate aminoacid identities, whereas dahes indicate gaps.

FIGURE 2: Phylogenetic relationship between TTV-N22 aminoacid sequences obtained from Brazilian non-human primates and sequences isolated from humans, non-humans primates and chickens. The phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1997). Isolates determined in this study are presented in bold. The database-derived isolates and their accession numbers is given in the legend to fig. 1.

Table 1:

№ sample	Specie	Sex	Age	Origin
14	*Cebus apella*	F	A	Parque Ecológico do Tietê, SP
17	*Cebus apella*	F	A	Parque Ecológico do Tietê, SP
19	*Cebus apella*	M	A	Parque Ecológico do Tietê, SP
45	*Leontopithecus chrysomelas*	M	A	Zoo Sorocaba, SP

FIGURE 1:

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   5         15         25         35         45         55
TGP96          QGFSPYEQTC LPEDITRWYP M--YQYQTRE QNKILQTGPF TPYIQNLQSE NLCMYYKFRF
Mf-TTV3        TGNKVWIDPV TKKDTKFQPP Q--SLVLLEG QPLWLLLFGY TDWIKKFYA- DRNPGTTYRV
Mf-TTV9        IGNVVWTEPL TKKTCDYASQ ---AYNVIKD APLYLALFGY IDICSKLAK- DKSFYLSNRV
Pt-TTV6        TGNKIWCDSL TKADMKYTEG --RSKYLIEN LPLWAAVWGY LDYCTKTSG- DAAFHYNYRV
Pt-TTV8-II     DGQSPYTEEL DPTDKQNWHP M--VQYQQQT INNILTCGPG TPKYNGKNTV EAKLEYSFKF
TTV SANBAN     VGNHVWFQYN TKADTQLIVT GGSCKAHIED IPLWAAFYGY SDFIESELGP FVDAETVGLI
So-TTV2        REDQGWGNKV RLWTRECRTD IPEETLGIEN MPLYVLMNGY IDYVTNHST- --HSPLNWVV
At-TTV3        NRFAICDWPY KDQHGEQTTP LN-FDLEIKD APLWWCVTAA YDWAIRQGK- ---NPANWSF
TLMV-CBD203    TGQSPYIEVL SQSDEKHWYP K--GSFQIKT LNTITSCGPG TVKLDNDKSC EAHFEYDFRF
TLMV-CBD231    PEGQLYKTEL SETDKVHWHP K--YSMQTEQ LELISETGPA APKINNTKQI EAHLNYDFLF
TUS01          KGNKIWFQYL SKKGTDYNEK --QCYCTLED MPLWAICFGY TDYVETQLGP NVDHETAGLI
TA278          EGNMLWIDWL SKKNMNYDKV --QSKCLISD LPLWAAAYGY VEFCAKSTG- DQNIHMNARL
Lc-TTV45       ---MLWIDWL TKDDSQYSKT --QSKCLIEN LPLWASVYGY TEYCSKVTG- DTNIEHNCRC
Ca-TTV14       SGILPFPK-L EFRN--MWSL R--GAIPSPS LS-ITCC--- ---------- ----------
Ca-TTV17       ---------I ASRG------ ------RVPE VSLHTGVKGQ FGL------- ----------
Ca-TTV19       SGILPFPK-L EFRN--MWSL R--GAIPSPS LS-ITCCH-- ---------- ----------
CAU65414       QRDPDWYRWN YNHSIAVWLR -----ECSRS HAKICNCGQF RKHWFQECAG LEDRSSQASL
M55918         QRDPDWYRWN YNHSIAVWLR -----ECSRS HAKICNCGQF RKHWFQECAG LEDRSTQASL

               ....|....| ....|....| ....|....| ...
                   65         75         85
TGP96          QWGGSPPKTV NVENPSHQNI FPIPRNEHET TSL
Mf-TTV3        TLLSPWTYPK LTN--KDLYG YVPLGDDFCA GRH
Mf-TTV9        CVKCPYTVPQ LLSKTNATLG HVILSENFMR GLV
Pt-TTV6        TLISPYTSPM LFNPQDPTKG FVPYSLNFGL GKM
Pt-TTV8-II     KFGGCPPPMA ALEDPCKQPT YQIPQT---T TML
TTV SANBAN     CVICPYTKPP MYNKTNPMMG YVFYDRNFGD GKW
So-TTV2        SVFCPYTDPP MTNVIPVGKD WFIQNVEPGE NKY
At-TTV3        FVRSDYTWPR ADNIILAYSS Y--FADNVVK NKY
TLMV-CBD203    KIGGCPPAME KLCDPSKQNK YPIPNTKLQT TSL
TLMV-CBD231    KWGGSPAPME AITDPAEQEK FPSPSNQLQG LQI
TUS01          IMICPYTQPP MYDKNRPNWG YVVYDTNFGN GKM
TA278          LIRSPFTDPQ LLVHTDPTKG FVPYSLNFGN GKM
Lc-TTV45       VIRSPYTVPQ LLDHNNPLRG YVPYSFNFGN GKM
Ca-TTV14       ---------- ---------- ---------- ---
Ca-TTV17       ---------- ---------- ---------- ---
Ca-TTV19       ---------- ---------- ---------- ---
CAV/U65414     EEAILRPLRV QGKRAKRKLD YHYSQPTPNR KKV
M55918         EEAILRPLRV QGKRAKRKLD YHYSQPTPNR KKA
```

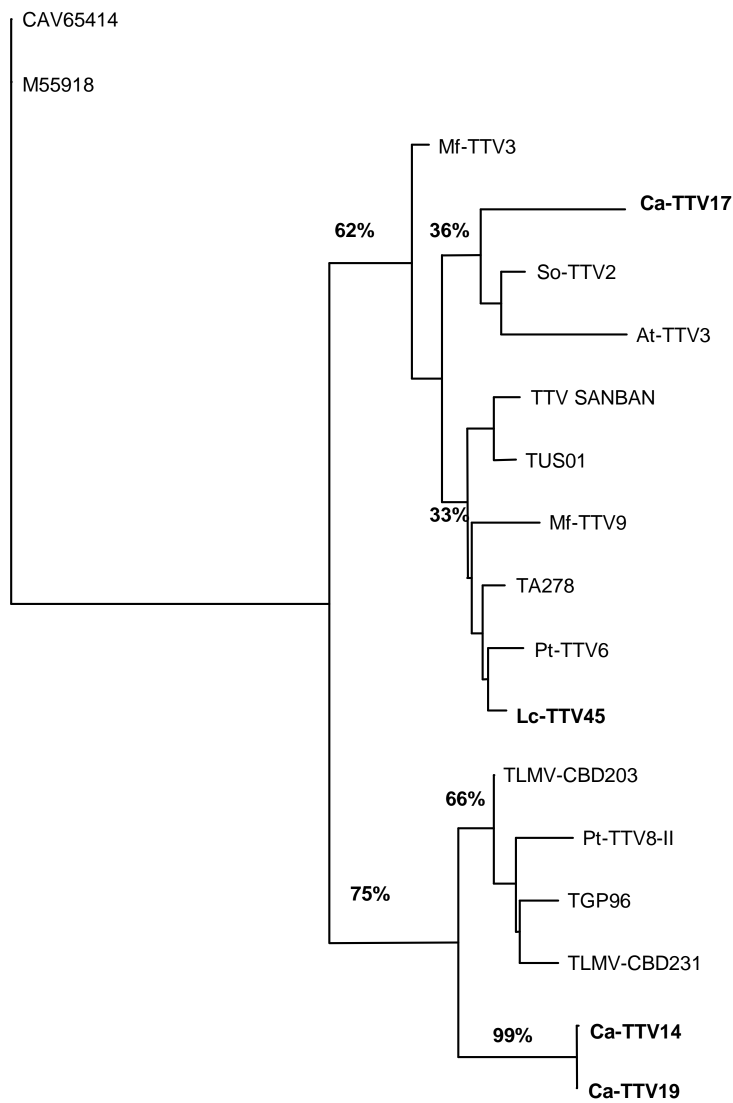

Fig. 2- Phylogenetic relationship between TTV-N22 aminoacid sequences obtained from Brazilian non-human primates and sequences isolated from humans, non-humans primates and chickens. The phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1997). Isolates determined in this study are presented in bold. The database-derived isolates and their accession numbers is given in the legend to fig. 1.

3. Discussão

Através da aplicação da Nested-PCR-UTR o DNA do TTV foi detectado no soro de 11 amostras de primatas não humanos do novo mundo, sendo o macaco-prego a espécie mais prevalente. A análise filogenética revelou que as seqüências detectadas em 8 amostras apresentaram maior identidade com as seqüências de TTV isoladas de macacos japoneses do novo mundo. Três seqüências de primatas não humanos mostraram similaridade com uma de Torque Teno Mini Vírus (TTMV) de humano. O DNA do TTV da região ORF2 foi detectado em duas amostras de primatas não humanos e em uma de frango doméstico de corte. As seqüências amplificadas pela região ORF2 não mostraram diferenças entre as de humano, primatas não humanos e de frango doméstico. Pela Semi-Nested-PCR da região codificadora (N22), o DNA do TTV foi detectado no sangue total de 4 primatas não humanos. A análise filogenética revelou que uma amostra de primata não humano agrupou-se com seqüências de primatas não humanos japoneses do novo mundo; duas amostras mostraram similaridade com uma seqüência de Torque Teno Mini Vírus (TTMV) de humanos e outra com uma seqüência de chimpanzé e com uma seqüência de TTV de humano.

Interessantemente, somente dois estudos detectaram o DNA do TTV no soro de primatas não humanos do novo mundo, pela PCR da região UTR. O primeiro obteve positividade de 23,5% no soro de *Sagüínus labiatus* e 20% em *Aotus trivirgatus*, enquanto que o outro estudo obteve 100% em 4 *Sagüínus labiatus*, 83% de 6 *Sagüínus oedipus* e 100% de 5 *Aotes trivirgatus* (Leary et al., 1999; Okamoto et al., 2000a). Pela PCR da N22 nenhum outro estudo obteve positividade em amostras de primatas do novo mundo (Abe et al., 1999; Okamoto et al., 2000a; Pujol et al., 2005). Nenhuma referência foi encontrada na literatura quanto à presença do TTV em amostras de frangos domésticos de corte, pela PCR da região ORF2. Utilizando PCR da UTR, Thom et al. (2003) verificaram que todas as amostras de soro de frangos domésticos que estudaram eram todas negativas, enquanto que Leary et al. (1999) encontraram 19% de positividade em 4 de 21 amostras de frangos domésticos que submeteram à PCR da UTR.

Ressalta-se que, oito das 11 amostras caracterizadas em nosso estudo, amplificadas pela UTR e duas pela N22, agruparam-se na análise filogenética com 2 cepas isoladas de primatas não humanos do novo mundo (Okamoto et al., 2000a), sugerindo que embora algumas cepas de primatas não humanos sejam similares àquelas encontradas em humanos (Leary et al., 1999), algumas podem ser específicas para primatas não humanos (Okamoto et al., 2000a). Embora alguns estudos mencionem que a infecção com ambos TTV e TTMV tenha sido detectada em uma ampla gama de primatas não humanos do velho mundo (Verschoor et al., 1999; Abe et al., 2000; Okamoto et al., 2000 a, b; Romeo et al., 2000; Thom et al., 2003), os resultados de nosso estudo mostram que esta é a primeira ocorrência de TTMV em primatas do novo mundo. Da mesma maneira, nossos achados constituem o primeiro relato da detecção do TTV em amostra de frango doméstico, pela região ORF2, cuja seqüência mostrou na análise filogenética, similaridade com uma cepa de humanos, em concordância com o estudo de Leary et al. (1999). Curiosamente, uma seqüência obtida de amostra de macaco-prego, amplificada pela região N22, mostrou similaridade com uma seqüência de humanos do genótipo 1, resultado também obtido em amostras de gibons e de chimpanzés (Okamoto et al., 2000a, b; Nopporpanth et al., 2001); a identidade entre seqüências de humanos e de primatas não humanos, sugere que o TTV seja um agente zoonótico.

O estudo das infecções virais em primatas não humanos se reveste de extrema importância tanto para a preservação da biodiversidade quanto para a prevenção de epidemias de grande impacto em saúde humana (Pujol et al., 2006). Os resultados de nosso estudo, portanto, poderão ser de grande valia e contribuir em futuras pesquisas para elucidar diversos aspectos relativos ao ciclo de disseminação e patogenicidade do Torque Teno Vírus, tanto na espécie humana quanto em outras espécies animais.

4. Conclusões

1. Pela nested-PCR dirigida a região não codificadora (UTR) o DNA do TTV foi detectado no soro de 4 (5,3%) de 75 *Cebus apella*, 2 (40%) of 5 *Alouata fusca*, 1 (20%) of 5 *Alouata caraya*, 1 (5.2%) of 19 *Callithrix penicilata*, 1 (4%) of 25 *Callithrix jacchus*, 1 (20%) of 5 *Saimiri sciureus* e 1 (25%) of 4 *Leontopithecus chrysomelas*. Não se obteve amplificação por PCR em nenhuma amostra de sangue total.

2. A maior prevalência de TTV foi encontrada em *Cebus apella* (macaco-prego) (4 de 75 – 5,3%), espécie de maior distribuição nas Américas.

3. Não foi detectado o TTV por PCR-UTR em nenhuma amostra de plasma de frangos domésticos de corte (*Gallus g. domesticus*).

4. Obteve-se a amplificação da região ORF2 do TTV em três amostras analisadas: uma de *Cebus apella* (macaco-prego), uma de *Callithrix jaccus* (sagüi-de-tufo-branco) e uma de frango doméstico de corte (*Gallus g. domesticus)*.

5. A análise filogenética revelou que as seqüências detectadas em 8 amostras apresentaram maior identidade com as seqüências de TTV isoladas de primatas-não humanos japoneses do novo mundo (So-TTV2 de *Sagüínus oedipus* e At-TTV3 de *Aotes trivirgatus*), sendo que três seqüências (uma de *Callithrix penicilata*, uma de *Leontopithecus crysomelas* e uma de *Cebus apella*) mostraram similaridade com TTMV (Torque Teno Mini Vírus) de humanos. As seqüências amplificadas pela região ORF2 não mostraram diferenças entre as de humanos, primatas não humanos e de frangos domésticos.

6. Pela semi-nested PCR dirigida a região codificadora (N22) o DNA do TTV foi detectado no sangue total de 3 (4%) de 75 *Cebus apella* e de 1 (25%) de 4

Leontopitecus crysomelas. Não se obteve amplificação por PCR de nenhuma amostra de soro.

8. A análise filogenética mostrou que uma amostra obtida a partir de primata não humano (*Cebus apella*) agrupou-se com seqüências de TTV isoladas de primatas não-humanos japoneses do novo mundo: (So-TTV2 - *Sagüínus oedipus* e At-TTV3 -*Aotes trivirgatu*s); duas amostras de *Cebus apella* com TTMV de humanos e uma de *Leontopitecus crysomelas,* mostrando similaridade com uma cepa de chimpanzé e com uma cepa protótipo humana denominada TA278.

9. Não se obteve amplificação por PCR-N22 de nenhuma amostra de plasma de frangos domésticos de corte (*Gallus g. domesticus*).

10. Os resultados apresentados mostraram que este é o primeiro relato da ocorrência de Torque Teno Vírus (TTV) e de Torque Teno Mini Vírus (TTMV) em primatas não humanos do novo mundo e em frangos domésticos de corte (*Gallus g. domesticus*), no Brasil.

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