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Authors: Barnett, Oliver E., Worobey, Michael, Holmes, Edward C., and Cooper, Alan

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DETECTION OF TT VIRUS AMONG CHIMPANZEES IN THE WILD USING A NONINVASIVE TECHNIQUE

Oliver E. Barnett,[1,3] Michael Worobey,[2] Edward C. Holmes,[2] and Alan Cooper[2]

[1] Department of Biological Sciences, The Open University, Walton Hall, Milton Keynes, Buckinghamshire MK7 6AA, UK

[2] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

[3] Corresponding author (email: O.E.Barnett@open.ac.uk)

ABSTRACT: Zoonotic transmission and emergence of pathogens are serious threats to endangered populations of free-ranging primate species. Recent discovery of a nonpathogenic yet highly prevalent virus in human populations, TT virus (TTV), has prompted studies into the presence of this virus among captive individuals of other species of nonhuman primates. In this study, we screened captive primate species for TTV. In addition, we provide the first data on TTV infection in free-ranging primates by noninvasive screening of three chimpanzee (*Pan troglodytes sweinfurthii*) communities. Phylogenetic relationships between virus isolates and those previously reported from human populations, captive primates, and domesticated species are inferred. Our findings are discussed with respect to potential zoonotic events that may result from increased levels of human encroachment into wild habitats.

Key words: Chimpanzee, nonhuman primates, *Pan troglodytes sweinfurthii,* survey, TT virus.

INTRODUCTION

Threats to endangered primate species are well documented and frequently stem from a single common factor: human encroachment into habitats of free-ranging populations (Bowen-Jones and Pendry, 1999; Dupain et al., 2000). Whether this encroachment is in the form of habitat destruction, hunting for the bush meat trade, or fragmentation of depleted populations, there is an increased level of contact between humans and other primate species.

Such increased interaction has been shown to greatly elevate the occurrence of zoonotic events where pathogens are transmitted to novel hosts from other "reservoir" species (Warren et al., 1999). For example, studies on parasite levels of chimpanzees (*Pan troglodytes*) around the Gombe National Park found a strong correlation between parasite diversity and prevalence with the proximity of chimpanzee communities to humans (Wallis and Lee, 1999). The majority of emergent pathogens observed in free-ranging populations stem from humans (Dobson and Foufopoulos, 2001), and such anthropozoonotic transmission can have devastating effects on depleted populations. Emerging infectious diseases are considered a serious threat to endangered and threatened primate species (Wolfe et al., 1998), and almost two thirds of human diseases are considered potentially zoonotic (Cleaveland et al., 2001). Although the exchange of pathogens from an abundant host to other closely related species is a predictable scenario (Woodroffe and Ginsberg, 1999), diseases that are nonpathogenic in the original host may be highly pathogenic in the "spillover" population (Wallis and Lee, 1999).

There is a lack of primary data about risk of human pathogens to threatened primates (Wolfe et al., 1998; Woodroffe and Ginsberg, 1999; Jones-Engel et al., 2001). Recorded examples of pathogen transmissions to free-ranging populations include the transfer of influenza and parainfluenza-1 from humans to free-ranging macaques (*Macaca tonkeana*) in Sulawesi, Indonesia (Jones-Engel et al., 2001), and canine distemper virus from domestic dogs to Serengeti lions (*Panthera leo*; Roelke-Parker et al., 1996). However, little is known of the species at greatest risk, the diseases most capable of crossing the species barrier, or the frequency at which such exchanges occur. Similarly, it is generally unknown which pathogens might be

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expected in a normal healthy population (Ferber, 2000).

TT virus (TTV) is an ideal virus for the study of pathogen transmission between primate species. It is a single-stranded, unenveloped DNA virus with a circular genome (Nishizawa et al., 1997) resulting in its tentative designation into the family *Circinoviridae* (Mushahwar et al., 1999). The virus particles are excreted into the bile and subsequently shed in feces of infected individuals (Okamoto et al., 2000a). Consequently, TTV can be transmitted both parentally and via the fecal-oral route. The dual mode of transmission enhances the spread of infection through a community (Okamoto et al., 2000b), making zoonotic events detectable with minimum levels of sampling. Also, since TTV can be detected in feces via polymerase chain reaction (PCR; Okamoto et al., 2000b), noninvasive sampling is possible, minimizing unnecessary contact and social disruption of wild communities and permitting multiple sampling of both captive and free-ranging individuals.

At present, all TTV genotypes are thought to be nonpathogenic in humans (Leary et al., 1999), although it is unclear whether this is always the case or if it holds true for nonhuman species. Until recently it was thought there was large variation in prevalence among human populations (e.g., 92% in Japan [Takahashi et al., 1998] and 16.4% in Colombian native Indians [Tanaka et al., 1999a]). However, contemporary thinking based on the work of several research groups is that infection is almost, if not completely, universal (Thom et al., 2003). The 28 or more reported isolates possess levels of sequence divergence in excess of 30% (Simmonds et al., 1998; Okamoto et al., 1999a; Tanaka et al., 1999b). Indeed, further classification applied to those strains exhibiting >50% divergence has identified four distinct genetic groups common still in their genome organization (Thom et al., 2003).

Studies into the zoonotic exchange of TTV have so far been carried out using captive primates and domesticated farm animals such as sheep, chicken, and pigs (Leary et al., 1999; Okamoto et al., 2000a; Abe et al., 2000), illustrating the large number of species TTV can potentially infect. Furthermore, Okamoto et al. (2002) highlighted the diversity of such strains among individuals representing several domesticated species. Chimpanzee-specific strains have been described by Okamoto et al. (2000b), as well as a simian-specific strain detected in captive crab-eating macaques (*Macaca fuscicularis*) and chimpanzees (Inami et al., 2000). In this study, we investigate zoonotic transmission of TTV between humans and nonhuman primates of both free-ranging and captive origin within varying degrees of human interaction.

MATERIALS AND METHODS

Samples

Fecal samples were collected from two chimpanzee (*Pan troglodytes sweinforthii*) communities thought to be adjacent to one another within the Kisingani region of the Democratic Republic of Congo. A total of 11 samples were collected from one of the Congonese communities, Congo community 1 (CC1), whereas five were collected from the second Congo community (CC2). Samples were also taken from three members of a habituated chimpanzee community, the Sonso community, within the Budongo Forest Reserve of Uganda, and also from a number of primate rehabilitation centers and wildlife parks in Chile and the UK.

The Sonso community is one of three habituated groups within the Budongo Forest Reserve, and it is regularly observed at close hand for behavioral studies. Consequently, these individuals have experienced elevated levels of human contact. Members of the two Congonese communities are not expected to have experienced human contact at levels comparable with the Sonso community. However, the rise in hunting for the bush meat trade across tropical Africa (Dupain et al., 2000; Bowen-Jones and Pendry, 1999) and the overlapping ranges of humans and chimpanzees within the Kisingani region is likely to have exposed them to considerable levels of human contact.

Samples obtained from the Centro de Rehabilitation de Primates in Santiago, Chile, included 18 samples from seven New-World spe-

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cies: woolly monkey (*Lagothrix lagothricha*), grey woolly monkey (*Lagothrix* l. *cana*), black spider monkey (*Ateles paniscus*), moustached tamarin (*Saguinus mystax*), squirrel monkey (*Saimiri scuireus*), tufted capuchin (*Cebus apella*), and black howler monkey (*Alouatta caraya*).

The Monkey Sanctuary in Looe, Cornwall, UK, houses a colony of woolly monkeys, and samples were obtained from five individuals. The Cotswold Wildlife Park, Oxon, UK, similarly provided samples from 10 individuals spanning nine species: siamang (*Hylobates syndactylus*), squirrel monkey (*Saimiri scuireus*), lar gibbon (*Hylobates lar*), Geoffroy's tufted-eared marmoset (*Calthrix geoffroyi*), red-bellied tamarin (*Saguinus labiatus*), black tamarin (*Saguinus midas*), cotton-top tamarin (*Saguinus oedipus*), black and white ruffed lemur (*Varecia* v. *variegatus*), and brown-headed spider monkey (*Ateles fusciceps*).

All samples were stored in RNA later storage buffer (Ambion, Austin, Texas, USA), except for those from the Budongo Forest Reserve, which were stored in Falcon tubes containing silica beads. Care was taken to prevent cross-contamination of samples by sterilizing apparatus and changing gloves between each sampling.

DNA extraction and PCR amplification

Viral nucleic acids were extracted from fecal samples of 52 individuals representing 14 primate species using a QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, California, USA). The manufacturers' protocol for optimizing extraction of pathogen DNA was employed, and the extracted nucleic acids were stored in 200 μl of storage buffer. To control for the possibility of operator contamination and sample cross-contamination, the sample set was split into extraction batches, each of which were comprised of six samples and two extraction blanks.

Several attempts were made at amplifying TTV DNA using single round PCR. However, successful amplification was only possible through careful optimization of a nested PCR protocol. Two sets of nested primers (designated B and C), each consisting of an external and internal primer pair, have been reported for adjacent 220 base pair segments of an untranslated region (UTR) of the TTV genome (Leary et al., 1999). A third and larger fragment (A), consisting of both segments B and C, is defined by the 5′ primers of set B and the 3′ primers of set C. Previous studies have demonstrated these primers can detect the most divergent known strains of TTV. Leary et al. (1999) showed set B detected 76.3% of positive samples examined, whereas set C detected 60.5%. When samples were screened with both nested primer sets, 94.7% of TTV positives were identified (Leary et al., 1999).

Polymerase chain reaction cocktails and cycling parameters were modified from those given by Leary et al. (1999). Initial amplifications were performed on 25-μl volumes using 1× PCR reaction buffer (ABgene, Surrey, UK), 2.0 mM magnesium chloride, 1.0 μM each external primer, 2 mg/ml bovine serum albumin (Sigma, St. Louis, Missouri, USA), 1.5 U Taq polymerase (ABgene), and 2.0 μl of extracted nucleic acids. Reamplification reactions used 0.5 μM of each internal primer, 0.9 U Taq polymerase, and 1.0 μl of the initial amplification reaction as a template. Other parameters were as above. Cycling parameters for both rounds were the following: extended denaturation phase of 94 C for 2 min, followed by 35 cycles of 94 C for 20 sec, 57 C for 30 sec, 72 C for 30 sec, and a final extension of 72 C for 10 min. The annealing step was altered to 55 C for 40 sec for reactions using the larger primer set A.

Molecular cloning, sequencing, and phylogenetic analysis

Products obtained from nested PCR were characterized on 2% GTG® agarose gel (NuSieve, East Rutherford, New Jersey, USA) and amplified products purified using a QIAquick™ PCR Purification Kit (Qiagen). Of the A, B, and C UTR segments, only B could be amplified from all identified TTV-positive individuals. To screen these individuals for possible infection with multiple strains, and as an added control for operator contamination, PCR products of the TTV B segments were cloned. Purified products were ligated into pGEM®-T Easy Vector (Promega, Madison, Wisconsin, USA) and used to transform *Escheria coli* JM109. The resulting cloned products were sequenced using Big Dye (Applied Biosystems, Foster City, California) and analyzed using an ABI 310 automated sequencer.

The phylogenetic positioning of strains detected in this study was investigated using the 29 homologous sequences available from GenBank (http: //www.ncbi.nlm.nih.gov/Genbank/). These include all known genotypes from humans, other primates, and domesticated species for which UTR sequences are available. Sequences were aligned using the ClustalX program (Thompson et al., 1997). Maximum-likelihood (ML) phylogenetic trees were estimated for each of the three UTRs under the general time-reversible (GTR) substitution model with invariant sites and a gamma model of among-site rate variation. The val-

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TABLE 1. Infection status of chimpanzees from populations known to be infected with TT virus (TTV). Also listed is the geographic origin of each individual and amplification status at each of the three targeted regions of the TTV genome (regions B, C, and A).

Sample identification	Origin	Region B	Region C	Region A
Katia	Sonso community	+[a]	−[a]	−
Bwoga	Sonso community	+	−	−
Duane	Sonso community	+	−	−
E5	CC2	+	+	+
E7	CC2	+	+	+
E13	CC2	+	+	+
E22	CC2	−	−	−
E24	CC2	+	+	+

[a] +=amplification positive; −=amplification negative; CC2 = Congo community 2.

ues of each parameter were estimated from the empirical data (parameter values available from the authors on request). The support for each node of the tree was estimated using bootstrap resampling (1,000 replications) under the maximum likelihood substitution model described above. All phylogenetic analyses were performed using the PAUP* 4.0b package (Swofford, 2000).

RESULTS

TT virus DNA was detected in seven samples from two free-ranging chimpanzee communities (100% of the Sonso community of the Budongo Forest Reserve and 80% of CC2; Table 1). The 11 samples taken from CC1 revealed no TTV DNA. Similarly, all 18 samples representing seven new-world species, collected from the Centro de Rehabilitation de Primates in Santiago, Chile, and all five samples collected from the Monkey Sanctuary in Cornwall, UK, revealed no sign of TTV infection.

The TTV-positive individuals of the Sonso community could only be detected using primer set B, whereas all three regions (A, B, and C) were successfully amplified from each of the TTV-positive individuals of CC2. This suggests a sequence change had occurred in one of the two 3′ primers of set C in the Sonso community. Comparison of cloned fragment sequences revealed no individuals to be carrying more than a single strain of the virus. Similarly, all extraction controls proved free of contamination when subjected to PCR at all three targeted regions of the TTV genome, thus eliminating the possibility of operator contamination.

The ML phylogenetic analyses (Fig. 1) show the TT viruses detected within this study cluster strongly with the human TTV strains JA1, TUS01, US35, and TJNO1, as well as a strain found in a captive chimpanzee (CH1). Only the tree generated from the B segment of the UTR is included here (similar trees were reconstructed from the A and C segments; taxa are available from the authors on request). Interestingly, Bwoga, an individual of the Sonso community within the Budongo Forest Reserve, was found to harbor a strain of TTV phylogenetically distinct from all other TTV strains except Pt-TTV6, a chimpanzee-specific strain (Okamoto et al., 2000b) and s-TTVCH65-1, a simian-specific strain (Inami et al., 2000).

It should be noted that the proximity of strains TUSO1 (genotype 11) and JA1 (genotype 2) in the phylogenetic tree is a consequence of the recombinant nature of strain TUSO1, as previously reported (Worobey, 2000).

DISCUSSION

We demonstrated that viruses in free-ranging primate populations can be detected using noninvasive techniques and that free-ranging chimpanzee communities harbor the TTV. All but one of the

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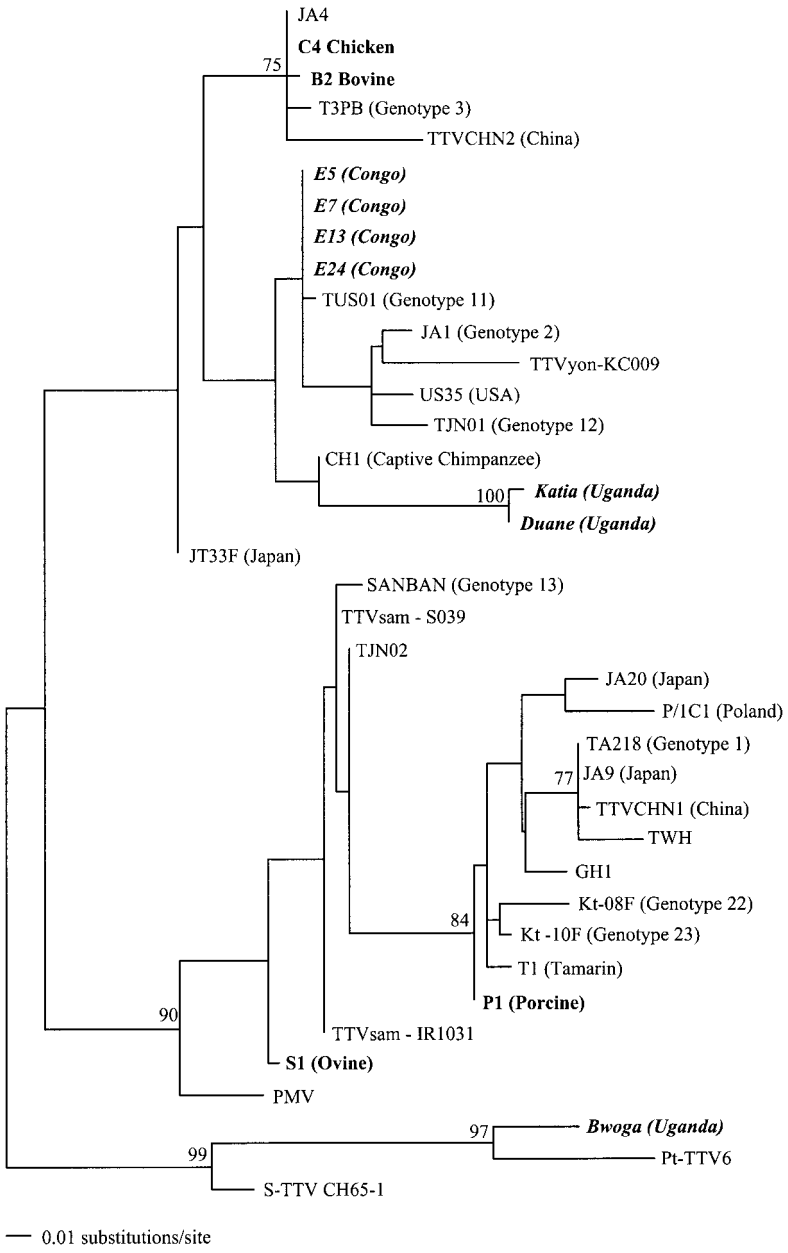


FIGURE 1. Phylogenetic tree showing the evolutionary relationship between the TT virus (TTV) genotypes detected in humans, free-ranging chimpanzees, various captive primates, and farm animals. The tree was constructed using maximum likelihood analyses of 202 base pair segments of sequence from segment B of the untranslated region (UTR). Geographic origin of samples collected for this study have been indicated for each individual. TTV strains detected in this study are highlighted in bold italic typescript; TTV strains previously detected in nonprimate species (taken from GenBank) are highlighted in bold typescript. Nodes with ≥70% bootstrap support are shown. Horizontal branch lengths are drawn to scale, and the tree is midpoint rooted for purposes of clarity.

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TTV strains detected in this study share close phylogenetic relationships with several human strains and do not cluster with the simian-specific isolates. These strains are present among individuals from two of the three free-ranging chimpanzee communities studied, each of which is experiencing increasing contact with humans. The unusual strain detected in Bwoga, from the Sonso community, is closely related to chimpanzee and simian-specific strains Pt-TTV6 and TTVCH65-1 previously detected in captive chimpanzees taken from the wild in 1979 from West Africa (Inami et al., 2000). The results do not allow an assessment of the prevalence of this strain in free-ranging African primate populations, but confirm that the virus is present among free-ranging chimpanzees of both West and East Africa. Whether the strain is genuinely specific to nonhuman primates is still uncertain, as simian-TTV may prove to represent a currently unidentified strain present in African human populations. However, for the time being, the simian TTV described by Inami et al. (2000) and that detected in our Ugandan sample set show little phylogenetic similarity to any human isolates.

Lack of TTV infection among captive individuals from the wildlife parks in the UK is perhaps related to a lower incidence of TTV among individuals of the UK human population. Early studies detected TTV in just 2% of UK blood donors (Simmonds et al., 1998), and the majority of primates at both wildlife parks have been captive bred in the UK for several generations. It is interesting, however, that TTV was not detected among any of the individuals held in the Centro de Rehabilitation de Primates in Santiago, Chile. These individuals have a variety of backgrounds, with many rescued from the pet trade, and with such close human contact we expected some degree of TTV infection. However, the negative results may simply be an artefact of the small sample set and do not necessarily mean that TTV is not present among New World primates, rehabilitated or otherwise.

To date, most studies on the zoonotic exchange of TTV have concerned captive primates or domesticated farm animals (Leary et al., 1999; Okamoto et al., 2000a). The presence of human TTV genotypes has already been reported among chimpanzees held in captivity (Okamoto et al., 2000a), identifying the likely occurrence of cross-species transmission. Okamoto et al. (2000b) also describe two chimpanzee-specific strains (Pt-TTVs). However, some of these species-specific isolates were found to have UTR sequences very similar to those of human isolates. Consequently, the authors suggested that these may represent as yet undescribed human TTV strains, especially given the large number of human TTV genotypes (at least 28) and the high level of human contact experienced by these chimpanzees.

The close phylogenetic relationship between all but one of the TTVs detected in this study and several human strains and lack of infection in CC1 might suggest a recent anthropozoonotic transmission of the virus to CC2; lengthy exposure of the positively infected CC2 to the virus might be expected to have facilitated spread of TTV to adjacent communities (such as the noninfected CC1) via boundary disputes or the female philopatry observed in this species. However, the limited number of samples available from CC1 restricts drawing firm conclusions regarding TTV infection status of this community. Hence, our suggestions, based on this assumed negative infection status, are purely speculative.

The threat of pathogenic viral transmissions to free-ranging primate populations is likely to increase as humans encroach yet further into their habitats. Consequently, a model to evaluate the potential for future zoonotic events between humans and free-ranging primates would be of considerable value. The ease of detection of TTV from noninvasively collected material promotes it as an appropriate

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model for such a task. However, future research will need to focus on determining the importance of host range overlap as a parameter in transmission of the virus. We will also need to determine the actual extent of genetic diversity among the various isolates and identify which of those are associated with the human populations encroaching upon free-ranging primate populations.

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