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Non-human primates in outdoor enclosures: Risk for infection with rodent-borne hantaviruses

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ABSTRACT

Different species of non-human primates have been exploited as animal disease models for human hantavirus infections. To study the potential risk of natural hantavirus infection of non-human primates, we investigated serum samples from non-human primates of three non-human primate species living in outdoor enclosures of the German Primate Center (GPC), Göttingen, located in a hantavirus endemic region of central Germany. For that purpose we used serological assays based on recombinant antigens of the bank vole (*Myodes glareolus*) transmitted *Puumala virus* (PUUV) and the common and field vole (*Microtus arvalis*, *M. agrestis*) associated *Tula virus* (TULV) which are both broadly geographically distributed in Germany. In 24 out of 251 (9.6%) monkey sera collected in 2006 PUUV- and/or TULV-reactive immunoglobulin G (IgG) antibodies were detected. Investigation of follow-up sera from 13 animals confirmed for two animals a

seroconversion due to hantavirus exposure at the GPC. To prove the origin of the infection, wild rodents from the surrounding regions were analyzed by hantavirus-specific reverse transcriptase PCR analysis. In 6 of the 73 investigated bank voles and 3 of the 19 investigated *Microtus* spp. PUUV- and TULV-specific nucleic acid sequences, respectively, were detected. In conclusion, our investigations demonstrate for the first time natural infections of non-human primates in outdoor enclosures in Germany. These findings highlight the importance of hantavirus surveillance in those primate housings and corresponding preventive measures against wild rodents, particularly in hantavirus endemic regions.

Keywords: Non-human primates, haemorrhagic fever with renal syndrome, hantavirus, rodent, bank vole, common vole, field vole

1. Introduction

In natural infections hantaviruses are transmitted from persistently infected reservoir hosts such as rodents or insectivores to humans (and other non-reservoir species) by inhalation of virus-contaminated aerosols (for review see Schönrich et al., 2008). Human infections with viruses indigenous in Europe and Asia can result in haemorrhagic fevers with renal syndrome (HFRS) of different severity levels and case fatality rates (for review see Schönrich et al., 2008). As hantaviruses can cause life-threatening diseases in humans, non-human primates have been employed to establish suitable disease models. In a first attempt, three cynomolgous monkeys (*Macaca fascicularis*) and a chimpanzee (*Pan troglodytes*) were intravenously inoculated with *Prospect Hill virus* (PHV), a hantavirus believed to be non pathogenic to humans. Surprisingly it caused acute nephropathy with mild, transient proteinuria and azotemia (Yanagihara et al., 1988). Later, an experimental intratracheal infection of cynomolgus macaques with cell culture-adapted *Puumala virus*

(PUUV) resulted in signs of lethargy followed by mild proteinuria and microhematuria and histopathological abnormalities in hantavirus RNA- and antigen-positive kidneys (Groen et al., 1995). Infection of cynomolgus macaques with a PUUV strain, that was exclusively replicated in its natural host, the bank vole (*Myodes glareolus*), induced some clinical symptoms such as loss of appetite, apathetic behaviour, fever, proteinuria, biochemical markers and immunological characteristics of HFRS typically observed in human patients (Klingström et al., 2002a; Sironen et al., 2008). A first indication for a naturally acquired hantavirus infection of non-human primates was reported for *Macaca mulatta* in China (see Clement et al., 1994).

Two Arvicolinae-associated hantaviruses, i.e. PUUV and *Microtus*-associated *Tula virus* (TULV), have a broad geographical distribution in Germany (Ulrich et al., 2004; Schmidt-Chanasit et al., 2010). Large numbers and clusters of human PUUV infections have been recorded during the outbreaks in 2005 and 2007, mainly affecting the federal states Baden-Wuerttemberg, Bavaria, Lower Saxony and North Rhine Westphalia (Essbauer et al., 2006, 2007; Hofmann et al., 2008). Whereas PUUV is causing the majority of human HFRS cases in Germany, little is known about the relevance of TULV as a human pathogen. Besides a single description of a HFRS case in north-eastern Germany (Klempa et al., 2003), TULV-specific antibodies were detected in human samples from a few seroprevalence studies (Ulrich et al., 2004; Mertens et al., unpublished data). A recent longitudinal study revealed a sympatric occurence of TULV in common (*Microtus arvalis*) and field voles (*M. agrestis*) from Sennickerode, district Göttingen, federal state of Lower Saxony (Schmidt-Chanasit et al., 2010).

As this site is in close vicinity of the German Primate Center (see Fig. 1) we wanted to prove if non-human primates in outdoor enclosures are at risk to get infected by hantaviruses circulating in the vole populations close to the husbandry. For this purpose we investigated the prevalence of hantavirus-reactive antibodies in three different simian

species and compared the findings with the results from molecular hantavirus investigations in the local rodent populations.

2. Materials and methods

2.1. Breeding colonies of non-human primates

The German Primate Center (GPC) is housing and breeding the Old World monkey species *Macaca mulatta, M. fascicularis* and *Papio anubis* in in- and outdoor units. Each unit is composed of an indoor area, a heated and/or roofed outdoor room and a large outdoor exhibition bordered by fences. Contacts to any other animal species except wild birds or small mammals are efficiently prevented. About one half of the investigated animals were born at the GPC, whereas the other animals originated from other husbandries in Germany, France or the USA. All animals are kept in accordance with the guidelines of the European Union for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EG, D-AFF 008-EWG). The primate husbandry is controlled by local and regional veterinary authorities in accordance with the German Animal Protection Law. All procedures are supervised by an animal welfare officer and the ethical committee for experiments using animals in the federal state of Lower Saxony. During the yearly routine health check 295 serum samples were collected in 1999 -2007 from 254 monkeys including 211 rhesus macaques (*M. mulatta*), 26 cynomolgus monkeys (*M. fascicularis*) and 17 olive baboons (*P. anubis*). This panel contains also serum samples from two *M. mulatta* and one *M. fascicularis*, that died in 2004 or 2005 due to tularemia. The blood collection was carried out in Ketamin-anesthesia (Ketavet®, Pfizer, Karlsruhe, Germany) in a dosage of 10 mg/kg.

2.2. Serological analysis of monkey serum samples

The serological investigations of the samples for hantavirus-specific antibodies were performed following the recently described scheme for seroepidemiological investigations (Mertens et al., 2009). Briefly, the sera were initially investigated in parallel by PUUV- and TULV-based screening IgG-ELISAs. For confirmation, ELISA-positive sera were tested in the corresponding PUUV- and TULV-IgG immunoblot tests (Mertens et al., 2009; Schmidt-Chanasit et al., 2010), but using a horseradish peroxidase-labeled rabbit anti-monkey IgG (Heavy and Light chain, H+L) (Nordic Immunology, Offenbach, Germany). Finally, selected ELISA-reactive, immunoblot-confirmed sera were investigated by chemiluminescence focus reduction neutralization test (cFRNT) using PUUV, strain Sotkamo, TULV, strain Moravia, and *Apodemus flavicollis*-borne *Dobrava-Belgrade virus* (DOBV), strain Slovenia, as a control (Heider et al., 2001).

2.3. Rodent trapping and hantavirus analysis

The trapping of rodents in Sennickerode and at an area of approximately 30,000 m^2 around the GPC of Göttingen, both in district Göttingen (see Fig. 1), as well as the TULV investigations of *Microtus arvalis* and *M. agrestis* from Sennickerode have already been described in detail (Kaysser et al., 2008; Schmidt-Chanasit et al., 2010). Hantavirus-S-segment-specific reverse transcriptase-PCR (RT-PCR) of animals trapped around the GPC and of bank voles from Sennickerode followed previously described detailed protocols (Essbauer et al., 2006; Schmidt-Chanasit et al., 2010). Retrieved novel partial PUUV and TULV S-segment sequences were submitted to GenBank with accession numbers GU300138-GU300143 and GU300135-GU300137, respectively.

3. Results

Using recombinant PUUV- and TULV-antigen-based IgG screening ELISA and confirmation immunoblot assays, 24 of the 251 (9.6 %) investigated animal serum samples

collected during 2006 were positive for hantavirus-specific IgG antibodies with at least one of the used antigens (Table 1). Out of these 24 samples 17 exclusively reacted with PUUV antigen, three with TULV antigen and four with both antigens to the same extent. A total of 16 of the 209 (7.7 %) sera from 13 female and three male rhesus macaques (*M. mulatta*) were seropositive with isolated reaction to PUUV antigen for 13 samples, to TULV antigen for two samples and a cross-reactivity to both antigens for one sample. In the olive baboons (*P. anubis*) serum panel 6 of 17 (35.3 %) were reactive with at least one of the antigens. Out of these 6 samples, with five of six originating from female animals, three reacted with the PUUV, one with TULV and two with both antigens. The level of seroreactivity in the cynomolgus monkey (*M. fascicularis*) panel was 8.0 % (2/25) with one serum reacting only with PUUV antigen and one with the two antigens. The two seroreactive sera originated from female individuals (Table 1). Subsequent analysis of five selected ELISA-positive, immunoblot-confirmed sera from *M. mulatta* by PUUV, TULV and DOBV cFRNT revealed one PUUV/TULV cross-reactive, but DOBV non-reactive serum originating from a six year-old female animal (#2116) born at the GPC. The endpoint titer to PUUV (640) was slightly higher than that to TULV (320).

Next, follow-up sera of 12 animals including 11 *M. mulatta* and one *M. fascicularis*, which were in the initially investigated serum samples from 2006 positive, were tested in the same way as described above (Table 1). From four *M. mulatta* (#4986, #9290, # 9796 and #9821) all serum samples were found to be positive, with at least one antigen. Four additional *M. mulatta* (#9291, #9292, #12083, #12103) demonstrated an initially negative or equivocal sample with all corresponding following samples being positive. The remaining three *M. mulatta* showed an oscillation of positive and negative reactivity (#2148, #13032) or vice versa (#11660). All three follow-up samples from *M. fascicularis* #9858 reacted with both antigens. Finally, the medical records were retrospectively checked for potential indications of a hantavirus infection. None of the seropositive animals

developed symptoms typical for a hantavirus infection like apathy, vomiting, diarrhea or hematuria. At the time of blood sample collection a complete health check was done. All animals were clinically healthy and kidney and liver enzymes were within the normal range. Six of the seropositive animals died during the investigation period. There was no correlation between the cause of death and the former hantavirus infection. Two animals (#2039, #2242) had to be humanly euthanasized because of severe injuries after heavy ranking fights. One animal died after severe aspiration pneumonia (#9292). One of the baboons (#13315) died because of several changes due to old age. Systemic echinococcosis was the cause of death in the last two animals (#9858, #9870). The histological samples of the deceased monkeys were screened for signs of a hantavirus infection but none of the animals showed kidney alterations in form of interstitial nephritis.

In addition, serum samples from two *M. mulatta* and one *M. fascicularis*, that died due to tularemia, tested in the PUUV and TULV ELISA revealed for all investigated samples of one *M. mulatta* PUUV-reactive antibodies (Table 1; #9411). The other two animals did not show any reactivity in the PUUV and TULV IgG screening ELISAs (data not shown).

Interestingly, in a recent study TULV infections have been described in *M. arvalis* and *M. agrestis* from Sennickerode, district Göttingen, about 20 km apart from the GPC (Schmidt-Chanasit et al. 2010; see Fig. 1). In addition, herein we investigated lung tissue samples from 60 bank voles (*M. glareolus*) from the same trapping site. Novel partial PUUV S-segment-specific fragments were detected by RT-PCR in 6 of the lung samples (10%). These sequences are closely related to each other, but can be differentiated at the nucleotide level from PUUV sequences originating from other regions in Germany, e.g. Baden-Wuerttemberg, northern and southern Bavaria and Cologne (see supplementary Table 1).

Further, during 2005 a total of 46 rodents, including 8 *M. agrestis*, 11 *M. arvalis* and 13 *M. glareolus,* have been trapped in the immediate vicinity and surrounding coppice of the GPC (Kaysser et al., 2008). RT-PCR analysis of lung tissue samples from *M. arvalis*, *M. agrestis* and *M. glareolus* revealed 3/11 (27.3 %) TULV-positive *M. arvalis*, but no indication for hantavirus infections in field and bank voles (data not shown). A pairwise comparison of the derived TULV sequences from Göttingen revealed a close relationship to the TULV sequences recently described from Sennickerode (see supplementary Table 2).

4. Discussion

Spillover infections of non-reservoir rodent species are believed to be a rare event (Klingström et al., 2002b), but represent a pre-requisite for a subsequent establishment of a transmission cycle in a novel reservoir host as well as genetic reassortment processes between different hantaviruses. In line, detection of multiple spillover infections of *M. agrestis* with *M. arvalis*-associated TULV has been discussed as the beginning establishment of a novel reservoir host transmission cycle (Schmidt-Chanasit et al., 2010). Similarly, multiple spillover infections of *A. agrarius*-borne DOBV to *A. flavicollis* have been observed in northern Germany (Schlegel et al., 2009). Detection of hantavirus-reactive antibodies in carnivores, like foxes, cats and dogs, and in other wildlife species, such as wild moose, demonstrated the infectivity of the pathogen for other species (for review see Zeier et al. 2005).

Here we demonstrated a stable hantavirus seropositivity over a period of up to seven years in all investigated longitudinal samples of six animals including five *M. mulatta* (#4986, #9290, #9411, #9796, #9821) and one *M. fascicularis* (#9858). These data suggest a long-term immunity induced by naturally acquired hantavirus infection in these primates. Interestingly, for two rhesus monkeys (#9291 and #12083) an obvious

seroconversion for PUUV was observed between 2003/2005 and 2005/2006 suggesting an exposure to PUUV at the GPC during 2003-2006. Due to the hantavirus outbreak in Germany in 2005 with a total of 447 HFRS cases and an increased number of cases (n=11) in the district Göttingen (Robert Koch-Institut: SurvStat, www.rki.de; data as of February 10, 2009), one may speculate on a high infection burden in the bank vole populations resulting in increased numbers of human cases and the potential exposure to some primates in the outdoor enclosures. The serological findings of TULV-reactive antibodies in *M. mulatta* animal #9292 may also suggest a seroconversion with an exposure in 2003 or before. Although for some seroreactive monkeys in this study the exposure and infection with PUUV, TULV or PUUV/TULV-cross-reactive hantaviruses, i.e. PHV (Chu et al., 1995), might have occurred at their breeding origin at other places in Germany, France or the USA, this, nevertheless, again confirms the susceptibility of non-human primates, i.e. *M. mulatta*, *M. fascicularis* and *P. anubis* for natural hantavirus infection.

Experimental hantavirus infection of non-human primates resulted in disease symptoms which resemble those observed in humans (Yanagihara et al., 1988; Groen et al., 1995; Klingström et al., 2002a). A retrospective analysis of the clinical records of all animals investigated here did not show hints for clinical symptoms after hantavirus exposure. In addition, for three animals that died in 2004/2005 due to tularemia no signs of a co-morbidity of hantavirus and *Francisella* infections could be found. Additional studies are needed to prove whether naturally acquired hantavirus infections in different non-human primate species may result in an asymptomatic or mild course of disease with unspecific symptoms.

The herein reported detection of hantavirus-reactive antibodies, and a seroconversion in particular, in non-human primates in outdoor enclosures and the parallel demonstration of PUUV- and TULV-infected reservoir hosts in the surroundings suggest

that infection events occurred in the past. The exposure of monkeys in the GPC to wild rodents from the surroundings was previously already suggested due to the death of a group of cynomolgus monkeys (*M. fascicularis*) and the parallel detection of *Francisella tularensis* in rodents from the surroundings (Mätz-Rensing et al., 2007; Kaysser et al., 2008).

In conclusion, this is, to our knowledge, the first report of natural infections of non-human primates in outdoor enclosures in Europe raising important questions on future hantavirus surveillance in such husbandries. In addition, the results of the study accentuate the need of an efficient rodent combat and management in primate husbandries to protect susceptible primate species from hantavirus infection.

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Legends to Figures

Fig. 1. Maps of Germany showing the federal state Lower Saxony (marked in black; A) and the localization of the rodent trapping sites at the German Primate Center (GPC) and in Sennickerode (SEN) in the administrative district Göttingen (B).

Table 1. Serological reactivity of non-human primate sera from the German Primate Center (GPC) in *Puumala virus* (PUUV) and *Tula virus* (TULV) IgG-ELISA and -immunoblot assay (IB).

Animal description					Results of IgG-ELISA/IB[b]	
Species	Animal no.	Origin	Age[a] (years)/ sex/ year of death	Year of blood collection	PUUV	TULV
Macaca mulatta	2039	GPC	8/f/2008	2006	-	+
Macaca mulatta	2116	GPC	6/f	2006	+	+
Macaca mulatta	2148	GPC	1/f	2002	E	+
			2/f	2003	-	-
			4/f	2005	+	-
			5/f	2006	(+)	-
Macaca mulatta	2242	GPC	3/f/2008	2006	+	-
Macaca mulatta	2305	GPC	2/m	2006	+	-
Macaca mulatta	2323	GPC	1/f	2006	+	-
Macaca mulatta	4986	USA, since 1998 in GPC	13/m	2005	+	+
			14/m	2006	+	+
			15/m	2007	+	+
Macaca mulatta	9290	Strasbourg, since 1998 in GPC	6/f	2003	+	+
			8/f	2005	+	E
			9/f	2006	+	-
Macaca mulatta	9291	Strasbourg, since 1998 in GPC	7/f	2003	-	-
			9/f	2005	+	-
			10/f	2006	+	-
			11/f	2007	+	E
Macaca mulatta	9292	Strasbourg, since 1998 in GPC	11/f/2008	2003	-	E
			13/f/2008	2005	-	+
			14/f/2008	2006	-	+
			15/f/2008	2007	-	+

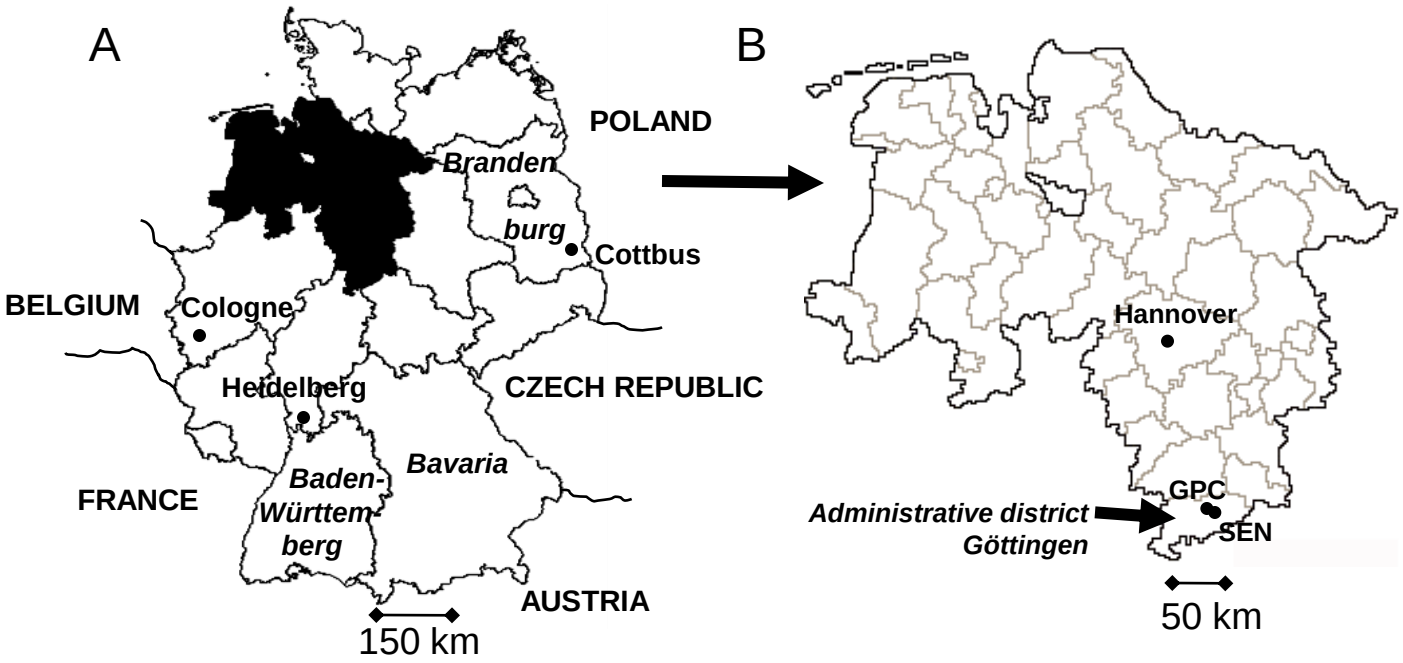
Macaca mulatta	9796	GPC	4/f	2003	+	-
			6/f	2005	+	-
			7/f	2006	+	-
			8/f	2007	+	-
Macaca mulatta	9821	GPC	4/f	2003	+	+
			6/f	2005	+	+
			7/f	2006	+	-
			8/f	2007	+	-
Macaca mulatta	11660	GPC	1/f	2003	-	-
			3/f	2005	-	-
			4/f	2006	+	-
			5/f	2007	-	-
Macaca mulatta	12083	GPC	2/f	2005	-	-
			3/f	2006	+	-
			4/f	2007	+	-
Macaca mulatta	12103	GPC	2/f	2005	E	-
			3/f	2006	+	-
			4/f	2007	+	-
Macaca mulatta	13032	GPC	1/m	2006	+	-
			2/m	2007	-	-
Macaca mulatta	9411	Strasbourg, since 1998 GPC	10/m/2004	2001	+	-
			12/m/2004	2003	+	-
			13/m/2004	2004	+	-
Macaca fascicularis	9858	Marburg, since 1999 in GPC	11/f/2009	1999	+	+
			15/f/2009	2003	+	+
			18/f/2009	2006	+	+
Macaca fascicularis	9870	Marburg, since 1999 in GPC	8/f/2007	2006	+	-
Papio anubis	13300	University Munich, since 2005 in GPC	2/m	2006	+	-
Papio anubis	13298	University Munich,	5/f	2006	+	-

		since 2005 in GPC				
Papio anubis	13307	University Munich, since 2005 in GPC	3/f	2006	+	+
Papio anubis	13311	University Munich, since 2005 in GPC	14/f	2006	-	+
Papio anubis	13312	University Munich, since 2005 in GPC	7/f	2006	+	+
Papio anubis	13315	University Munich, since 2005 in GPC	20/f/2009	2006	+	-

[a]Age at the time point of blood collection.
[b]The serological investigations and the subsequent result evaluations were performed following the recently described scheme for seroepidemiological investigations using PUUV and TULV IgG-ELISA as screening tests and the corresponding IgG-IB for confirmation (Mertens et al., 2009).
-, negative, i.e. “negative” result in the ELISA screening test
+, positive, i.e. “positive” or “weakly positive” result in one test and “positive” result in the other test
(+), weakly positive, i.e. reaction in both tests “weakly positive” or “weakly positive”/“positive” in one test and “equivocal” in the other
E, equivocal, i.e. “equivocal” result in both tests.
m, male; f, female.

Figure

Fig. 1


A
POLAND
Branden
burg
Cottbus
BELGIUM
Cologne
Heidelberg
CZECH REPUBLIC
Bavaria
FRANCE
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Württem-
berg
AUSTRIA
150 km
B
Hannover
GPC
Administrative district
Göttingen
SEN
50 km