EPIDEMIOLOGICAL STUDY OF SELECTED VIRAL PATHOGENS AND ASSESSMENT OF RESPONSES TO POLIO VACCINES IN SEMI- CAPTIVE CHIMPANZEES IN UGANDA

BY

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DECLARATION

I LAWRENCE MUGISHA, declare that the approved thesis hereby submitted to the School of Graduate Studies, Makerere University for the PhD degree and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university for a degree.

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DEDICATION

This work is dedicated to my wife Agnes and my lovely children Agaba Mark Mugisha and Aturinda Nelly Mugisha

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GENERAL REMARKS

PhD history and collaborations

The PhD concept was prepared by me based on my personal experience of working with semi-captive wild-born chimpanzees with the background information on potential risks for disease transmission between caretakers and chimpanzees. The concept was in line with established Great Ape Health Monitoring Unit (GAHMU) to monitor the health of great apes and react promptly whenever epidemics threaten ape populations (http://www.eva.mpg.de/primat/GAHMU/). Hence collaboration was established with Dr. Fabian Leendertz, coordinator GAHMU based at Robert Koch Institute, special pathogens laboratory where all laboratory work was undertaken supported by other laboratory departments in the same institution.

Organisation of the thesis

This thesis has been organized in manuscript format with each chapter being one manuscript. The manuscripts start from chapter two to chapter six. The third, fourth, fifth and sixth chapters have been published in the *Journal of Medical Primatology , Open Veterinary Science Journal* and *Virus research* while the second chapter has been submitted and is under review in the *Journal of Medical Primatology.*

Chapter one covers introduction, literature review and analyses risk factors for disease transmission between humans and non-human primates. The same chapter covers the purpose and specific objectives of the study, overview of subspecies information, existence of sanctuaries and general methods. Chapter two presents general results of the serological and polymerase chain reactions (PCR) findings on viral pathogens in wild-born semi-captive chimpanzees and presents some of the behavioral sanctuary management practices that might facilitate disease transmission.

Chapter three presents results of PCR findings on retroviruses and discusses their implications in sanctuary management and re-introductions.

Chapter four presents results of a novel herpes virus and phylogenic relationship with other known herpes virus of humans and primates and their implications.

Chapter five presents results of the nearly full genome of Hepatitis B Virus infection in chimpanzees and results of analysis for recombination among HBV genotypes alongside reviews on the current knowledge on hepatitis infection in chimpanzees and on the primates.

Chapter six presents the results of the immunological responses to polio vaccination in chimpanzees and provides evidence of induced immunological protection in chimpanzees for over 10 years

Chapter seven summarizes the thesis findings and their implications, recommendations and focus for future research.

LIST OF ACRONYMS

ALP	Alkaline phosphatase
BGH	Board on Global Health
CDC	Center for Disease Control and Prevention
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CoV	Coronavirus
CSWCT	Chimpanzee Sanctuary & Wildlife Conservation Trust
DNA	Deoxyribonucleic Acid
EHP	Employee Health Program
EID	Emerging Infectious Diseases
ELISA	Enzyme Linked Immunosorbent Assay
GAHMU	Great Ape Health Monitoring Unit
GPT/ALT	Alanine aminotransferase
GT/GGT	γ-glutamyl transferase
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HEP	Hepatitis E Virus
HIV	Human Immunodeficiency Virus
HMPV	Human Metapneumovirus
HRSV	Human Respiratory Syncytial Virus
HTLV	Human T-Lymphotropic Virus
IHR	In-House Reference serum
IOM	Institute of Medicine
IPV	Inactivated Poliovirus Vaccine
MMR	Measles, Mumps and Rubella
MYA	Million Years Ago
NHP	Non-Human Primates
NIC	Ngamba Island Chimpanzee Sanctuary
OPV	Oral Poliovirus Vaccine
PASA	Pan African Sanctuary Alliance
PCR	Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
SFV	Simian Foamy Virus
SIV	Simian Immunodeficiency Virus
STLV	Simian T-Lymphotropic Virus
TB	Tuberculosis
tMRCA	time since Recent Common Ancestor
UNCST	Uganda National Council of Science and Technology
UVRI	Uganda Virus Research Institute
UWA	Uganda Wildlife Authority
UWEC	Uganda Wildlife Education Centre
VPP	Veterinary Preventive Program
WHO	World Health Organisation

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PUBLICATIONS

Mugisha, L., Leendertz, H.F., Opuda-Asibo, J., Ehlers, B. (2010). A novel herpesvirus in the sanctuary chimpanzees on Ngamba Island in Uganda. *J med Primatol* 39, 71-74.

Mugisha, L., Pauli, G., Opuda-Asibo, J., Joseph, O.O., Leendertz, H.F., Diedrich, S. (2010). Evaluation of poliovirus antibody titers in orally vaccinated semi-captive chimpanzees in Uganda. *J Med Primatol*.39, 123-128.

Mugisha, L., Kucherer, C., Ellerbrok, H., Opuda-Asibo, J., Joseph, O.O., Pauli, G., Leendertz, H.F (2010). Retroviruses in wild-born semi-captive East African sanctuary chimpanzees (*Pan troglodytes schweinfurthii*). *The Open Veterinary Science Journal,* 4, 6-10.

Ehlers, B., **Mugisha, L**., and Leendertz, FH. (2010). Letter to the Editor: Reply to the comment by Gessain et al. on Mugisha et al.. *J.Med.Primatol*, 1-2.

Mugisha, L., Kaiser, M., Ellerbrok, H., Opuda-Asibo, J., Joseph, O.O., Pauli, G., Leendertz, H.F (2010). The "original" Hepatitis B virus of Eastern chimpanzees (*Pan trogrodytes schweinfurthii*)**.** *Virus Res,* **155**(1):372-5.

Mugisha, L., Ehlers, B., Ellerbrok, H., Opuda-Asibo, J., Joseph, O.O., Pauli, G., Leendertz, H.F (2010). Multiple viral infections in confiscated wild born semi-captive chimpanzees (*Pan trogrodytes schweinfurthii*) in a Sanctuary in Uganda: Implications for sanctuary management and conservation. Submitted *J.Med.Primatol*

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ABSTRACT

The high influx of non-human primates (NHP) including apes into sanctuaries that have increased in numbers in the last two decades across Africa's equatorial region is of great concern. There were no studies conducted previously to establish the health status of NHP including the potential viral pathogens of zoonotic concern in these sanctuaries. The main goal of this study was therefore to screen the chimpanzees for the main viral pathogens, characterize them at molecular level and establish phylogenetic relationship with strains from other primates to generate baseline data on these pathogens important for monitoring the health of the chimpanzees in the sanctuary and as basis for reviewing of standard operating procedures. The study also evaluated effectiveness of vaccination of chimpanzees against measles and poliovirus. In order to achieve this, blood and fecal samples were collected from 42 wild-born captive chimpanzees on Ngamba Island Chimpanzee Sanctuary (NIC) during annual health checks in 2007 and 2008. Retrospective samples from previous health checks were included in the analyses. Serum was subjected to enzyme-linked immunoassays for detection of antibodies directed against simian/human immunodeficiency virus (SIV/HIV), human T-lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) and Hepatitis B Virus (HBV). Nucleic acids (DNA and RNA) were extracted from all samples and amplified by Polymerase Chain Reaction (PCR) and genotyped for specific viral pathogens. Viruses were characterized by phylogenetic analysis.

The study has revealed that chimpanzees NIC sanctuary are infected with multiple viral pathogens with zoonotic potential. The viral infection prevalence in chimpanzees were 85.7%, 73%, 60.5% and 32.4% for the Adenoviruses, Simian Foamy virus (SFV), gammaherpesviruses and Hepatitis B Virus (HBV), respectively. They were also screened for simian/human immunodeficiency virus (SIV/HIV), human T-lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) antibodies, Hepatitis C virus (HCV), Hepatitis E Virus (HEV), Flavivirus, human metapnuemovirus, and Chikungunya viruses but were negative for all these parameters.

Phylogenetic analysis of the herpersviruses found that chimpanzees were infected with members of the *Gammaherpesvirinae* with one novel herpesvirus in the same family tentatively named *Pan troglodytes* rhadinovirus 3 (PtroRHV-3) in two chimpanzees that fell into a clade of primate rhadinoviruses and Kaposi sarcoma herpesvirus (human herpesvirus 8).

SFV sequences obtained in this study formed four sub clusters within the specific SFV *P. t. schweinfurthii* clade with significant variability among the newly described SFVs strains. This gives evidence for an on-going SFV transmission among chimpanzees within the sanctuary most likely through horizontal routes leading to co-infection of individuals with more than one strain.

PCR amplification of chimpanzee samples positive for antibodies to hepatitis B (core) antigen (ant-HBc) detected chHBV DNA in one captive wild born (Mika) and one wild chimpanzee (Kiiza). The HBV clustered closely with HBV isolate FG published previously for *P.t.schweinfurthii*. Retrospective analysis of samples from Mika for HBV genome provided evidence of persistence HBV infection with high viral load in the infected chimpanzee and proves that HBV circulates naturally in chimpanzees. Analysis of the nearly complete HBV genomes indentified from Mika and Kiiza for recombination with human HBV subtype C, as earlier reported for the FG isolate there was no recombination with human HBV subtypes or strains from other sub species.

Adenovirus infection was widely spread in chimpanzees with 85.7% prevalence and this is most probably shed as infectious virions in substantial quantities in feces in apparently healthy population. This poses the risks of intra-species transmission and intra-species recombination of adenoviruses.

This study also presents applicability of using some of the established non-invasive methods in detection of some of viral pathogens as evidenced by the detection of viral DNA by PCR for adenoviruses, herpespesviruses and hepatitis B virus in feces of chimpanzees. This is a very valuable tool especially for health monitoring of wild ape populations.

This study also shows that chimpanzees vaccinated against poliomyelitis using oral polio vaccine (OPV) had neutralizing antibodies against polio virus type 1, 2 and 3 nine years post vaccination. Conversely only a few chimpanzees vaccinated against measles had detectable protective titers. This shows that preventive measures employed in sanctuaries especially vaccinations need to be monitored and evaluated for effectiveness over time. OPV mounts

protection among vaccinated chimpanzees but its continued use should be evaluated along the global eradication program following the established WHO global action for laboratory containment of wild polioviruses.

Findings of these viral pathogens coupled with the close phylogenetic relationship between chimpanzees and humans plus high levels of interactions during sanctuary operations, presents a high potential for pathogen exchange. Some of the viral pathogens are shed in body fluids in large quantities like SFV in saliva and adenovirus in feces which presents high risk for zoonotic transmission to animal keepers in sanctuaries coming into contact with chimpanzee fecal matter and other body fluids while cleaning housing facilities. Results of this study give insights onto implications of management of primates in sanctuaries and cautions for the improvement of occupational health and safety protocols to minimize risks of pathogen exchange.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction and Literature Review

Emerging and re-emerging infectious diseases (EIDs) are significant burdens to global economies and public health (Daszak et al., 2000; Furgerson et al., 2005; Jones et al., 2008; Tabish, 2009; Aluwong and Bello, 2010). At least 17 million people die annually from infectious diseases (Tabish, 2009). Some of these diseases gained worldwide public interest like the highly pathogenic H5NI avian influenza, severe acute respiratory syndrome (SARS), Ebola virus, food-and waterborne illnesses, and a range of antibiotic-resistant bacterial diseases like multidrug-resistant and extremely drug-resistant tuberculosis (TB). Most of the EIDs (60.3%) are zoonoses and the majority of these (71.8%) originate from wildlife hosts (Tabish, 2009). Infectious agents from non-human primates (NHP) in particular have caused a number of new human diseases, leading to calls for international surveillance to monitor the human-nonhuman primate interface (Jones et al., 2008). The best known examples of primate-human transmission are the emergence of HIV-1 and-2 which originated from simian variant of the virus SIV (Gao et al., 1999; Hahn et al., 2000; Hirsch et al., 1995; Peeters et al., 2002; Weiss and Wrangham, 1999) as well as discovery of novel HIV-1 lineage (HIV-1 group P) distinct from HIV-1 groups M, N, and O closely related to gorilla simian immunodeficiency (SIVgor) in a Cameroonian woman (Plantier et al., 2009). Other known examples are: HTLV-1 from STLV-1 (Crandall, 1996; Gessain and the De The, 1996; Leendertz et al., 2006; Koralnik et al., 1994; Makuwa et al., 2004; Meertens et al., 2001; Slattery et al., 1999; Voevodin et al., 1997); transmission of simian foamy virus (Wolfe et al., 2005) and Ebola virus (Rouquet et al., 2005; Towner et al., 2008).

Likewise infectious diseases of human origin have caused various degrees of mortalities in wild great apes leading to population decline (Boesch-Achermen, 2000; Goodall and Ferber, 1983; Ferber, 2000; Leendertz et al., 2006; Köndgen et al., 2008; Wolfe et al., 1998). These together with other reported emergence of infectious diseases in great ape populations like Ebola in chimpanzee and gorillas (Formentry et al., 1999); Georges et al., 1999; Huijbregts et al., 2003; Le guenno et al., 1999; Leroy et al., 2005; Rouquent et al., 2005; Walsh et al., 2003; Wyers et al., 1999) and anthrax in chimpanzee and gorillas (Leendertz et al., 2004 and Leendertz et al., 2006) have presented significant challenges and emerging threat to the survival of the remaining great ape populations throughout their home range. The deadly Ebola virus

is seen as one of the biggest disease threats to wild apes and over the last two decades, it has caused extensive gorilla and chimpanzee die-offs in Gabon and Republic of Congo which were home to some of the largest protected great ape populations in the world (Bermejo et al., 2007; Walsh et al., 2003). Recent human outbreaks of the Sudan and the newly described Bundibugyo strain of Ebola virus occurred close to some of the major habituated chimpanzee and gorilla populations in Uganda and Rwanda (Okware et al., 2002; Towner et al., 2005). It is estimated that by to date, Ebola virus strain Zaire has killed one third of the world gorilla populations and large number of chimpanzees leading to the World Conservation Union to upgrade western gorillas to the Critically Endangered status on its Red List of Threatened Species (Walsh et al., 2007).

Several factors leading to the appearance of EIDs have been described by the Board on Global Health (BGH) and the Institute of Medicine (IOM) and other authors (Aluwong and Bello, 2010; Morse, 2004) to include: microbial adaptation and change, human demographics and behaviour, international travel and commerce, economic development and land use, technology and industry, breakdown of public health measures, human susceptibility to infection, climate and weather, changing ecosystems, poverty and social inequality, war and famine, lack of political will, and intent to harm. Some of these factors have brought about unprecedented high levels of interactions between human and NHPs leading to trans-species transmission of pathogens. Apes compared to other animals are particularly more susceptible to human pathogens due to close phylogenetic relationship of more than 97% DNA similarity (Ruvolo et al., 1994).

Most African Equatorial rain forests, the geographical home range of great apes are under intense pressure of destruction from expanding industrial logging with 30% of the pristine Central African dense humid forests under logging concessions (Laporte et al., 2007) and increasing human population pressure demanding land for settlement and agriculture. The disturbance alters ecosystem composition and biodiversity (Malcom et al., 2000), opens remote areas for poaching (Robinson et al., 1999) and modifies numerous functional attributes of the ecosystem (Hall et al., 2003). It has been noted that these human-induced land changes are primary drivers for a range of infectious disease outbreaks and modifiers of the transmission of

endemic infections (Patz et al., 2000). Habitat destruction and fragmentation increases levels of primate-human interactions and promotes exchange of pathogens as documented for Lyme disease (Glass et al., 1995).Furthermore, occurrence of some gastrointestinal microbes in primates is associated with anthropogenic habitat disturbance (Chapman et al., 2006; Gillespie and Chapman, 2006; Kawalewski and Gillespie, 2008). Recently forest fragmentations have been shown to increase bacterial transmission rates between humans, NHPs and domestic livestock (Goldberg et al., 2008). Salzer et al., 2007 found *Cryptosporidium* sp. And *Giardia* sp. infections in red colobus in forest fragments but not in undisturbed forest.

As with forest destruction, hunting of primates for bushmeat has been identified as one of the threats to apes and other primate population declines. In Central Africa alone, it is estimated that 1 metric ton of wildlife is consumed every year (Wilkie and Carpenter, 1999). The Congo Basin is the biggest centre for bushmeat hunting worldwide and in many areas bushmeat makes up 10% of people's protein intake (Pearce**,** 2005). The local demand for and consumption of bushmeat is high (Lahm, 1993.) and the extent and intensity of commercial hunting has increased dramatically over the past decade (Auzel and Wilkie, 2000, Bowen-Jones, and Pendry, 1999; Wilke et al., 1992). In Uganda, hunting and consumption of bushmeat has not been well documented until recently (Olupot et al., 2009) providing analysis and underlying influences in Uganda's premier parks. Still hunting remains illegal except for spot hunting around Lake Mburo National Park and supervised control of three species declared as vermin. Uncontrolled hunting is disseminating wildlife populations in addition to providing avenues for disease transmission as hunters come into close contact with broad range of body fluids and tissues. Hunting of NHPs has been linked to transmission of Ebola, monkeypox (WHO, 2003) and simian foamy virus (Wolfe et al., 2004). Recent studies have provided more evidence of naturally acquired SFV in hunters living in Cameroon (Wolfe et al., 2004) and persons with frequent contacts to NHPs in several South and Southeast Asian Countries (Engel et al., 2006; Jones-Engel et al., 2005; Wolfe et al., 2004). In one of the studies, the seroprevalence of SFV infections was highest 24.1% (7/29) in studied persons that had contacts with apes (gorillas and chimpanzees) compared to 3.6% (2/56) that had contact with monkeys (Calittini et al., 2007). A large proportion of these positive individuals (36%) reported to have been severely bitten and injured while hunting wild chimpanzees and gorillas had SFVcpz or SFVgor sequences detectable in their blood (Calittini et al., 2007).

In Uganda and elsewhere in Africa, many groups of chimpanzees and gorillas have been and are still being habituated for ecotourism and research as a sustainable way for their conservation (Butynski and Kalina, 1993; Butynski et al., 1990). Currently nine groups of gorillas in Uganda are habituated/undergoing habituation for ecotourism. The process of habituation and associated activities for ecotourism and research thereafter promotes close interactions between humans and apes. These interactions have been implicated in the transmission of infectious diseases and pose an additional risk to ape population survival (Golberg et al., 2007; Graczyk and Cranfield, 2001; Sleeman et al., 2000). This is evidenced by recent findings of human metapneumovirus (HMPV) and human respiratory syncytial virus (HRSV) that cause respiratory diseases in humans as commonest cause of death among chimpanzees in Tai forest (Köndgen et al., 2008). Forty nine percent of chimpanzee deaths observed over 47 years of study at Gombe, Tanzania were attributed to respiratory diseases (Williams et al., 2008). Further, respiratory disease outbreaks causing death in habituated chimpanzee populations have been reported in Bossou, Guinea (Sugiyama, 2004) and Mahale, Tanzania (Hanamura et al., 2008). Respiratory infections have been found to be common in habituated groups of mountain gorillas (*Gorilla gorilla beringei*) requiring clinical intervention and are suspected to be of tourist origin (Cranfied and Minnis, 2007). Cases of aggression with direct attacks on tourists and local communities leading to injuries and death in some circumstances have been observed and associated with habituation and habitat fragmentation that also presents high risk for potential disease transmission. Macaques have been known to attack tourists leading to trans-cutaneous exposure to infectious agents present in the body fluids of macaques (Jones-Engel et al., 2006).

Poaching and pet trade has led to high number of NHPs being rescued and cared for in primate sanctuaries throughout Africa. To date there are 20 sanctuaries in Africa caring for a range of NHPs (more than 800 chimpanzees, 80 gorillas, 55 bonobos and an estimated over 1000 other endangered primates (PASA Workshop Report, 2006). However, care of captive NHPs is associated with frequent risks of aggressive attacks leading to bites and scratches to animal keepers/caregivers and this has led to the transmission of a range of infectious agents. Simian Foamy Virus (SFV) from chimpanzees, baboons, African green monkeys and macaques to humans occupationally exposed to these NHPs in captivity and biomedical research centers

has been described (Brooks et al., 2002; Heniene et al., 1998; Meiering et al., 2001; Sandstrom et al., 2000). Other reports include herpes B virus (herpes virus simiae) (Huff and Barry, 2003), primate malaria (Coatney et al., 1971), and tuberculosis (Kalter et al., 1978). A growing practice in some sanctuaries of walking with rescued chimpanzees or gorillas into the forest as part of rehabilitation with staff and more recently tourists presents un- investigated risks of pathogen exchange. Increasingly tourists continue to get into close contact to NHPs both in the wild and in captivity.

Similarities in pathogen susceptibility have made NHPs ideal laboratory models. During the 20th century, laboratory research on captive primates has elucidated the life cycle and pathogenesis of many infectious agents and facilitated drug and vaccine development. However, laboratory handling of tissues or fluids of NHPs led to transmission of a range of infections to humans, including simian immunodeficiency virus (SIV) (Khabbaz et al., 1994) and SV40, which might have been subsequently distributed through oral polio vaccine to humans (Shah et al., 1971).

Keeping NHP pets has been linked to transmission of a variety of micro-organisms (Renquist and Whitney, 1987) and the people involved are not aware of the consequences. Exposure to sick or dead primates represents a risk of infection as in the case of a student who was infected by Ebola virus while performing an autopsy of a chimpanzee carcass in Taï National Park, Côte d'Ivoire (Rouquet et al., 2005).

Most of the documented transmissions are result of close contact between humans and NHPs from bushmeat handling and consumption, human contact with great ape cadavers (Rouquet *et al*., 2005) and bites and scratches during close contact in captive and research facilities. A number of important human diseases, including adult T-cell leukaemia (HTLV-1) and malaria (*Plasmodium* spp.), are discussed to have emerged as the results of ancient or contemporary cross-species transmissions from NHPs (Slattery, et al., 1999; Wolfe, et al., 2005).

Despite of this knowledge, the risks of disease transmission in captive facilities especially in newly established sanctuaries in Africa is not well addressed and documented. Sanctuary management involves a wide range of activities ranging from rescue, hand-raising during rehabilitation, feeding and veterinary care all which present high potential risks of disease transmission due to close contact. This high level of interaction facilitate transmission of micro-organisms form NHPs to humans (Wolf et al., 1998,) as well as from humans to NHPs with consequences to individual health, public health and conservation of apes in general (Wolfe et al., 1998). The current study has given an insight into some of the potential pathogens circulating in NHPs in sanctuaries.

The study has attempted to elucidate the immune responses to vaccinations using oral polio vaccine (OPV) as most NHPs in sanctuary apes are vaccinated with human based vaccines especially against measles, polio and tetanus. The vaccinations are based on the documented recommendations for disease prevention in captive and wildlife primates (Jungle, 1995; Wallis and Lee, 1999) and are part of occupational health and safety management in such facilities. However, no studies have been undertaken in these sanctuaries to establish the duration and levels of induced immunological responses. Chimpanzees on Ngamba Island provided this unique opportunity with already existing samples obtained during earlier annual health checks.

Furthermore, the study has generated a better understanding of the disease dynamics in the great apes and their epidemiological relationship to humans. Hence it is important to establish the disease status of captive primates to able to prevent transmission of occupational diseases among employees and proper management of apes in sanctuaries.

1.2 Purpose of the study

The purpose of the study was to establish baseline data on the prevalence of viral pathogens in sanctuary chimpanzees and to develop and implement diagnostic methods in order to formulate strategic control measures that will help to make appropriate decisions in sanctuaries and preparedness for potential reintroductions and management of captive primates.

The specific objectives of this thesis were therefore as follows:

1. To establish and describe selected viral pathogens in wild-born captive chimpanzees on Ngamba island.

2. To analyse the phylogenetic relationship of identified viral pathogens with strains from other primates in other regions.

3. To measure antibody responses to measles and polio vaccines, by age and sex among captive chimpanzees on Ngamba island.

4. To explore non-invasive methods of disease diagnostic for selected viral pathogens in faeces of chimpanzees as non-invasive diagnostic material

1.3 Chimpanzees subspecies and their distribution

The evolutionary history of the common chimpanzee, *Pan troglodytes* has been clearly classified recently based on complete *P. troglodytes* mitochondrial genomes (Bjork et al., 2010). Current genetic data on complete *P.troglodytes* mitochondrial genomes support existence of four chimpanzee subspecies with two major lineages. The western African lineage contains *P.t.verus* found in Senegal, Guinea-Bissau, Guinea, Sierra Leone, Liberia, Mali and Ivory Coast and *P.t.ellioti* (Oates et al., 2009) formerly known as *P.t.vellerosus*, in Nigeria and Cameroon, both of which are monophyletic. The other lineage (the Central/Eastern African group) contains *P.t. schweinfurthii* found in Tanzania, Rwanda, Uganda, Democratic Republic of Congo, Sudan and the Central African Republic, a monophyletic clade nested within the *P.t.troglodytes* found in Congo, Gabon, the Central African Republic, Equatorial Guinea and Cameroon (Bjork et al., 2010)(Fig 1.1a and b).

Genetic as well as some morphological data, suggest strong population within chimpanzees that correlates with subspecies boundaries demarcated by river and habitat boundaries and reinforced by dispersal patterns (Becquet et al., 2007; Gonder et al., 2006; Guy et al., 2003).

Chimpanzees (*Pan troglodytes)* and bonobos (*Pan paniscus*) are our closest species phylogenetically and their genetic variations shed light on their evolutionary histories as well as a comparison of our own history. Estimation of time since recent common ancestor

(tMRCA) based on relaxed molecular clock indicate that the human and chimpanzee plus bonobo lineages diverged 6.5-4.2 million years ago (MYA) (Bjork et al., 2010; Kumar et al. 2005), while chimpanzees and bonobos diverged more recently with estimate of 2.149 (1.684-2.657) MYA (Bjork et al., 2010; Caswell et al. 2008). Within *P.troglodytes*, the tMRCA is now estimated at 1.026 (0.811-1.263) MYA for the four subspecies with two major lineages. One of these lineages (tMRCA=0.510 [0.387-0.650]) contains *P.t.verus* (tMRCA=0.155 [0.101-213] MYA) and *P.t.ellioti* (tMRCA=0.157 [0.102-215] MYA). The other major lineage contains *P.t. schweinfurthii* (tMRCA=0.111 [0.077-146] MYA) a monophyletic clade nested within the *P.t.troglodytes* (tMRCA=0.380[0.296-476MYA]) (Bjork et al., 2010; Stone et al., 2010). In respect to this genetic relationship and geographical interactions, chimpanzees are faced with threats of extinction throughout their home range and recent specific subspecies studies indicate that the Southwest Nigeria chimpanzees are close to extinction (Greengrass, 2009). A dramatic decline in chimpanzee subspecies populations had been documented for *P.t.verus* in Ivory Coast (Campbell et al., 2008) and for *P.t. troglodytes* in Gabon (Walsh et al., 2003) mainly due to uncontrolled forest conversion and natural resource exploitation. The population continues to decline throughout their home range due to commercial hunting facilitated by rapid expansion of mechanized logging, infectious diseases and pet trade (Walsh, 2003).

1.4 Pan African Sanctuaries Alliance

There are 20 primate sanctuaries in Africa caring for confiscated orphaned chimpanzees, gorillas, bonobos and other primates. The influx of these orphaned primates into sanctuaries is alarming and most of the sanctuaries have exceeded their carrying capacity and are considering or have taken on the option of reintroduction. The sanctuaries are unified by the voice of the African primate sanctuary movement referred to as Pan African Sanctuaries Alliance (PASA), which was formed in Entebbe, Uganda, in 2000. The organisation works to ensure premium on standards of health, welfare, husbandry, education, conservation and accountability of member sanctuaries (Farmer, 2002). In 2000, PASA collectively cared for 422 chimpanzees, 60 gorillas and 10 bonobos, along with a unspecified number of baboons, monkeys and other primates. Ten years later, those numbers have risen dramatically to over 800 chimpanzees, 80 gorillas, 55 bonobos and an estimated of over 1000 other endangered primates outside PASA sanctuaries signaling a serious problem (PASA Handbook, 2007). By 2006, the population of

chimpanzees in sanctuaries was growing at a rate of 15% with average of 56 arrivals per year and the population is project to increase to 1800 in 20 years (Faust et al., 2011)

1.4 1. Ngamba Island Chimpanzee Sanctuary (NICS).

Ngamba Island Chimpanzee Sanctuary started in 1998 and is located on Ngamba Island (S 000 06/E 32°39, 0.46km^2, 1160m above sea level) which lies 23km off Entebbe in the north-west of Lake Victoria, and is part of the Koome group of islands, in the Mukono District, Uganda (Fig 1.2).

Ngamba island chimpanzee sanctuary was established to care for orphan confiscated chimpanzees in Uganda. The majority of chimpanzees at Ngamba were once held as pets or sold as pets and were confiscated by Uganda Wildlife Authority (UWA) and other stakeholders, and brought to the sanctuary for long term care. Ngamba Island is a home to forty-four rescued orphan chimpanzees living in semi-captive environment roaming freely on 100acres of forested island every day. However, the island is not big enough to provide their dietary needs and hence their food is 80% supplemented everyday with domestic fruits and vegetables. In addition, all the chimpanzees are brought back into the holding facility which is a large indoor enclosure consisting of several adjacent rooms for night accommodation, management and veterinary care. They have unlimited exposure and contact to staff who provide for their extra needs on daily basis. Because of the sudden influx of orphan chimpanzees in 2000-2001, the sanctuary is almost surpassing its adjusted carrying capacity of 45 individuals. Like any other sanctuary in Africa, Ngamba's resources and capacity has been overstretched by size of the chimpanzee population requiring routine care. NIC runs an ecotourism program as part of conservation education and awareness as well as raising revenue to meet the ever-increasing long term financial needs of managing sanctuary. The tourist turnover is about 5000 tourists per year from all over the world interested in viewing chimpanzees in close proximity including day and overnight visitors. Forest walks are conducted as part of the rehabilitation process where the rescued infant/juvenile chimpanzees are led into the forest by caregivers along with tourists with high levels of close interactions like mutual grooming, play sessions and some infants' trenking on the back/shoulder of tourists and caregivers. This "rehabilitation process" increases levels of human contact with apes and poses a health risk of trans-species transmission of diseases. NIC

implements a Veterinary Preventive Program (VPP) for the chimpanzees and Employee Health Program (EHP) for staff. Prior to arrival of any newly confiscated chimpanzee on the island, they first undergo quarantine at Uganda Wildlife Education Centre (UWEC) for three months during which period they are screened for helminths and protozoa infections and vaccinated against measles, polio and tetanus and screened for tuberculosis at least two times. While on the island, annual health checks are carried out for clinical, biochemical and parasitological parameters. Booster vaccine doses are given at intervals. Sampling is carried out during health checks. One sample for each idividual is bio-banked with the Uganda Virus Research Institute (UVRI), Entebbe for future reference and research. At the same time staff are screened and vaccinated against the same diseases. The tourists, veterinarians and researchers who come into close contact are required to have up to date vaccinations against measles, mumps and rubella (MMR), polio, tetanus, yellow fever and meningiococcal meningitis. Tourists who keep a minimum distance to the chimpanzees are not obliged to have the above vaccinations.

1.5 Methods and Materials

The study was conducted on 42 orphan chimpanzees living in semi-captive management at NICS. The group consisted of 23 females and 19 males with age group categorized as follows: 4 Infants (1 to 5 years), 6 Juveniles (6-8 years), 12 sub-adults (9 to 11 years) and 20 adults (12 years and more). The chimpanzees were brought to the sanctuary at different times after being rescued from illegal traders and poachers since 1998. Rescued individuals are held in quarantine for 90 days during which period they are vaccinated against polioviruses using Oral Polio Virus Vaccine (OPV)- 0.1ml of Sabin Polio (SB Biologicals 52904, 4A) and also vaccinated against measles and tetanus. Routine management of the chimpanzees on the island exposes them to direct human contact by caregivers and veterinarians and indirectly to tourists, school groups, local community members and researchers. They are fed on locally grown fruits and vegetables purchased from local markets.

Samples (blood and faeces) were collected from all chimpanzees resident in the sanctuary under general aneasthesia using a drug combination of Ketamine 3mg/kg and Meditomidine 0.03mg/kg during annual medical health checks in Febuary, 2007 and 2008. Blood (7.5ml) was taken by inguinal venipuncture using EDTA Vacutainer tubes. Serum harvested by centrifugation

and stored at –80^{0}C at the Uganda Virus Research Institute till transported on dry ice to the Robert Koch-Institut, Berlin, Germany for analysis. Retrospective blood and feacal samples collected in 2001 and 2005 and stored at –40^{0}C were additionally investigated in this study.

Serology was performed on serum samples for simian immunodeficiency virus (SIV), human T-lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) and Hepatitis B (HBV). A qualitative enzyme immunoassay kit for the detection of antibodies against human T-lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) was performed on 42 chimpanzee serum samples (Murex Biotech Limited, Central road, Temple hill Dartford DA1 5LR, UK). SIV/HIV antibody detection was perfomed using Murex HIV-1.2.0 kit (Abbot Murex Boitech limited) and GENESCREEN HIV 1.2 version 2 (BIO-RAD) by enzyme immunoassay while Enzygnost Anti-HBc monoclonal (Dade Behring Marburg GmbH, Germany) enzyme immunoassay kit was used for qualitative determination of antibodies to Hepatitis B (core)-antigen in serum obtained from chimpanzees.

Nucleic acids (DNA/RNA) were extracted from blood by the blood and body fluids spin protocol using QIAamp DNA Blood Mini Kit as per manufacturer's instructions and stored at –20°C until use in further analyses. Faecal DNA was extracted using the GeneMatrix Stool DNA Purification Kit (Qiagen). Purified DNA was subjected to Polymerase Chain Reaction (PCR) using specific primers as described in the specific chapters. In some cases PCR product was cloned using the Topo TA Cloning Kit (Invitrogen^{TM}) according to the instructions of the manufacturer. Transformation was done with competent *Escherichia coli* TOP10 cells provided by the manufacturer. The transformed cells were then plated onto Luria-Bertani agar plates supplemented with ampicillin and the plates were incubated overnight at 37°C.

Purified PCR products were sequenced in both directions by using the BigDye terminator cycle kit (Amplied Biosystems) on the ABI Prism 3100 automated sequencer (Applied Biosystems). The obtained sequences were aligned with the ClustalW program, manually edited and imported into PHYLIPversion3.67(http://evolution.genetics.washington.edu/phylip.html). Distance-based trees were generated by using the Kimura two-parameter model in conjunction with the Neighbour Joining (NJ) and minimum-evolution methods in the PHYLIP

software package (Felsenstein, 1989). The topology of the tree was confirmed by Maximum Likelihood analyses. The reliability of the inferred tree was evaluated by bootstrap analysis on 1000 replicates.

1.6. Research Approvals and Permits

Chimpanzee Sanctuary & Wildlife Conservation Trust (CSWCT) granted me permission to undertake the study and use samples collected during annual health checks. Uganda Wildlife Authority (UWA) and Uganda National Council of Science and Technology (UNCST) approved my research proposal and granted me permits to conduct this research under permit reference nos: UWA/TBDB/RES/50 and NS71 respectively. C**onvention on International Trade in Endangered Species** of Wild Fauna and Flora (CITES) under their respective offices in Uganda and Germany granted import and export permits as a requirement to transport biological samples from endangered species to Robert Koch Institute, Berlin, Germany for the purpose of this research. Copies of the research approvals and permits are included as appendix at the end of this thesis.

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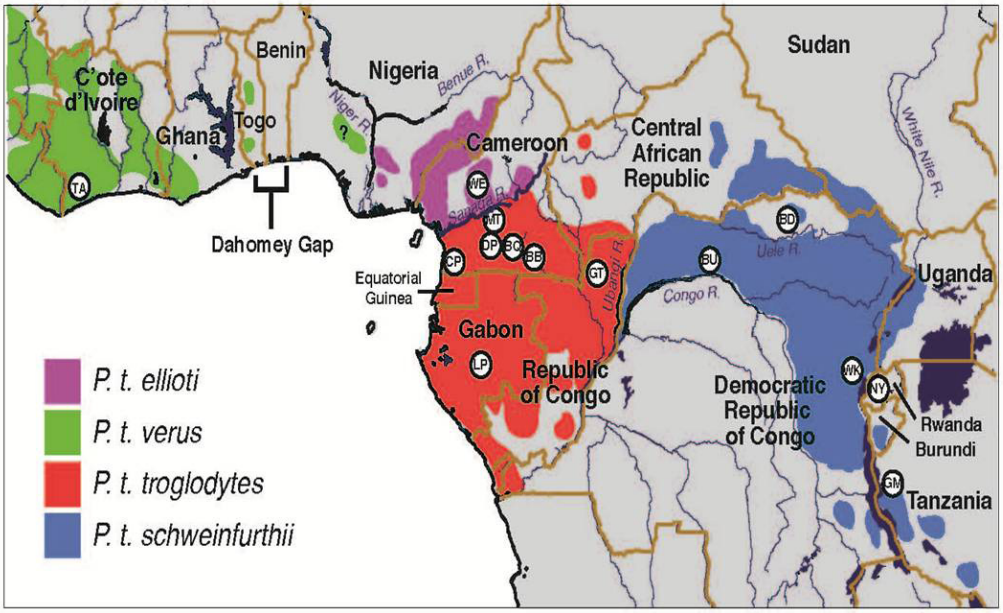
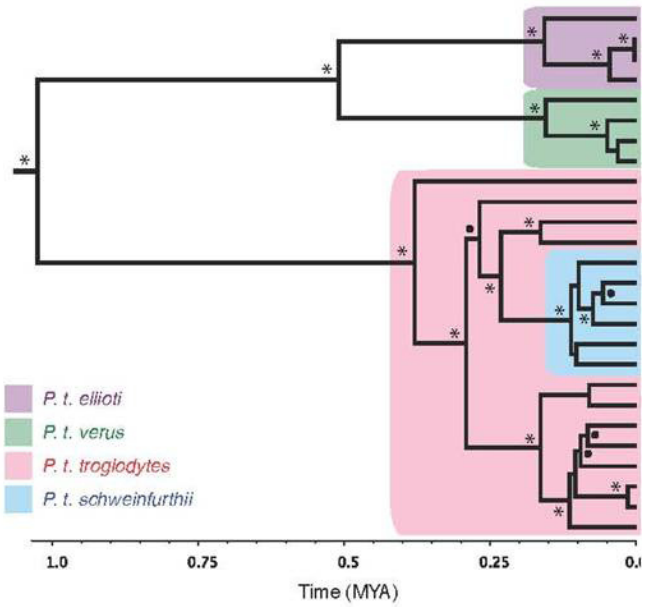
Figure 1. 1a and b. Geographic distribution of chimpanzee's subspecies and their phylogenetic relationship

a)

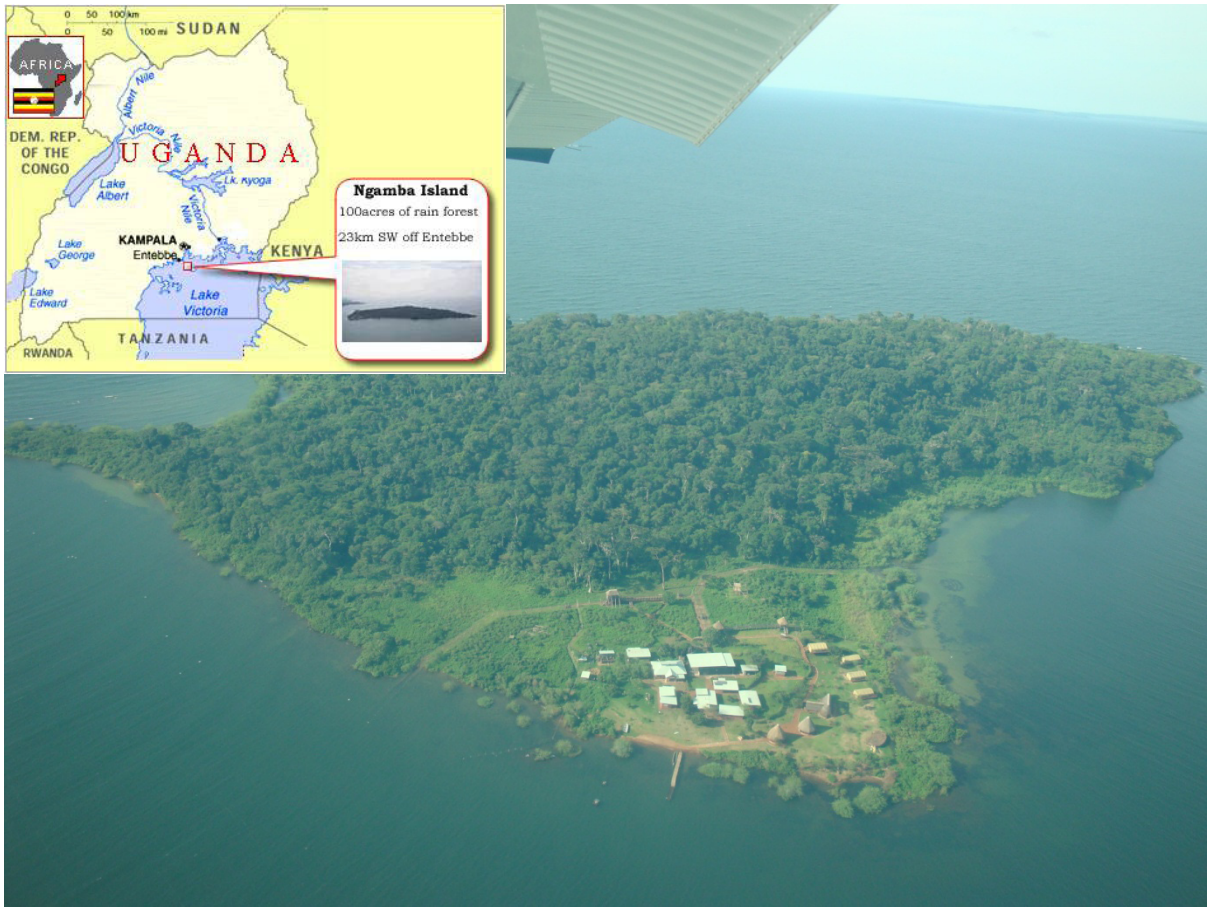


b)



Source: Bjork et al., 2010

Figure 1.2. Map of Uganda showing the location of Ngamba Island and aerial photographic view of Ngamba Island Chimpanzee Sanctuary.



CHAPTER TWO

MULTIPLE VIRAL INFECTIONS IN CONFISCATED WILD BORN SEMI-CAPTIVE CHIMPANZEES (PAN TROGLODYTES SCHWEINFURTHII) IN A SANCTUARY IN UGANDA: IMPLICATIONS FOR SANCTUARY MANAGEMENT AND CONSERVATION

Multiple viral infections in confiscated wild born semi-captive chimpanzees (*Pan troglodytes schweinfurthii*) in a Sanctuary in Uganda: Implications for sanctuary management and conservation.[1]

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2.0 Abstract

Confiscated 42 wild-born semi-captive chimpanzees living in a sanctuary on Ngamba Island, Uganda were screened for a broad range of viral pathogens to determine the prevalence of specific viral infections some of which may be cross reacting with human viruses. Specific Polymerase Chain Reaction (PCR) and serological assays performed on chimpanzee samples (blood and faeces) revealed multiple viral infections with zoonotic potential. The viral infection prevalence in chimpanzees were 85.7%, 73%, 60.5% and 32.4% for adenoviruses, simian foamy viruses, gammaherpesviruses and hepatitis B virus, respectively. They were negative for simian/human immunodeficiency virus (SIV/HIV), human T-lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) antibodies, hepatitis C virus (HCV), hepatitis E virus (HEV), flavivirus, human-metapneumovirus (HMPV), and chikungunya viruses. These results indicate that wild-born captive chimpanzees are infected with multiple viral pathogens with potential for inter- and intra-species transmission. The data has implications for sanctuary management and conservation efforts and implies usefulness of incorporating sanctuary primates into emerging infectious disease research programs.

2.1. Introduction

Newly emerging diseases can become major threats to public health and animal reservoirs are implicated as the major sources of these emerging diseases (Aluwong and Bello, 2010; Daszak et al., 2000; Jones et al., 2008; Pederson et al. 2007). Viruses emerging from wildlife hosts have caused high-impact diseases as severe acute respiratory syndrome (SARS), Ebola fever and influenza in humans. Infectious agents from non-human primates in particular have caused a number of new human diseases, leading to calls for international surveillance to monitor the human-nonhuman primate interface. This is attributed to the close phylogenetic relationship between humans and nonhuman primates and associated anthropogenetic disturbances of primate habitats resulting in a high potential for pathogen exchange (Davies and Pederson, 2008; Gillespie et al., 2008; Wolfe et al., 1998). Indeed, approximately 75% of emerging human infectious diseases are zoonotic from wildlife, livestock or their products and frequently carnivores serves as sources of infections (Taylor et al., 2001; WHO, 2007).

Primate-associated zoonotic diseases have received dramatic global attention. A novel HIV-1 lineage (HIV-1 group P) distinct from HIV-1 groups M, N, and O closely related to gorilla simian immunodeficiency (SIVgor) was discovered in a Cameroonian woman (Plantier et al., 2009). HIV 1 and 11 emerged from a simian variant of the virus SIVcpz (Gao et al., 1999; Hahn et al., 2000; Keele et al., 2006; Peeters et al., 2002) and caused global HIV/AIDs pandemic. Other primate retroviruses have since been shown to have crossed the barrier and infected humans as for example simian foamy virus (Calattini et al., 2007; Wolfe et al., 2004).

The emergence of these new viruses and many other human diseases have occurred after an established animal virus switched from its animal hosts into humans and subsequently transmitted within human populations. Transfers between different animal hosts lead to the analogous emergence of epizootic diseases (Parrish et al., 2008). Three likely stages of viral disease emergence leading to successful host switching have been described (Woolhouse et al., 2005): (i) initial single infection of a new host with no onward transmission (spill-overs into "dead-end hosts"), (ii) spillovers that go on to cause local chains of transmission in the new host population before epidemic fade out (outbreaks), and (iii) epidemic or sustained endemic host –to-host disease transmission in the new host population. These stages are affected by a number of variables that affect successful disease emergence such as: the type and intensity of contacts between the reservoir (donor) host or its viruses and the new (recipient) host; host barriers to infection at the level of the organism and cell; viral factors that allow efficient virus spread within the new host population (Morse, 2004). The risks imposed by such initially unrecognized viruses are highlighted by the emergence of SARS, coronavirus (CoV), hantaviruses and human immunodeficiency virus type 1 (HIV-1) and HIV-2. These established enzootic viruses switched hosts beyond the species border being unknown before their emergence in humans (Holmes et al., 2005; Wolfe et al., 2005; Wong et al., 2007). Other important human viruses (e.g measles and small pox), may have originated in wildlife or domesticated animals in prehistoric times (Wolfe et al., 2007).

At the same time great ape populations are being driven to extinction by emergence of infectious diseases in great ape populations some of which are of human origin (Gillepsie et al., 2008; Leendertz et al., 2006). Polio and measles have caused high mortalities in chimpanzees

and gorilla populations respectively (Ferber, 2000; Goodall, 1983; Kortland, 1996). Human respiratory syncytial virus (HRSV) and metapneumovirus (HMPV) caused mortalities in chimpanzees in Tai Forest in Cote d'Ivoire (Kondgen et al., 2008) following habituation for research. HMPV was also implicated in chimpanzee respiratory disease mortalities at Mahale, Tanzania, which suffered a decline in the population of habituated chimpanzees (Kaur et al., 2008). 49% of chimpanzee deaths documented during 47 years of study at Gombe, Tanzania were attributed to respiratory diseases (Williams et al., 2008). Other confirmed transmissions of potential pathogens from humans and domestic animals to wild primates have been documented. For example, gorillas and chimpanzees in Uganda have recently been shown to be infected with human strains of bacteria (Goldberg et al., 2008; Rwego et al., 2009). New *streptococcus pnuemonie* clones were found in deceased chimpanzees in the Tai Forest of Cote d'Ivoire (Chi et al., 2007). Great ape populations are also being driven to extinction from exposure to novel pathogens from wildlife reservoirs. Ebola virus has caused 80% decline of gorilla and chimpanzee populations at the Gabon and Republic of Congo border in the last two decades (Bermejo et al., 2007; Thibault et al., 2003; Huijbregts et al., 2003; Leroy et al., 2004; Leroy et al., 2005 Walsh et al., 2003). In addition, Anthrax caused by *Bacillus anthracis* has led to epidemics in chimpanzee populations (Leendertz et al., 2004b; Leendertz et al., 2006a). Transmission of human pathogens to NHPs has been recognized in captive facilities since decades (Ruch, 1959; Brack, 1987) but recent documentations of disease outbreaks in wild great apes from human calls for concerted efforts to save great apes from extinction. The human-primate interactions have increased dramatically in the recent past due to exponential human population growth, habitat destruction and fragmentation with high risks of inter- species pathogen transmission (Gillispie et al., 2005b; Gillespie and Champman, 2006, 2008). A variety of primate behaviors (ranging, inter-individual and inter-group associations, foraging, grooming, and interspecies contacts such as preying upon other primates) are a contributing factor to the observed disease patterns. For example, West African chimpanzees have been shown to have acquired the retrovirus Simian T-Cell leukemia Virus and Foamy viruses from their main prey, the red colobus monkey (*Procolobus badius*) (Leendertz et al., 2004c; 2008). Recently, Uganda red colobus from Kibale (*Procolobus*[*Pilocolobus*] *ruformitratus tephrosceles*) were found to be infected with simian retroviruses representing distinct and divergent strains in the *Spumavirus*

(SFV) and *Lentivirus g*enera (SIV) and novel *Deltavirus* lineage (STLV) (Goldberg et al., 2009) which further shows implications of predator-prey relationship in disease transmission.

Pan Africa Sanctuaries Alliance (PASA) sanctuaries (currently 20 sanctuaries) were created over the last three decades to accommodate and care for high numbers of orphaned chimpanzees, gorillas, bonobos and other endangered primates in Africa. These are located outside the natural habitats and some outside the known home range of wild primates close to human communities. Disease management is not or not well addressed in sanctuaries often overshadowed by other ethical and welfare requirements for the rescued individuals. Hence the management of these sanctuaries presents an additional risk of disease transmission that exposes naïve population of primates to human pathogens and vice versa. The role of sanctuaries as potential hotspots for emergence of infectious diseases and cross-species transmission of dangerous pathogens is underestimated and has received little attention. For example, screening for viral pathogens of wild-born captive chimpanzees at Ngamba Island has not been done since establishment of the sanctuary in 1998 with 17 individuals and now with 44 chimpanzees on 43 hectares of forest on Ngamba Island on L. Victoria. The results from this study presents the first extensive results of viral pathogens carried by wild-born-captive chimpanzees within sanctuaries and will provide baseline data for a wide range of diseases to be monitored in sanctuaries during the entire process of quarantine, management, re-introduction and post-reintroduction and by wild great ape health monitoring projects.

2.2. Methods and Materials

2.2.1. Sample collection and nucleic acid extraction

Blood and faeces were collected from chimpanzees at Ngamba Island in Uganda during the annual health checks under general aneasthesia which composed of 23 females and 19 males (Table 2.1). Blood was carefully drawn from femerol vein into vacutainer EDTA, SST and CPT tubes. The samples in CPT tubes were processed using a cell preparation procedure within 12 hours to extract leucocytes from buffy coat and stored under liquid nitrogen. The samples in SST and EDTA tubes were was centriguged to extract serum and plasma respectively and stored stored at –80^{o}C. Faeces were collected rectally and stored at -80 C. All samples were transported on dry ice to the Robert Koch Institut, Berlin, Germany for analysis.

2.2.2. Serology

Serology was performed for SIV, human T-lymphotropic virus types I and II (HTLV-I and HTLV-II) and Hepatitis B (HBV). A qualitative enzyme immunoassay kit for the detection of antibodies against human T-lymphotropic virus types I and II (HTLV-I and HTLV-II) was performed on 42 chimpanzee serum samples (Murex Biotech Limited, Central road, Temple hill Dartford DA1 5LR, UK) as previously shown to detect STLV-1 effectively in wild chimpanzees (Leendertz et al., 2004). SIV/HIV antibody detection was perfomed using Murex HIV-1.2.0 kit (Abbot Murex Boitech limited, Dartford, UK) and GENESCREEN HIV 1.2 version 2 (BIO-RAD,Munchen, Germany) by enzyme immunoassay while Enzygnost Anti-HBc monoclonal (Dade Behring Marburg GmbH, Germany) enzyme immunoassay kit was used for qualitative determination of antibodies to Hepatitis B (core)-Antigen in serum obtained from chimpanzees as per manufacturer's instructions. Antibody titers of all vaccinated chimpanzees against measles and polio using human based vaccine and schedule was analysed using established serological assay and microneutrisation test (WHO, 2004) test respectively.

2.2.3. Polymerase Chain Reaction (PCR)

A number of single and multiplexPCR were carried out on nucleic acids (DNA and RNA) extracted from blood cells and faeces to identify a number of viruses as previously described with details in specific chapters: Simian foamy viruses (Hussain et al., 2003); Herpesviruses (Ehlers et al., 2008; Goltz et al., 2002); adenoviruses (Chmielewics et al., 2005); Hepatitis Viruses (B, C, E) (Adlhoch et al., 2009) and Human metapnuemovirus (Mackay et al., 2004).

2.3. Results

All wild-born semi-captive chimpanzees screened in the study were infected with more than one virus both previously described and novel viruses in some individual chimpanzees. We report for the first time results of extensive study using both PCR and ELISA methods for detection of viral pathogens and antibodies from chimpanzees in sanctuaries in Africa with viral prevalence of 85.7%, 73%, 60.5% and 32.4% for adenoviruses, simian foamy viruses, gammaherpesviruses and hepatitis B virus respectively. Screening for SIV/HIV, human T-lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) antibodies and Hepatitis C virus (HCV), Hepatitis E Virus (HEV), Flavivirus, Human metapnuemovirus, Chikungunya virus

nucleic acids was negative (Table 2.2). The results of Hepatitis C virus (HCV), Hepatitis E Virus (HEV), Flavivirus, Human metapnuemovirus, Chikungunya virus which were negative are not included in the table of results.

In this study we extended our search by using some of the established non-invasive methods in comparison with other methods by analyzing both genomic DNA and proviral RNA extracted from buffy coat and faecal samples of individual chimpanzees along with enzyme immunoassay on serum (Table 2.2). Interestingly, all samples were negative for adenovirus on PCR from genomic DNA extracted from blood but 85.7% positive on PCR performed on DNA extracted from faecal samples. For gammaherpesviruses, only three were detected on DNA extracted from faeces including two individuals (Asega infected with PtroLCVI and Kidogo with PtroRHV-1) that were negative on genomic DNA from blood (Mugisha et al., 2010b). For HBV, Mika was both infected on the analysis of genomic DNA from faeces and blood. We were not able to detect the virus on PCR from DNA extracted from blood and faeces from the rest of 11 chimpanzees reactive for HBV on ELISA.

To assess some of the disease preventive measures using vaccination employed by most sanctuaries, the chimpanzees were screened for antibody titers for polio and measles post vaccination in 2001 and 2007. Only 4 chimpanzees had detectable protective titers for measles for 2001 and 2007 screened samples. Conversely, more than 80% of chimpanzees had neutralizing antibodies against polio virus type 1, 2 and 3 (Mugisha et al., 2009) in chapter six.

Statistical results using 1-sided Fisher's exact test and Exact logistic regression did not reveal any effect/ association of identified viral infections with age, sex and origin except for HBV (1-sided fishers exact analysis (P=0.004)) where HBV was more prevalent in adults than non-adults. There was no link or effect of infection of one virus on another virus indentified in the same individuals.

2.4. Discussion

The Ngamba sanctuary wild born semi captive chimpanzees were found infected with a wide range of viruses with known and unknown zoonotic potential. We found viral infection prevalence of 85.7%, 73%, 60.5% and 32.4% for the adenoviruses, simian foamy viruses, gammaherpesviruses and hepatitis B virus respectively. Characterisation of the indentified viruses phylogenetically in this study is discussed in the proceeding chapters (Mugisha et al., 2010a, b, c). No evidence of SIV and STLV infections was found in these chimpanzees in contrast to other studied captive and wild chimpanzees mainly from central and west Africa in which these viruses are known to be enzootic for nonhuman primates (Corbet et al., 2000; Hahn, et al., 2000; Leendertz et al., 2007; Santiago et al., 2002; Worobey, 2010). Both these viruses are old viruses that are thought to have coevolved with nonhuman primates species over millions of years. It is known that more than 70 species of primates inhabiting sub-Saharan Africa harbor over 40 SIV-related lentiviruses (Bibollet-Ruche et al., 2004; Keele et al., 2006; Peeters et al., 2002; Worobey, 2010), three of which have crossed the species barrier and generated human immunodeficiency virus types 1 (HIV-1, HIV-1 variant and HIV-2) (Hahn et al., 2000; Sharp et al., 2005; Plantier et al., 2009). SIVcpz seem not to have coevolved with chimpanzees unlike in other primate hosts since as of to date only two chimpanzee subspecies, the central *P. t. troglodytes* and eastern *P. t. schweinfurthii*, are naturally infected by SIVcpz. (Hahn, et al., 2000; Sharp et al., 2005; Van Heuverswyn et al., 2007). Recently SIV strain designated SIVgor was found in wild living gorillas in West Africa living nearly 400km apart suggesting that SIV is endemic in gorillas as in chimpanzees (Van Heuverswyn et al., 2006). The gorilla SIV viruses clustered together, forming a monophyletic lineage within the SIVcpzPtt radiation that was much more closely related to HIV-1 group O than was any other known SIV hence discovery of HIV-1 group O-like viruses in wild gorillas (Van Heuverswyn et al., 2006). Hence it is surprising that all 42 wild born chimpanzees of eastern *P. t. schweinfurthii*, were negative for SIVcpz antibodies. This can probably be explained by the fact that most of the apes in sanctuaries were taken out of the forests when they were still young, well protected by their mothers and most likely had not encountered any aggressions risky for transmission of this virus. The most recent findings that SIVcpz, the immediate precursor of HIV-1, is pathogenic causing immunodefiency syndrome (AIDS) in free-ranging chimpanzees (Keele et al., 2009) and identification of a new human immunodeficiency virus (HIV-I group P) in a Cameroonian

woman closely related to SIVgor (Plantier et al., 2009) highlights the significance of testing all rescued primates on arrival and presents new challenges to the management of sanctuaries. Our study reveals multiple viral infections in most of the individual chimpanzees screened with at least more than one virus including a novel herpes virus (Mugisha et al., 2010). This shows that chimpanzees and other apes harbour a lot of viruses some of which are still unknown. Recent studies in primates have revealed many viral pathogens with unknown pathogenicity as well as consequences after cross-species transmission to humans. Examples include discovery of 2 novel gammaherpesviruses in captive gorilla and monkey (Ehlers et al., 2008); 10 betaherpesviruses in wild great apes (Leendertz et al., 2009); 30 adenoviruses from apes and 3 adenoviruses from macaques (Roy et al., 2009); 1 gammaherpersvirus in chimpanzees (Mugisha et al., 2010).

In this study, we found 85.7% prevalence of adenovirus infection in wild captive chimpanzees which is in agreement with recent findings that healthy populations of great apes (chimpanzees, bonobos, gorillas and orangutans) shed substantial quantities of infectious adenoviruses in stool (Roy et al., 2009). Their study also revealed evidence for intra-species recombination between adenoviruses, high degree of phylogenetic relatedness across their primate hosts providing evidence of cross species transmission. The shedding of live adenoviruses in stool presents high risks of zoonotic transmission to caregivers in sanctuaries coming into close contact with primate faecal material during cleaning of housing facilities. Adenoviruses are classified into into six subgroups A to F, currently referred to as species HAdV-A through HAdV-F (Bailey and Mautner, 1994). Lethal adenovirus infections have been observed in human patients who are immune suppressed (Hierholzer, 1992; Kojaoghlanian et al., 2003). Infections with HAdV-C are a common cause of pediatric upper respiratory infections worldwide; later in life HAdV-B has been found to cause epidemics in adults (Kolavic-Gray et al., 2002).

We also document 73% prevalence of simian foamy virus infection in chimpanzees (Mugisha et al., 2010). SFV has received increasing attention following recent findings of SFV human infections from a wide variety of primates (Sandstrom et al., 2000; Jones-Engel, et al., 2005; Wolfe et al., 2004; Switzer et al., 2004; Jones-Engel et al., 2006, 2008; Calattini et al., 2007; Goldberg et al., 2009). This poses high risks of SFV transmission to caretakers present in sanctuaries which need to be addressed through review of management protocols.

The documented viruses in chimpanzees in this study with pathogenic zoonotic potential plus other already described viral pathogens in primates can easily be cross transmitted to humans given the current levels of interactions. Contact between the donor and receipt hosts is a precondition for virus transfer affected by geographical, ecological and behavior factors. Trade in wildlife, bushmeat hunting, human population expansion, environmental factors like deforestation and agriculture expansion promote viral emergence and viral host switching from animals to humans and vice versa. Animal husbandry practices in sanctuaries promote close interactions of staff with apes under their care especially through feeding and rehabilitation processes (Fig. 2.1a, b). In some sanctuaries this has even extended to tourists as part of raising awareness for the great apes. There is high level of interaction during rehabilitation of newly rescued baby chimpanzees and in some cases involve interaction with domesticated animals (Fig. 2.1 c, d) All these behavioral activities are being done by individuals without the knowledge of the potential risks of infectious disease acquisition and transfer between the animal and person undertaking rehabilitation process.

Regarding tourists, it has been noted that a large proportion of travelers to tropical regions are not protected against vaccine-preventable diseases and the majority of the travelers demonstrate poor recall of actual vaccination status (Muehlenbein et al., 2008). The described behaviours plus the health status of tourists visiting primate sanctuaries and participating in some rehabilitation programs poses risks of anthropozoonotic transmission of infectious diseases with un predetermined threat to survival of apes and other wildlife. It is already known that travel is a potent force of disease emergence. Migrations of humans has been the pathway for disseminating infectious diseases throughout recorded history and will continue to shape the emergence, frequency and spread of infections in geographic areas and populations (Wilson, 1995). This close proximity plus other stress related factors like overcrowding increases chances of disease cross transmission.

Apes or other primates in some of the sanctuaries/captive facilities are vaccinated against major communicable diseases where vaccines are available and recommended like measles, polio, tetanus. Unfortunately little or no information is available on vaccines for apes and in most cases human based vaccines and recommendation have been adopted and assumed to

confer immunity in vaccinated apes. In our current long term evaluation of polio and measles vaccination in chimpanzees only 4 of 42 chimpanzees vaccinated against measles in 2001 had protective immunity. Therefore it seems measles vaccine in chimpanzees only offers limited protection and is variable but this needs further studies to have mere conclusive results. Conversely more than 70% of chimpanzees vaccinated with oral polio vaccine had protective immunity to all poliovirus serotypes 9 years post vaccination (Mugisha et al., 2010). With this background, it seems that the most appropriate way is to enforce vaccination in humans where the responses and levels of protection are established for most vaccines as more studies are undertaken in great apes. Periodic check ups post quarantine should be undertaken preferably 2 to 3 years and should include complete physical examination, body measurements, full blood count, kidney and liver functional tests, urinalysis, TB screening, screening for helminthes and protozoa and samples should be submitted to appropriate laboratory for screening major viral and bacterial pathogens. The information generated from these examinations will help performing disease risk analysis, management of group dynamics (re-socialization and integration) and planning for conservation programs like re-introductions. The results from this study presents the first extensive study for viral pathogens carried by wild-born-captive chimpanzees within sanctuaries and will provide baseline data for a wide range of diseases to be monitored in sanctuaries during the entire process of quarantine, routine management, re-introduction and post-reintroduction and by wild great ape health monitoring projects.

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Table2.1. Age and sex distribution of Ngamba chimpanzees considering only 3 categories due to difficulties of age estimation for juveniles and sub adults

Categorisation	Age group	Female	Male	Total
Infants	1 to 5 yrs	1	3	4
Sub adults	6 to 11yrs	9	10	19
Adults	12 yrs and above	13	6	19
Total		23	19	42

Table 2.2. Shows results of viruses investigated using ELISA and PCR methods in wild-born captive chimpanzee on Ngamba Island.

Virus type	No of chimps tested	Detection methods			Total Positive	
		ELISA	PCR (Blood)	PCR (Feacal)		
SIV/HIV	42	Nd	0	Nd	0	Mugisha et al., 2010
HTLV I &II	42	Nd	0	Nd	0	
SFV	38	Nd	28	0	28	
HBV	37	12	2	1*	12	Mugisha et al., 2011
Adenoviruses	39	Nd	0	32	32	In this study
Gammaherpesviruses	40	Nd	22	3	22	Mugisha et al., 2010
Poliovirus 1,2,3	42	>28	Nd	nd	>28	Mugisha et al., 2009
Measles	42	2	Nd	nd	2	In this study
Others	40	Nd	0	Nd	0	In this study

Others, (Hepatitis C Virus (HCV), Hepatitis E Virus (HEV), Flavivirus, Human-metapneumovirus (HMPV), and Chikungunya virus); .nd, not done

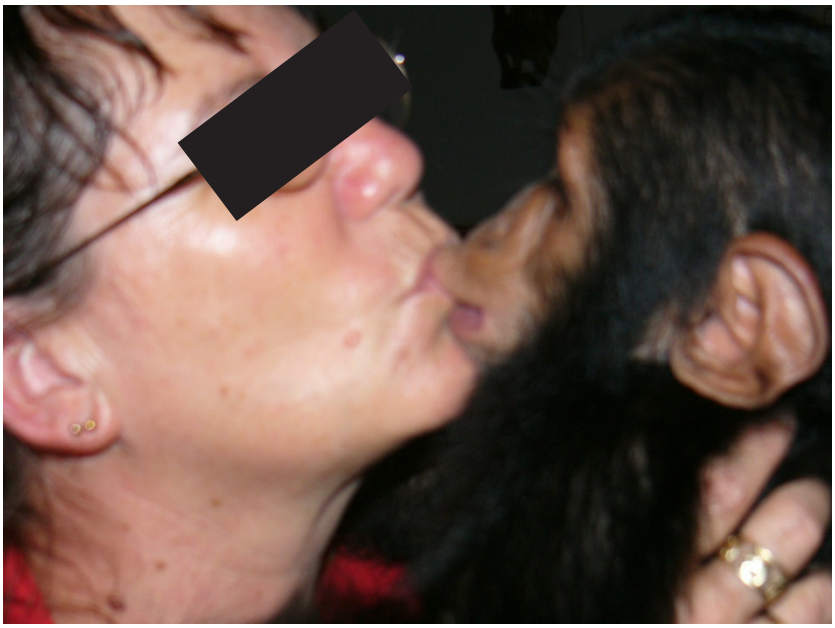
*only feacal samples was available from Mika and not from Kiiza, a wild chimpanzee positive for HBV DNA

Figure 2 1 (a, b, c, d). Interactions of chimpanzees with humans and domestic animals

a)

b)

c)



d)

CHAPTER THREE

RETROVIRUSES IN WILD-BORN SEMI-CAPTIVE EAST AFRICAN SANCTUARY CHIMPANZEES *(PAN TROGLODYTES SCHWEINFURTHII).*

Running title: Retroviruses in Sanctuary Chimpanzees

Retroviruses in wild-born semi-captive east African Sanctuary Chimpanzees (*Pan troglodytes schweinfurthii*)[2].

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Keywords: SIV, STLV, SFV, Orphan Chimpanzees

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3.0. Abstract

Information on retroviruses infections in great apes is scarce, especially for apes kept in sanctuaries throughout Africa. To investigate the prevalence of retroviruses and possible transmission of different retroviruses originating from chimpanzees of different origin (Uganda, Congo and Rwanda), 38 wild-born captive orphan chimpanzees residing in a sanctuary on Ngamba Island were analyzed for retroviral infections. Samples from sanctuary chimpanzees were analyzed using enzyme-linked immunoassays and polymerase chain reactions (PCR). Viruses were characterized by phylogenetic analysis. All chimpanzees were negative for antibodies against Simian Immunodeficiency Virus (SIV) and Simian T-cell Leukemia Virus (STLV). However, 28/38 (73%) chimpanzees were positive to Simian Foamy Virus (SFV) by analysis of a 425-bp DNA segment obtained by PCR using generic *integrase* primers homologous to highly conserved portions of the *polymerase* gene. Phylogenetic analysis of SFV sequences obtained in this study formed four sub clusters within the specific SFV *P. t. schweinfurthii* clade with significant variability among the new SFVs strains.

We provide evidence of on-going cross-transmission of SFV among chimpanzees within the sanctuary mostly likely through horizontal routes. We propose to test all chimpanzees introduced into sanctuaries for retroviral and other infections. This will help avoid the spread also of pathogenic viruses in captive populations.

3.1. Introduction

Simian Immunodeficiency Virus (SIV), Simian T-cell Leukemia Virus (STLV) and Simian Foamy Virus (SFV) are known to occur in wild populations of chimpanzees. However, the prevalence of these viruses varies significantly. SIV is found only in some specific groups of wild chimpanzees with low prevalence within the group (Keele et al., 2009; Peeters et al., 2008). SFV in contrast was present in all wild chimpanzee groups studied. The prevalence rates ranged from 44% to 100% with several individuals infected with more than one strain (Leendertz et al., 2008; Liu et al., 2008). An age-dependent prevalence was revealed, with older individuals showing a higher prevalence (Lui et al., 2008). Not much is known about the prevalence of STLV in wild chimpanzees. Data from Côte d'Ivoire suggest a significantly higher prevalence among chimpanzees older than nine years with a seroprevalence of 71.4% compared to 9.7% in individuals younger than nine years (Leendertz et al., 2004).

To understand the age distribution of animals brought to chimpanzee sanctuaries, it has to be mentioned that in order to capture one infant chimpanzee from their community in the natural habitat, poachers have to shoot three to five adults. The adults are used for commercial/ domestic bushmeat trade while the babies are sold for pet trade. The latter are those ending in many cases in sanctuaries throughout Africa. Capturing of wild chimpanzees is strictly forbidden in all great ape range countries but still every year many infant chimpanzees are confiscated from illegal dealers or private owners. Between 2001 and 2005, 5 to 95 infant chimpanzees were received through confiscations per year in 19 Pan African Sanctuary Alliance (PASA) primate sanctuaries though the trend is declining in the last years (PASA, unpublished data). The age of rescued infant chimpanzees ranges from one to five years with some being rescued after staying with their illegal owners for two to three years.

Here we show investigations on the prevalence of SIV, STLV and SFV in wild-born chimpanzees of the Eastern subspecies (*P. t. schweinfurthii*) at the Ngamba Island Chimpanzee Sanctuary (NICS), Uganda. Animals originated mainly from Uganda and the Democratic Republic of Congo and a few from Rwanda (Table 3.1). Furthermore we provide molecular data on the SFV strains detected.

3.2. Materials and Methods

The chimpanzee sanctuary started in 1998 and is located on Ngamba Island (S 000 06/E 32˚39, 0.46 km^2, 1160 m above sea level). Chimpanzees were brought to the sanctuary after being rescued from illegal traders and poachers. The island is 100 acres of secondary rain forest, lies 23 km off Entebbe in the north-west of Lake Victoria, and is part of the Koome group of islands in the Mukono District, Uganda. The samples investigated in this study were obtained from 38 orphan chimpanzees in semi-captive management at NICS, following research approval from the relevant national authorities. The individuals tested consist of 21 females and 17 males. Table 3.1 summarizes the age at the time of introduction into the sanctuary and origin of the chimpanzee. Apart from the initial group of 17 chimpanzees that had lived together before being brought to the island, most of the chimpanzees arrived at the sanctuary at the age range from three to five years. The chimpanzees underwent a three-month quarantine period but no serological or PCR analyses were done to screen for viral pathogens.

During an annual medical health check in February, 2007, blood was collected from all chimpanzees under general anesthesia. Whole blood (10 ml) was taken by inguinal venipuncture using CPT Vacutainer tubes. 8 ml of blood was centrifuged at 1600 g (2800 rpm) for 30 minutes. Two aliquots of the buffy coat were refrigerated at 4°C for 15 minutes and then stored in cryotubes in liquid nitrogen at the Uganda Virus Research Institute till transported on dry ice to the Robert Koch-Institut in Berlin/Germany for analysis. Serum was stored at -20°C. The permit to export biological samples from endangered species was obtained from Uganda and German offices for the Convention on International Trade in Endangered Species (CITES).

DNA was extracted from the buffy coat using the QIAamp DNA Blood Min Kit (Qiagen, Hilden, Germany). Amplification of SFV proviral genomic region (425 bp; 6086 – 6492 on SFV reference sequence NC_001364) was performed using generic primers targeting the viral integrase (*int*) region as previously described (Hussain et al., 2003). Standard PCR conditions were used for both rounds of amplification with the exception of the annealing temperatures of 60°C for 45 cycles for the first round and 55°C for 45 cycles for the second round, respectively. Generic and nested PCR products were visualized in 1.5% agarose gels stained with ethidium bromide under UV light.

The purified PCR products were sequenced in both directions with PCR primers using the BigDye terminator cycle kit (Applied Biosystems, Darmstadt, Germany) on an ABI Prism 3100 automated sequencer (Applied Biosystems).

Sequences were aligned with the ClustalW program, manually edited and imported into PHYLIPversion3.67. The following reference sequences were used: GenBank accession numbers were AY686179, AY583781; AY278782, X83293, AY686201, X83290, DQ354075, DQ354089, X83292; AY686203, AY686206, AY195688, AY278792, AY278776, AF049086, AY686195, AY195685, X83297, AY195682, X83296, AY639122, AY639141, AY639133, EU527588, AY639136, AY639123, AY639124, AY639138, AY639128, AY639130; EU527540, EU527532, X83294, EU527541, EU527501, EU527498, EU527637, AY195676, AY195675, EU527499, EU527496, EU527534, EU527502, X83298. Distance-based trees were generated using the Kimura two-parameter model in conjunction with the Neighbor Joining (NJ) and minimum-evolution methods in the PHYLIP software package (Felsenstein, 1989). The

topology of the tree was confirmed by Maximum Likelihood analyses. The reliability of the inferred tree was evaluated by bootstrap analysis on 1000 replicates.

For serological analyses a commercially available enzyme immunoassay kit for the detection of antibodies against human T-lymphotropic virus types I and II (HTLV-I/-II) was used (Murex Biotech Limited, Dartford, UK) according to the manufacturer's instructions. This test has been shown to efficiently detect STLV-1 in wild chimpanzees (Leendertz et al., 2004). Simian Immunodeficiency Virus (SIV) antibody detection was performed using the Murex HIV-1.2.0 kit (Abbott Murex Biotech Limited) according to the manufacturer's instructions and the GENESCREEN HIV 1.2 version 2 (BIO-RAD, München, Germany) which were designed for the detection of antibodies directed against human immunodeficiency virus and is widely used for detection of SIV in non-human primates (data will be shown elsewhere).

3.3. Results and Discussion

DNA samples from 38 chimpanzees were available, 28 (73.7%) were positive for SFV by PCR (Table 3.1). All serum samples were negative for SIV and HTLV/STLV antibodies. These results showed that none of the chimpanzees in the sanctuary was infected with SIV or STLV.

Int sequences from 23 out of 28 chimpanzee blood samples positive for SFV DNA could be used for sequence analysis. Five sequences were of poor quality due to low PCR signal and were therefore not included in this analysis. The nucleotide sequences obtained in this study were submitted to GeneBank (NCBI) and published under the accession numbers EU239510–EU239533.

Phylogenetic tree analysis was performed with the NJ method using the 23 SFV sequences generated in this study and published relevant SFV sequences from all chimpanzee subspecies, as well as from gorilla, bonobo, orangutan, African and Asian monkeys and one sequence of an SFV-infected human (Calattini et al., 2007; Hussain et al., 2003; Liu et al., 2008; Switzer et al., 2004; Wolfe et al., 2004).

The obtained sequences group fell within the SFV clade of the Eastern chimpanzee subspecies (*P. t. schweinfurthii*) (Fig. 3.1). The subspecies status of the chimpanzees studied plus their genetic diversity and association was established as *P. t. schweinfurthii* by analysis of mtDNA (data not included here). Within this clade, *the* 23 SFV sequences grouped in four subclusters with other recently published SFV strains from East African chimpanzee subspecies. While 22 sequences grouped in subclusters 1 to 3, one strain obtained from the individual "Umutama" clusters with recently published sequences obtained from wild chimpanzees in subcluster 4.

SFV strains from some individuals were genetically more closely related than others. The SFV strain from "Robbie", a former alpha male of the community, forms a subcluster with SFV strains from the chimpanzees "Kyewunyo", "Connie", "Baluku", "Umugezi" and "Nagoti" with 99.5 to 100.0 % nucleotide identity. "Mika", the current alpha male, has 99% to 100% nucleotide identity with "Rutoto", one of the new infant arrivals at the sanctuary, and "Becky". Also sequences of "Sunday", "Masiko" and "Ikuru" showed 99.9% nucleotide identity. It is noteworthy to mention that "Ikuru" has been frequently bitten by the other two chimpanzees. In order to investigate possible co-infections with different SFV strains, the PCR product of one chimpanzee ("Becky") was cloned and sequenced. The SFV sequences generated belong to two different sub clusters. This demonstrates a one case co-infection with SFV strains. Further investigations should show whether other SFV-positive individuals are infected with more than one strain.

Sanctuary chimpanzees are usually living in a limited habitat forming a large community, which leads to a high aggression level. In contrast to the natural situation, the individuals have no alternative to keeping out of a conflict or avoiding further conflicts by staying at a distance of each other. Aggressions are mostly associated with fighting leading to scratches and also deep bite wounds. Such injuries might play an important role in transmission of pathogens (Calattini et al., 2007; Jones-Engel et al., 2005). In sanctuaries this may contribute to multiple infections with strains with different genetic background. More analyses are needed to establish the frequency of infections with multiple SFV strains possibly resulting in virus recombination.

The SFV strains in this study showed a relatively high diversity and could be grouped into four main clusters. However, no clustering according to the geographic origin of the chimpanzees was found. The comparison of the strains described here with recently published SFV strains from wild *P. t. schweinfurthii* from Uganda, Congo and Tanzania (Leendertz et al., 2008) could also not identify clustering by geographical origin. These results are consistent with previous findings (Leendertz et al., 2008) which could not show region-specific clusters of SFV strains within one chimpanzee subspecies. However, this study shows in principle the possibility of pathogen introduction to great ape communities in sanctuaries which can cause co-infections with multiple virus strains.

This may have direct implications for sanctuary management since most apes are hand raised, and long-term interactions of animals and keepers with bites and scratches are frequently observed. In order to avoid pathogen transmission to humans, special precautions are needed, especially in sanctuaries where apes are SIV positive. Another concern may be that most sanctuaries aim at reintroducing great apes into the wild. The impact of these viruses on wild primates, which can again mix with regional strains, is unknown.

All wild-born captive chimpanzees on Ngamba Island were negative for SIV/HIV and STLV-1 and -2 antibodies, which might not reflect the situation in other ape sanctuaries in Africa. With the most recent findings that SIVcpz is pathogenic in free-ranging chimpanzees (Keele et al., 2009) it is obvious that all newly arriving chimpanzees at sanctuaries should be screened for defined pathogens including SIV. This will not only protect the health of the chimpanzee in the groups but also minimize the risk of infection for people in close contact with the animals. Furthermore, future reintroduction programs might benefit from such programs as only healthy or recombinant virus free animals would be released into the wild.

3.4. Acknowledgements

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Figure legend

Figure 3.1. Phylogenetic analysis of *int* sequences from SFV-infected chimpanzees of Ngamba and selected published sequences from four chimpanzee subspecies. The species' origins for the chimpanzee SFV sequences used are indicated. The phylogeny is based on the Neighbour-joining method using 2 Kimura distances performed in PHYLIP3.67 and the reliability of the inferred tree was evaluated by bootstrap analysis on 1000 replicates. The topology of the tree was confirmed by Maximum Likelihood Method. The scale bar represents an evolutionary distance of 0.1 nucleotide per site. The tree was rooted by using the new spider Monkey SFV sequence (X83298).

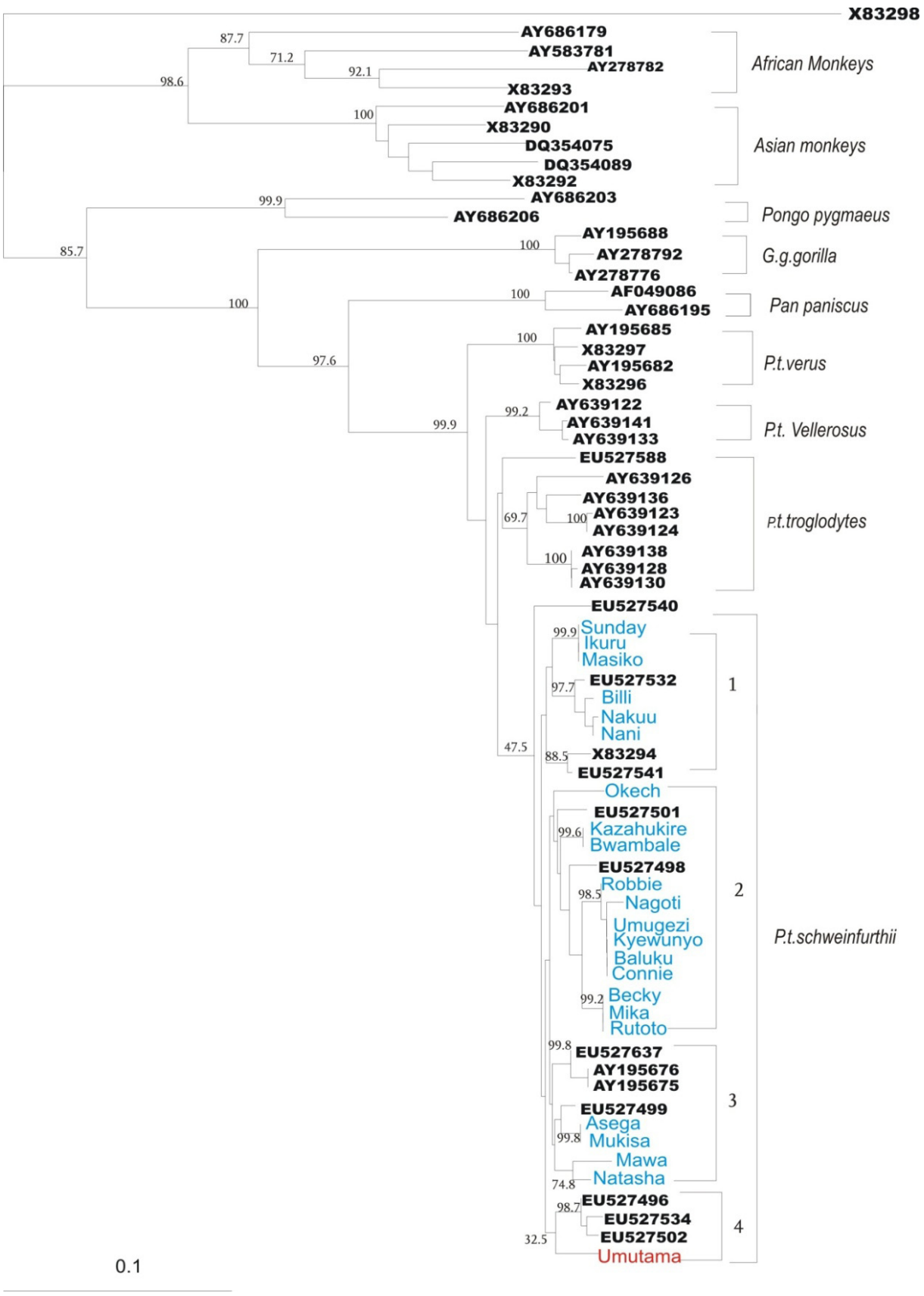
X83298
AY686179
AY583781
AY278782
X83293
AY686201
X83290
DQ354075
DQ354089
X83292
AY686203
AY686206
AY195688
AY278792
AY278776
AF049086
AY686195
AY195685
X83297
AY195682
X83296
AY639122
AY639141
AY639133
EU527588
AY639126
AY639136
AY639123
AY639124
AY639138
AY639128
AY639130
EU527540
Sunday
Ikuru
Masiko
EU527532
Billi
Nakuu
Nani
X83294
EU527541
Okech
EU527501
Kazahukire
Bwambale
EU527498
Robbie
Nagoti
Umugezi
Kyewunyo
Baluku
Connie
Becky
Mika
Rutoto
EU527637
AY195676
AY195675
EU527499
Asega
Mukisa
Mawa
Natasha
EU527496
EU527534
EU527502
Umutama
87.7
71.2
92.1
98.6
100
99.9
85.7
100
100
100
97.6
99.2
99.9
69.7
100
100
99.9
97.7
47.5
88.5
99.6
98.5
99.2
99.8
99.8
74.8
98.7
32.5
African Monkeys
Asian monkeys
Pongo pygmaeus
G.g.gorilla
Pan paniscus
P.t.verus
P.t. Vellerosus
P.t.troglodytes
1
2
3
4
P.t.schweinfurthii
0.1

Table 3.1. Name, origin, sex and results of foamy virus PCR of chimpanzees on Ngamba Island

Name	Sex	Est. yr of birth	Age (yrs) by 2007	Year of arrival	origin	First round PCR	Integrase Nested PCR
Kidogo	Female	1984	23	1998	Congo	-	-
Sophie	Female	1986	21	1998	Congo	-	-
Katie	Female	1987	20	1998	Congo	-	-
Ikuru	Female	1995	12	1999	Congo	+	+
Billi	Female	1998	10	2001	Congo	-	+
Mukisa	Female	1998	9	2001	Congo	-	+
Ndyakira	Female	1999	8	2002	Congo	-	-
Nakuu	Female	2001	6	2003	Congo	+	+
Peace	Female	1986	21	1998	Uganda	(+)	(+)
Connie	Female	1989	18	1998	Uganda	-	+
Bahati	Female	1990	17	1998	Uganda	-	-
Natasha	Female	1990	17	1998	Uganda	-	+
Becky	Female	1991	16	1998	Uganda	-	+
Sally	Female	1991	16	1998	Uganda	-	-
Cindy	Female	1993	14	1998	Uganda	-	(+)
Nkumwa	Female	1996	11	1998	Uganda	-	(+)
Kazahukire	Female	1999	8	2002	Uganda	+	+
Kyewunyo[1]	Female	2002	5	2002	Uganda	-	+
Megan	Female	1984	23	1998	Rwanda	-	(+)
Nagoti	Female	1986	21	1998	Unknown	-	+
Nani	Female	2001	6	2002	Unknown	+	+
Masiko	Male	1984	23	1998	Congo	+	+
Sunday	Male	1987	20	1998	Congo	-	+
Robbie	Male	1986	21	1998	Congo	-	+
Mawa	Male	1996	11	1999	Congo	+	+
Kalema	Male	1996	11	1999	Congo	-	-
Umutama	Male	1996	11	1999	Congo	+	+
Umugezi	Male	1997	10	1999	Congo	-	+
Baluku	Male	1998	9	1999	Congo	-	+
Asega	Male	1998	9	2000	Congo	-	+
Kisembo	Male	1999	8	2000	Congo	-	-
Indi	Male	1999	8	2001	Congo	-	(+)
Okech	Male	2001	7	2003	Congo	-	+
Rambo	Male	2004	3	2006	Congo	-	-
Tumbo	Male	1989	18	1998	Uganda	-	-
Mika	Male	1992	15	1998	Uganda	-	+
Bwambale	Male	1999	7	2002	Uganda	+	+
Rutoto	Male	2004	3	2006	Uganda	-	+

[1] *Kyewunyo is captive born*

( ) 5 sequences not analyzable

CHAPTER FOUR

A NOVEL HERPESVIRUS IN THE SANCTUARY CHIMPANZEES ON NGAMBA ISLAND IN UGANDA

Running Head: Herpesviruses in Sanctuary chimpanzees

A Novel Herpesvirus in the Sanctuary Chimpanzees on Ngamba Island in Uganda[3]

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Key words: Herpesviruse, chimpanzee, sanctuary, PCR, DNA polymerase

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4.0. Abstract

Recent studies in non-human primates have lead to discovery of novel primate herpesviruses. In order to get more information on herpesvirus infections in apes, we studied wild born captive chimpanzees. Hence, chimpanzees from Ngamba island sanctuary, Uganda, were analysed with pan-herpes PCR targeting the herpesvirus DNA polymerase gene and the glycoprotein B gene. The obtained sequences were connected by long-distance PCR, and analysed phylogenetically. Twenty-one out of 40 chimpanzees were infected with members of the *Gammaherpesvirinae*, two of them with a novel member of this subfamily. Phylogenetically, the novel virus fell into a clade of primate rhadinoviruses and the Kaposi sarcoma herpesvirus (human herpesvirus 8), representing a third distinct rhadinovirus in chimpanzees. Therefore, non-human primates harbor several herpesviruses many of which are still unknown. This has implications to management of primates in sanctuaries requiring continuous updates on the management protocols to deal with potential occupational pathogens.

4.1. Introduction

Little is known about the spectrum of infectious agents carried by wild non-human primates. However, such baseline data would be helpful to identify potential zoonotic threats and to better understand players involved in diseases threatening the health of non-human primates (Gillespie et al., 2009; Leendertz et al., 2006). Herpesviruses may play an important role in multi-factorial disease progressions and despite various discoveries of novel herpesviruses in wild great apes and other primates (Ehlers et al., 2008; Lacoste et al., 2005; Leendertz et al., 2009), the spectrum of herpesviruses is far from being described.

The members of the family *Herpesviridae* have been previously grouped into three subfamilies, designated *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Roizman et al., 1992), and phylogenetic lineages in these subfamilies were identified(McGeoch et al., 2000; McGeoch et al., 2001; McGeoch et al., 2005). In studies amplifying unknown herpes virus sequences from the DNA polymerase (DPOL) gene or – more recently - from two conserved genes by a bigenic PCR approach, novel herpesviruses were continuously discovered. For example, 14 novel gammaherpesviruses (GHV) were discovered in eight different host species from six mammalian orders (Ehlers et al., 2008), and ten novel betaherpesviruses were

identified in great apes (Leendertz et al., 2009). These discoveries were based on sequences encompassing two or more conserved genes, and determined from long-distance (LD)-PCR products (Ehlers et al., 2007, 2008; Leendertz et al., 2009; Prepens et al., 2007).

Herpesviruses are widespread in vertebrate species, sharing several moderately to well conserved genes, as determined from amino acid identity comparisons (e.g., DNA polymerase and glycoprotein B). Within the GHV, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated Herpesvirus (KSHV), classified as *Human herpesvirus* 4 (HHV-4) and *Human herpesvirus* 8 (HHV-8), are the human members of the *Lymphocryptovirus* genus and the *Rhadinovirus* genus, respectively. Both of these viruses play a critical role in human multistep carcinogenesis, especially in immunodeficient patients, leading to Burkitt's lymphoma (Magrath and Jude, 1996) and Kaposi's sarcoma (KS) (Chang et al., 1994; Schulz, 1998), respectively. GHV of the genus *Rhadinovirus* have also been detected in several mammalian species including New World monkeys (squirrel monkey, spider monkey) (Albrecht, 1990; 2000) and Old World monkeys (several macaque species, African green monkey, mandrill) (Auerbach et al., 2000; Desrosiers et al., 1997; Greensill et al., 2000; Lacoste et al., 2000; Rose et al., 1997; Strand et al., 2000). Comparison and phylogenetic analyses of available sequences support the existence of two distinct lineages among the Old World monkey rhadinoviruses, called RV1 and RV2 for the *Rhadinovirus* lineages 1 and 2 (Bosch et al., 1998; Goltz et al., 2002; Lacoste et al., 2001; Schultz et al., 2000.). KSHV belongs to the RV1 lineage, whereas no human virus has yet been discovered in the RV2 lineage.

Previous studies on African great apes from Cameroon and Gabon have shown that they harbor different rhadinoviruses closely related to KSHV. By serology and PCR, common chimpanzees were determined to be infected with rhadinoviruses of the RV1 and the RV2 lineage being termed Pan Rhadino-herpesvirus 1a, 1b, 2 (PanRHV1a, PanRHV1b, PanRHV2) (Lacoste et al., 2000, 2001). Using a bigenic LD-PCR approach, rhadinoviruses very similar to PanRHV1a and PanRHV2 were found in chimpanzees from Ivory Coast (PtroRHV-1 and PtroRHV-2) (Prepens et al., 2007). These studies demonstrate that wild animals, especially primates, harbor many unknown viruses, and further search will continue to reveal novel pathogens.

In this study, we analysed wild-born captive chimpanzees residing at Ngamba Sanctuary in Uganda to gain more insights into the herpesviruses harbored by this community. The results would fit into improvements of management protocols including employee health programs and future plans for re-introductions.

4.2. Materials and Methods

The study was conducted on 40 orphan chimpanzees (*Pan troglodytes schweinfurthii*) in semi-captive management at Ngamba Island Chimpanzee Sanctuary, Uganda. The total group consists of 23 females and 19 males with age group categorized as follows: 4 Infants (1 to 5years), 6 Juveniles (6-8 years), 12 sub-adults (9 to 11 years) and adults (12 years and more). These chimpanzees were brought to the sanctuary at different times after being rescued from illegal traders and poachers mainly from Uganda and Democratic Republic of Congo since 1998 when the sanctuary started.

Blood was collected from 40 chimpanzees under general anesthesia during our annual medical health checks in February 2007. Whole blood (10 ml) was taken by inguinal venipuncture using CPT Vacutainer tubes. Leucocytes / cells from fresh whole blood collected in CPT tubes were processed using a cell preparation procedure within 12 hours. The processed cells were stored in cryotubes under liquid nitrogen (-196^{o}C) and stored at Uganda Virus Research Institute till transported on dry ice to Robert Koch Institute for analysis.

4.2.1. DNA Extraction, Amplification, Purification, Cloning and Sequencing

DNA was extracted from buffy coat of the whole blood cells of 40 chimpanzees using QIAamp DNA Blood Min Kit (QIAGEN®, QIAamp®, Hilden, Germany) according to manufacturer's instructions.

Panherpesvirus consensus PCR for the amplification of 160 to 181 bp (excluding primer-binding sites) of the DPOL gene was carried out as described previously (Chmielewicz et al., 2001). Samples that did not yield an amplification product were rerun under more relaxed conditions; i.e. the ramp time between the annealing step and the extension step was prolonged 50-fold, and the final concentration of the polymerase was increased two-fold.

For the amplification of the gB genes of as yet unknown GHVs, two degenerate, deoxyinosine-containing primer sets (GH1 and GH2) were used (Ehlers et al., 2008).

ested LD-PCR was performed with the TaKaRa-Ex PCR system according to the instructions of the manufacturer (Takara Bio Inc., Otsu, Japan) by using virus-specific primers (not listed) as described previously (Goltz et al., 2002; Ehlers et al., 2008). PCR products were purified by using the PCR purification kit (QIAGEN) and directly sequenced with the Big Dye terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) in a 377 DNA automated sequencer (Applied Biosystems).

4.2.2. Multiple sequence alignment and phylogenetic analysis

Analysis was carried out at the level of encoded amino acid sequences. Sets of partial amino acid sequences from DPOL and gB were aligned using ClustalW (Thompson et al., 1994). Positions in was used for phylogenetic analyses with the Neighbor-Joining method as performed previously (Prepens et al., 2007).

4.2.3. Nucleotide sequence accession numbers

The following DPOL and gB sequences of known primate herpesviruses were used for comparative purposes: Epstein-Barr virus (EBV), Genebank accession number (acc.-no.) NC_009334; Pan troglodytes lymphocryptovirus (PtroLCV-1), herpesvirus pan = *Panine herpesvirus 1* (PnHV-1), acc.-no. AF534226; Rhesus lymphocryptovirus (RhHV-1) = *Macacine herpesvirus* 4 (McHV-4), acc.-no. NC_006146; Marmoset lymphcryptovirus (MaLCV) = Callithrichine herpesvirus 3 (CalHV-3), acc.-no. NC_004367; KSHV = HHV-8, acc.-no. NC_003409; Rhesus rhadinovirus (RRV) = Macacine herpesvirus 5 (McHV-5), acc.-no. NC_003401; Pan troglodytes rhadinovirus 1 (PtroRHV-1), acc.-no. AY138585; Pan troglodytes rhadinovirus 2 (PtroRHV-2), acc.-no. AY138585; Macaca fascicularis rhadinovirus 1 (MfasRHV-1), acc.-no. AY138583; Macaca fascicularis rhadinovirus 2 (MfasRHV2), acc.-no. EU085377; Retroperitoneal fibromatosis-associated herpesvirus (RFHV), acc no. AF204166; Herpesvirus Saimiri (SaHV-2) = *Saimirine herpesvirus 2*, acc.-no. NC_001350; Chimpanzee cytomegalovirus (CCMV) =*Papine herpesvirus 2*, acc.-no. AF480884. The nucleotide sequence of PtroRHV-3 was deposited in Genbank under the acc.-no. GQ995451.

4.3. Results and Discussion

Twenty-one of 40 samples from chimpanzees (*Pan troglodytes schweinfurthii*) analysed with pan-herpes DPOL PCR were tested positive for GHV. The sequences from five samples originated from PtroRHV-1 (acc.-no. AY138585) (Prepens et al., 2007). Fourteen samples were positive for the lymphocryptovirus PtroLCV-1 (Acc.-no. AF534226) (Ehlers et al., 2008). Two cases appeared to harbor a novel gammaherpesvirus which was tentatively named *Pan troglodytes* rhadinovirus 3 (PtroRHV-3). All results are listed Table 4.1.

With degenerate primers targeting the gB gene a 453 bp sequence was amplified from one of the PtroRHV-3-positive samples. From the partial gB and DPOL sequences of PtroRHV-3, specific nested primers were deduced and used in LD-PCR. The 3.3 kb product was sequenced and a final contiguous PtroRHV3 sequence was compiled spanning 3446 bp.

In a pair-wise nucleic acid sequence comparison, the 3.4 kbp sequence of PtroRHV-3 was found to be 69% identical to PtroRHV-1. The 3´end of the PtroRHV-3 sequence (0.4 kb) was 99% identical to a rhadinovirus (PanRHV1b) found in *Pan troglodytes troglodytes* (Lacoste et al., 2000). No close matches were found in GenBank for the 5'-part (3 kbp) of the PtroRHV-3 sequence, spanning a part of the DPOL and the gB gene. As the PtroRHV-3 sequence was detected in *Pan troglodytes schweinfurthii* from Uganda and the Democratic Republic of Congo, it was regarded as originating from a hitherto unknown *P. t. schweinfurthii* RHV, closely related to *P. t. troglodytes* RHV1b.

From the partial gB and DPOL open reading frames (1020 bp and 2376 bp, respectively) amino acid sequences were deduced, concatenated and aligned with corresponding aa primate GHV sequences from GenBank. Phylogenetic tree analysis was performed as described previously (Prepens et al., 2007), and aa sequences of the chimpanzee cytomegalovirus (GenBank acc.- no. AF480884) were used for rooting. In the tree, the novel virus clustered more closely with the human rhadinovirus (KSHV/HHV-8) and with PtroRHV-1 than with PtroRHV2 (Fig. 4.1).

This study confirms that there are three distinct rhadinoviruses within the *Gammaherpesvirinae* of chimpanzees (Lacoste et al., 2001). Although the human rhadinovirus HHV-8 is closely related to PtroRHV-1 (and PanRHV1a), no human rhadinoviruses with close similarity to PtroRHV-2 or PtroRHV-3 are known. Nevertheless, they could exist, since herpesviruses coevolve with their hosts.

The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus pose significant health risks worldwide, including the induction of cancer and lymphoproliferative diseases. They pose a risk of serious disease especially to those lacking a competent immune system, such as neonates, transplant patients and people suffering from AIDS [reviewed in (Pass, 2001)]. Although acute gammaherpesvirus infection is rapidly cleared by a strong immune response, the establishment of latent infection in hematopoietic cells allows these viruses to successfully evade the host immune response and maintain lifelong infection. To-date, the pathogenesis and immunity of gammaherpesvirus infections are still not well understood and therefore convincing strategies to prevent long-term latency have not been realized (Tibbetts et al., 2003).

It has been demonstrated in studies with mice that the level of latent infection with a gammaherpesvirus is independent of the infectious dose over at least a 104-fold range, regardless of whether inoculation occurs via a systemic or a mucosal route (Tibbets et al., 2003). Host factors like a compromised immune system have been implicated in the initiation of infection leading to substantial acute phase replication and subsequent establishment of a maximal level of latency. In addition, it is not known whether the gammaherpesviruses found in great apes present the same way as their human counterparts both in establishment of infection and pathogenicity. For example, it has now been shown that chimpanzees harboring simian immunodeficiency virus have a higher likelihood of dying and lower reproductive rate (Keele et al., 2009). Most likely, the underlying pathological processes are multi-factorial, and herpesviruses may play an important role. Therefore, detailed analysis in long term studies are needed.

Finally, not only co-evolvement with the host but also horizontal transmission appears to be an important component of beta- and gammaherpesvirus evolution (Ehlers et al., 2003; Leendertz et al., 2006; Bernhard Ehlers and Duncan McGeoch, unpublished data). These circumstances and uncertainties present challenges in management of occupational hazards in captive ape facilities (sanctuaries and zoos) where there is close interaction of employees with the apes.

4.4. Acknowledgements

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Figure 4.1. Phylogenetic analysis of the novel chimpanzee rhadinovirus. A phylogenetic tree was constructed using the amino acid (aa) sequences encoded by the gB-DPOL segments of the novel primate herpesviruses and of known human and non-human primate herpesviruses, available in GenBank. Abbreviations of common names are used, and those of species names according to the ICTV (International Committee on the Taxonomy of Viruses) are given in parentheses. A multiple alignment of concatenated 1100 aa was analysed with the neighbor-joining method. A rooted phylogram is shown. With CCMV as outgroup. The branch length is proportional to evolutionary distance (scale bar). Results of bootstrap analysis (1000 replicates) are indicated at the nodes of the tree. The novel PtroRHV-3 is highlighted in bold type. Full names of known viruses and their nucleotide sequence accession numbers are listed in the method section.

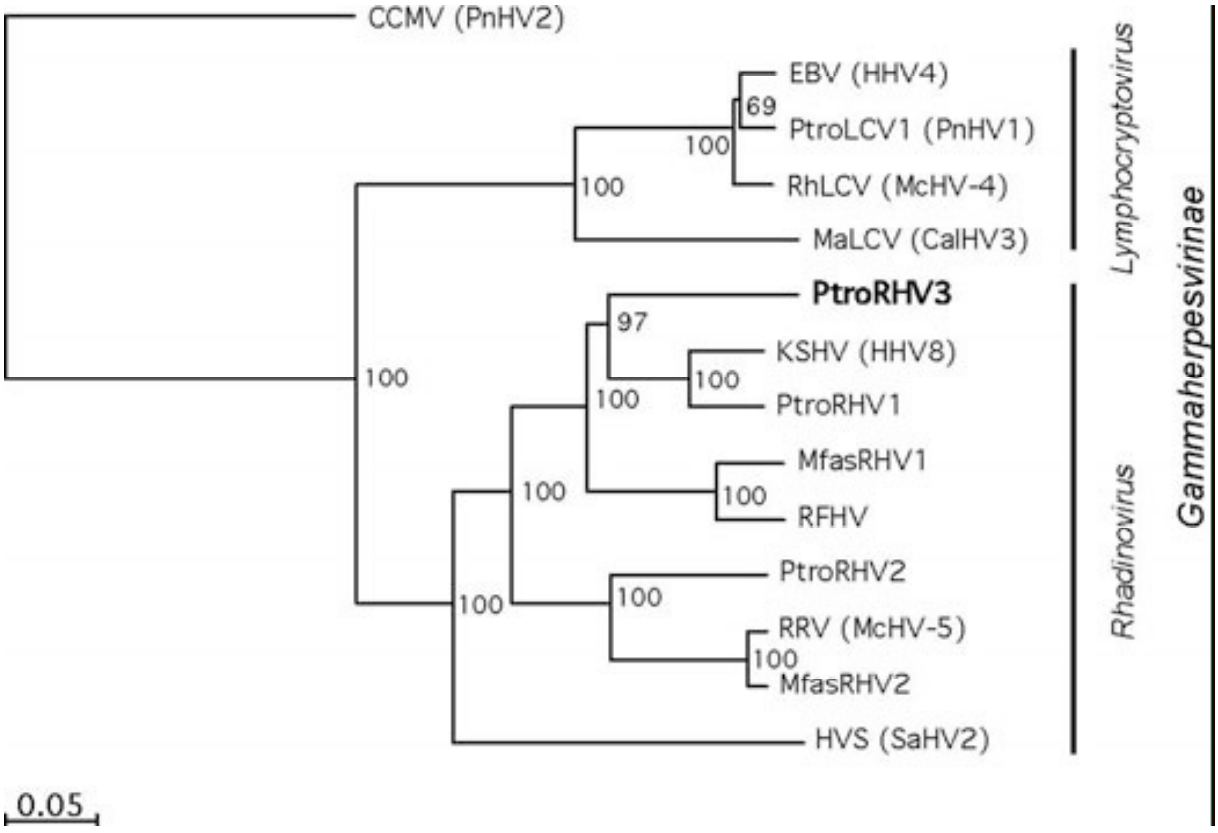

Table 4.1. Chimpanzee biodata and gammaherpesviruses detected

Chimpanzee biodata				*Gammaherpesvirinae*		
				Lymphocryptovirus	*Rhadinovirus*	
Name of chimpanzee	Sex	Age	Origin	PtroLCV-1	PtroRHV-1	PtroRHV-3
Kidogo	F	23	DRC [§]	-	-	-
Sophie	F	21	DRC	+	-	-
Katie	F	20	DRC	+	-	-
Ikuru	F	12	DRC	+	-	-
Billi	F	10	DRC	+	-	-
Mukisa	F	9	DRC	-	-	+
Ndyakira	F	8	DRC	-	-	-
Nakuu	F	6	DRC	+	-	-
Masiko	M	23	DRC	-	-	-
Sunday	M	20	DRC	+	-	-
Robbie	M	21	DRC	-	-	-
Mawa	M	11	DRC	-	-	-
Kalema	M	11	DRC	-	-	-
Umutama	M	11	DRC	-	-	-
Umugezi	M	10	DRC	+	-	-
Baluku	M	9	DRC	+	-	-
Asega	M	9	DRC	-	-	-
Kisembo	M	8	DRC	+	-	-
Indi	M	8	DRC	+	-	-
Okech	M	8	DRC	-	+	-
Rambo	M	3	DRC	-	+	-
Megan	F	23	Rwanda	-	-	-
Kyewunyo	F	5	Uganda	-	+	-
Peace	F	21	Uganda	-	-	-
Connie	F	18	Uganda	-	-	-
Bahati	F	17	Uganda	-	+	-
Natasha	F	17	Uganda	-	-	-
Becky	F	16	Uganda	-	-	-
Sally	F	16	Uganda	-	-	-
Cindy	F	14	Uganda	-	-	-
Nkumwa	F	11	Uganda	+	-	-
Kazahukire	F	8	Uganda	-	-	-
Tumbo	M	18	Uganda	-	+	-
Mika	M	15	Uganda	-	-	-
Bwambale	M	7	Uganda	-	-	+
Rutoto	M	3	Uganda	+	-	-
Nagoti	F	21	Unknown	+	-	-
Nani	F	6	Unknown	+	-	-
Total +ve samples				14	5	2

CHAPTER FIVE

THE "ORIGINAL" HEPATITIS B VIRUS OF EASTERN CHIMPANZEES (*PAN TROGLODYTES SCHWEINFURTHII)*

The "original" Hepatitis B virus of Eastern chimpanzees (*Pan troglodytes schweinfurthii)*[4]

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5.0. Abstract

Little is known about Hepatitis B Virus (HBV) infections in chimpanzees. Therefore, we investigated the prevalence of chimpanzee HBV (chHBV) infections in 37 captive, wild-born chimpanzees in the sanctuary on Ngamba Island, Uganda including one sample from a wild chimpanzee. In one third of the plasma samples (32.4%; 12/37) we detected antibodies to hepatitis B (core) antigen (ant-HBc). Amongst those individuals chHBV DNA was detected by PCR in one captive wild born and one wild chimpanzee. Phylogenetic analysis revealed that the sequences fell into the clade of *Pan troglodytes schweinfurthii.* Retrospective analysis of samples from PCR positive animal provided evidence of persistence of HBV infection with high viral load in chimpanzees. In contrast to the only available earlier described HBV sequence from the subspecies *Pan troglodytes schweinfurthii*, there was no evidence of recombination with human HBV. Our sequences therefore are likely to present the "original" chHBV from *P. t. schweinfurthii*.

5.1. Introduction

Hepatitis B virus (HBV) infection is an important public health problem. More than 350 million people worldwide are chronically infected with the HBV (Maddrey, 2000). The infection can cause acute and chronic liver disease, including cirrhosis and hepatocellular carcinoma. Human HBV is the prototype of the family Hepadnaviridae, a spherical enveloped particle containing partially double stranded DNA and RNA dependent DNA polymerase. The circular genome is approximately 3,200 base pairs long and can be differentiated into the genotypes A to H, with intergenotypic diversity of at least 8% (Arauz-Ruiz et al., 2002; Naumann et al., 1993; Norder et al,. 1994, Okomoto et al., 1988; Stuyer et al., 2000; Liu et al., 2006). HBV in non-human primate species including a divergent HBV-related strain in a New World Woolly Monkey has been identified in captive and wild primates (Grethe et al,. 2000; Lnaford et al., 1998; Makuwa et al., 2003; Makuwa et al., 2006; Noppornpanth et al., 2003; Sa-Nguanmoo et al., 2008; Vartanian et al., 2002; Voudin et al., 1998; Warren et al., 1999). HBV infections have been documented in subspecies of chimpanzees and seem to be asymptomatic in their natural hosts (Hu et al., 2001; MacDonald et al., 2000; Njouom et al. 2010; Takahashi et al., 2001; Vartanian et al., 2002.). For the chimpanzee subspecies *Pan troglodytes schweinfurthii* from East Africa, only one HBV sequence (Vartanian et al., 2002) is available which is interestingly

a recombination with human HBV type C (Magiorkinis *et al*., 2005). Recombination between different genotypes within human HBV is well documented between genotypes A and D, A and C, B and C or between D and C (Bollyky et al., 1996; Cui et al., 2002; Hannoun et al., 2000; Morozov et al., 2000; Owiredu et al., 2001). HBV genotypes E and G were shown to be recombinants consisting partially of genome regions from D and A, respectively (Bowyer and Sim, 2000; Fares and Holmes, 2002). However, little information exists about recombination of HBV in apes. More recently, evidence of recombination between HBV *Pan troglodytes troglodytes* and *Pan troglodytes ellioti* (formally known as *vellerosus*) was observed in two of the Cameroonian strains (Njouom et al. 2010). Then the recombination of chHBV-FG with human HBV genotype C was reported (Magiorkinis et al., 2005). It might be expected that there are more recombination events between chHBV and HBV genotypes A, D, and E known to circulate in Africa. However, at present the knowledge is limited due to the small number of chHBV sequences available from wild apes. Several cross-species transmissions have been reported which occurred in captivity between different primate species, examples are transmissions between gibbons and chimpanzees, chimpanzees and gorillas (Grethe et al., 2000; Norder et al., 1996) and between chimpanzees and humans (Hu et al., 2000; Takahashi et al., 2000). Hence we investigated for HBV chronic infections in captive wild-born chimpanzees on Ngamba island and tested for recombination phenomena.

5.2. Materials and Methods

Blood was collected in EDTA anticoagulant-coated test tubes from captive wild-born chimpanzees living on Ngamba Island Chimpanzee Sanctuary, Uganda, (n=37) in 2008 during the annual routine health checks under anaesthesia. In addition feacal samples were collected from the same individuals. Plasma was separated by centrifugation at 3000 rpm for 10 minutes at room temperature. Two ml aliquots of the plasma and feacal samples were initially stored under liquid nitrogen and later transferred to -80°C freezer until transported on dry ice to the Robert Koch-Institut for analysis. Additional samples collected from the same individuals since 2001 to 2007 were included in the study. A serum sample obtained from a wild chimpanzee (Kiiza) in Kibale National Park in Uganda in 2006 during snare wire removal was included in the analysis.

5.2.1 Serology

Enzygnost Anti-HBc monoclonal enzyme immunoassay kit (Dade Behring Marburg GmbH, Germany) was used for qualitative determination of antibodies to Hepatitis B (core)-antigen in plasma obtained from chimpanzees according to the manufacturers protocol.

5.2.2. DNA extraction, amplification and sequencing

DNA was extracted from plasma of 37 captive chimpanzees and one serum of the wild chimpanzee using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), which allows extraction of viral DNA and viral RNA. Feacal DNA was extracted using the EURx GeneMATRIX Stool DNA Purification Kit (Roboklon).

A real time qPCR was performed on extracted DNA previously established for the detection of HBV (Pas et al., 2000), HBV PreS F: 5´- ggACCCCTgCTCgTgTTACA-3´, HBV PreS R: 5´- gAgAgAAgTCCACCACgAgTCTAgA-3´ and HBV PreS TM: 5´-Cy5-TgTTgACAARAATCCTCACAATACCRCAgA-BBQ-3´. Each sample was analyzed in duplicate. Copy numbers were determined using a standard curve; the mean and standard deviation were calculated. The detection limit was 10 copies of target DNA per reaction.

From individual animals DNA from both, blood and feaces, was amplified by nested PCR as described by MacDonald et al. (2000). PCR products were separated by 1.5% agarose gel electrophoresis. DNA fragments were extracted with the QIAGEN QIA Quick Gel extraction Kit, following the manufacturer's instructions and used for sequence analysis with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Weiterstadt, Germany) on an ABI 310 automated DNA sequencer.

5.2.3. Amplification and sequencing of HBV genomes

For each PCR reaction, 2.5 µl 10 x buffer, 1.5µl $MgCl_2$ 50mM, 2µl dNTPs 10 mM, 0.5µl each primer 10 µM, 1µl DMSO, 0.2 µl ET SSB (600 ng/µl) (New England Biolabs, Ipswich, MA, USA), 0.3 µl (1.5 U) Platinum® Taq (Invitrogen) and 3 µl DNA were used. Reaction volume was adjusted with water (Molecular Biology Grade Eppendorf) to 25µl. The reaction was run in a thermocycler (Mastercycler epgradient, Eppendorf) at 95°C for 2 min, 45 cycles at 95 °C for 20 sec, at 58 °C for 20 s and at 72 °C for 3 min. The final extension was 72 °C for 10 min.

Primers shown in Table 5.1 were used for diagnostics and for sequencing of the near full-length genome of HBV. The following primer combinations were used HBV1802F/HBV803R, HBV PreS F/HBV1626R and HBV1446F/HBV1752R to amplify a 2.2kb, 1.4kb and 0.3kb fragment, respectively. PCR products were separated by 1.5% agarose gel electrophoresis, purified with QIAGEN QIA Quick Gel extraction Kit (Qiagen) and sequenced with the ABI PRISM Big Dye Terminator cycle sequencing kit, according to the manufacturer's protocol. Sequences were determined using the ABI 310 automated DNA sequencer and analyzed with the ABI PRISM DNA Sequencing Analysis Software (Version 3.7, Applied Biosystems).

5.2.4. Phylogenetic analysis

Nucleotide sequences were analyzed with the ABI PRISM DNA Sequencing Analysis Software (Version 3.7, Applied Biosystems), BioEdit v7.0.9 (www.mbio.ncsu.edu/BioEdit/bioedit.html). HBV sequences determined in this investigation were compared to published sequences using the BLAST network program (NCBI). Sequences were aligned with sequences from GenBank database using the CLUSTALW software program (Thompson et al., 1994).

The nearly complete HBV genomes (positions 1-1730 and 1850-3263 compared to the reference sequence V00866) identified from Mika and Kiiza (sequence accession number HQ018763 and HQ018764 respectively) were aligned to a selection of published complete HBV genomes using MUSCLE (Edgar, 2004) as implemented in SeaView (Gouy et al., 2010). Following visual inspection, the alignment was then manually edited, being only slightly modified. The resulting dataset comprised 50 sequences and 3181 nucleotide positions.

Likelihoods of models of evolution (JC, HKY and GTR; +F; +I, +G, +I+G) were estimated and then compared according to the Akaike information criterion using jModelTest (Posada, 2008). The model of evolution to which the dataset was a better fit was GTR+I+G.

A maximum likelihood (ML) phylogenetic tree was estimated under this model using PhyML (Guindon & Gascuel, 2003) as implemented on a dedicated webserver http://www.atgc-montpellier.fr/phyml/. Equilibrium frequencies, topology and branch lengths were optimized and the tree search was realized using a combination of hill-climbing algorithms (NNI & SPR). Branch robustness was assessed by non-parametric bootstrapping (500 pseudo-replicates). A complete genome sequence of woolly monkey hepatitis virus was used as outgroup based on

previous studies (Bartholomeuz and Schaefer, 2004). Bootstrapping with 1000 replicates of heuristic searches tested the confidence of the tree. The nodes with bootstrap values greater than 70% are significantly supported with >95% confidence (robustness) (Hillis and Bull, 1991).

Obtained sequences (from Mika and Kiiza) were analyzed for recombination with human HBV using boot scanning as described by Magiorkinis et al. (2005). Bootscan analysis for each strain was implemented in the SimPlot software program (Lole et al., 1999).

5.3. Results

Serological analyses revealed that 32.4% (12/37) of the chimpanzee samples tested had antibodies against hepatitis B (core) antigen (ant-HBc), including the sample of the wild chimpanzee (Kiiza). Of the positive chimpanzees, 5 were males and 7 females (Table 5.2). There were significantly more adults sero-positive than infants, juveniles and sub adults (Sided Fishers exact analysis (P= 0.004). To get information on the possible time point of infection, plasma samples collected between 2001 and 2007 from the same chimpanzees were investigated. All individuals positive for anti-HBV antibodies in the 2008 panel were also positive in the samples collected between 2001 and 2007. By PCR analysis for HBV-DNA amplification of 12/37 HBsAg-positive samples, only samples from Mika showed positive result for HBV genome. To follow the course of infection, Mika plasma samples from previous health checks in the years 2001, 2004, 2005 and 2007 were analyzed by qPCR for HBV. All Mika samples were positive for HBV DNA (Table 5.3). In addition all feacal samples from Mika were positive in PCR

In addition to the wild born captive chimpanzees, the investigation of one serum sample from Kiiza, a wild chimpanzee, revealed antibodies directed against HBV as well as HBV genome equivalents (Table. 5.3).

A real time multiplex qPCR performed on the same samples for concurrent detection of HCV and HEV) as previously described (Adlhoch et al., 2009) were negative for HCV and HEV infections in all samples (data not shown).

Phylogenetic analyses of the nearly complete HBV sequences (position 1-1730 and 1850-3263 compared to the reference sequence V00866) generated from Mika and Kiiza (Fig 5.2 and 5.3) shows that the HBV sequences cluster closely with the HBV isolate published

previously from *P.t. schweinfurthii*. The HBV sequence obtained from Kiiza is closely related to FG than the HBV sequence from Mika. It was reported that isolate HBV-FG was a recombinant of a chimpanzee sequence with human HBV C. Therefore, a 500 nt fragment (position 551-1050 spanning half of the RT domain of the pol gene which overlaps with half of the coding region of the small surface protein) was compared with the HBV sequences obtained from Mika and Kiiza. Both genomes were analyzed by bootscanning using SimPlot (Lole et al., 1999), which failed to identify any sign of recombination with human subtypes or strains from other species (Fig 5.4 a, and b).

5.4. Discussion

In 32.4% (12/37) of blood samples from chimpanzees antibodies directed against hepatitis B (core) antigen (ant-HBc) were detected. Two chimpanzees (Mika and Kiiza) were also positive by PCR for HBV. Anti Hepatitis B (core) antigen (ant-HBc) antibodies are generally the first antibodies to appear after infection with HBV shortly after the detectability of HBsAg and often persist lifelong (Niermeijer et al., 1978). Retrospective analysis was carried out to trace the time point of infection on serum samples from the same chimpanzees collected between 2001 and 2007. All individuals who had developed antibodies against HBc in 2008 were reactive in retrospective samples indicating that the animals were exposed to hepatitis B virus infection before the screening of the animals in the sanctuary was initiated. Of the positive chimpanzees, 5 were males and 7 females. There were significantly more adults sero-positive than infants, juveniles and sub adults (Sided Fishers exact analysis (P= 0.004)) (table 3). The current high seroprevalence of HBV infection reported in this paper corresponds with previous findings of 29.2% (n=156 chimpanzees and 14 gorilla) from Gabon and Congo (Makuwa et al., 2006); 38.6% (n=of 101) observed in captive gibbons from Thailand positive for at least one marker of HBV infection (Noppornpanth et al., 2003). Even higher seropositivity for at least one marker of HBV infections in non-/human primates in captivity was reported recently in gibbons (5/15: 33.3%) and orangutan (40/43; 93%) (Sa-nguannmoo et al., 2008).

Only Mika was positive for HBV DNA in the blood sample from 2008 and in retrospective samples and Kiiza a wild chimpanzee from Kibale National Park, Uganda as well as on feacal samples. This confirms the applicability of the non-invasive methods for detection of HBV by

PCR analysis as described before (Makuwa et al., 2006). Both chimpanzees are representatives of the Eastern chimpanzee subspecies (*Pan troglodytes schweinfurthii*) and hence our findings extend the data on HBV sequences in Eastern subspecies as only one sequence was available in data bases (accession no. AF498266; Vartanian et al., 2002). The two additional nearly full genome sequences will help to understand the evolution of HBV infection in chimpanzees. The HBV viral load was determined for Mika from 2001 to 2008 in plasma samples and the serological profile corresponds to an acute progressing to chronic highly replicative phase of HBV infection (Table 5.3 and Fig. 5.1). Viral loads for Mika were comparable to the HBV viral load found in wild-born chimpanzees in Central and West Africa (Makuwa et al., 2007). The viral load decreased in the observation period, which might be due to improvement of nutrition and medical care of chimpanzees in the sanctuary enhancing their immunity. The observed peak of viral load in 2005 however, corresponds to the hierarchy taking over demands as Mika established himself as an alpha male between 2004 and 2005 which was a very stressful period. Results from annual haematologic and serum biochemistry analyses from Mika revealed high level in 2007 and moderate increase in 2009 in enzyme levels of γ-glutamyl transferase (GT/GGT- 123U/l; 91u/l) and Alanine aminotransferase (GPT/ALT- 76u/l; 71u/l) respectively and a decrease/increase in Alkaline phosphatase (ALP-76u/l; 114u/l) in 2007 and 2009 respectively, compared to non infected chimpanzees (data not shown), an indicator of reduced liver function in reference to reported values from healthy chimpanzees (Stone *et al*., 2000). However, the chimpanzee Mika showed no obvious symptoms of an HBV infection or reduced activity levels and changes in serum clinical chemistry should be interpreted with caution as they can be affected by other factors like age and sex (Videan *et al*., 2008). We suggest that social behaviors in chimpanzee especially hierarchy may influence the HBV disease progression and transmission dynamics that is currently not known in apes but this explanation is limited by low numbers of cases available for long term follow up. HBV is transmitted in humans mainly through the sexual route and exposure to contaminated blood and other infectious fluids. Transmission in apes has not been described in detail but presumably occurs through the same routes. The role of an infected alpha male in transmission of HBV especially to females and other individuals requires extra study and may be useful in understanding epidemiology of HBV infections in wild great apes. However, none of the HBV antibody reactive females was PCR positive to allow molecular epidemiological tracing of these infections. It remains unclear as to why the

chimpanzees reactive to hepatitis B (core) antigen (ant-HBc) were negative in HBV PCR. Other factors may affect transmission of HBV in chimpanzees and sexual transmission may not be a major route of transmission. The clinical, serological and immunological responses following infection with HBV have been well described in humans and only 25-50% of cases of acute HBV infection are reported to be symptomatic, the remainders are asymptomatic or are associated with non-specific symptoms. The course of chronic HBV infection is characterized by the persistence of HBsAg in the serum and the failure to develop anti-HBs anibodies, which provides protective immunity. In chronic HBV infection, the persistence of HBeAg (and failure to develop HBeAb) is associated with high levels of viral replication, high levels of HBV DNA in serum and more severe disease (Paar, 2001; Jay et al., 1998; Price *et al*., 2001).

The two HBV sequences obtained from chimpanzees in this study cluster with the published isolate FG (accession number: AF498266) from a wild chimpanzee (*Pan troglodytes schweinfurthi*) from East Africa (Vartanian et al., 2002). Kiiza is a wild chimpanzee from a research habituated group of Kibale Chimpanzee Project in Kibale National Park, and belongs to the same community as the chimpanzee for whom the FG sequence was published. It is especially worthwhile noticing that the HBV sequence obtained from Kiiza is more closely related to FG's corresponding to the fact that Kiiza originates from the very same habituated chimpanzee group. These chimpanzees are followed daily by field assistants and visiting international researchers for behavioral data collection. These events probably led to transmission of human HBV genotype C from one of the researchers to chimpanzees resulting into recombination. The co-existence of a potential human-chimpanzee recombinant virus and a non-recombinant chimpanzee specific HBV strain in one and the same community is surprising and further investigations are needed to clarify the "natural" existence of the recombinant chHBV FG. However, the two new sequences described in this investigation may represent the "original" *P. t. schweinfurthii* sequence, which was at the origin of the recombination event leading to the published isolate FG (Vartanian *et al*., 2002).

In general transmission of HBV between humans and apes and the risk of associated recombination effects is a concern to the management of apes in sanctuaries where there is close contact of humans and apes through daily routine work with incidences of aggression and the risk of blood contact.

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Table.5.1. Amplification and sequencing primers

Oligo Name	Localisation*	Oligonucleotide sequence 5'-3'	Annealing temperatures [°C]
HBV1802F	1802-1820	CTCACCAgCACCATgCAAC	57.8
HBV803R	803-781	gTAACAgCggYAWAAAgggACTC	59
HBV PreS F	182-201	ggACCCCTgCTCgTgTTACA	59.9
HBV1626R	1626-1607	gTTCACggTggTCTCCATgC	59.9
HBV1446F	1446-1463	TCCTgCCgACgACCCTTC	59
HBV1752R	1752-1732	TCTCCTCCCCTARCTCCTCCC	59
HBV PreS R†	271-247	gAgAgAAgTCCACCACgAgTCTAgA	58.1
HBV365F†	365-386	ggYTATCgYTggATgTgTCTgC	59.3
HBV3205R†	3205-3186	gCCTTCCTgACTgCCgATTg	61.2

F: forward orientation; R: reverse orientation; *location of the primer position is based on reference sequence V00866; †additional primers used for sequencing

Table 5.2. Ngamba chimpanzee Age/sex Distribution 2007 and HBV ELISA results

	Age category	**Females tested/ positive**	**male tested/ positive**	**Total**
Infants	1 to 5 yrs	1/0	3/0	3
Juveniles	6 to 8 yrs	2/0	4/2	6
Sub adults	9 to 11yrs	4/0	6/1	10
Adults	>12yrs	12/7	6/2	18
Total		19/7	18/5	37

Table 5.3. Quantitative real-time PCR for HBV for chimpanzees Mika and Kiiza

Chimp	Sample year	Ct-.value	copies/µl DNA	copies/ml plasma
Mika	2001	19.2	1.8×10^5	1.0×10^8
	2004	22.1	2.7×10^4	1.5×10^7
	2005	19.8	1.2×10^5	7.1×10^7
	2007	27.6	$6{,}8 \times 10^2$	3.9×10^5
	2008	31.6	$1{,}5 \times 10^2$	2.2×10^4
Kiiza	2006	22.7	2.5×10^4	1.4×10^7

Figure. 5.1. Showing the viral load of HBV in plasma from the positive tested chimpanzees overtime.

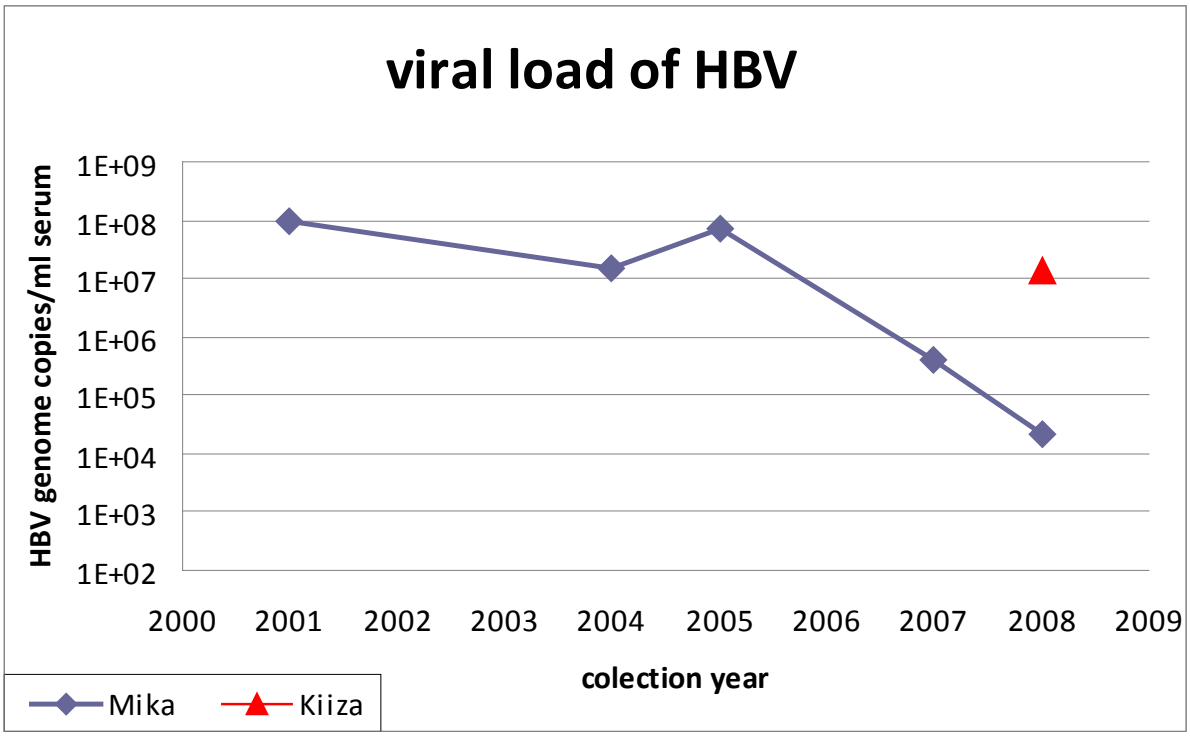


Figure legend

Figure 5.2: Maximum likelihood tree of HBV complete genomes. Colored external branches stand for genomes identified from African apes, Mika and Kiiza HBV branches being highlighted in red. Bootstrap values above 90 are shown for the African ape clade only. The tree was rooted using one HBV sequence isolated from a woolly monkey.

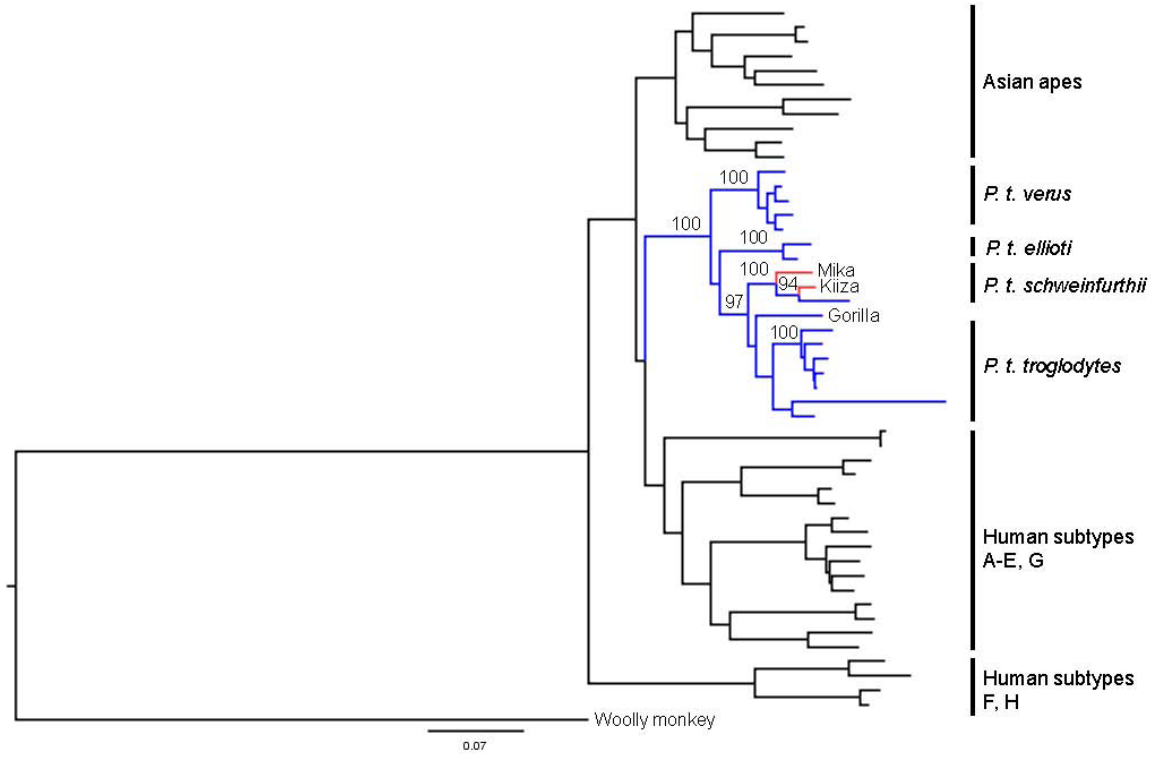


Figure 5.3. Phylogenetic comparison of a new chHBV sequences from HBV-infected chimpanzee (MIKA) of Ngamba Island and wild living chimpanzee (Kiiza) –color coded with previously described chHBV sequence (chHBV-FG) and recombinant region of chHBV-FG sequence previously grouped with HBV genotype C and other selected publicly available sequences from four chimpanzee subspecies. The species' origins for the chimpanzee HBV sequences used are indicated. The phylogeny is based on the Neighbour-joining method using 2 Kimura distances performed in PHYLIP3.67 and the reliability of the inferred tree was evaluated by bootstrap analysis on 1000 replicates. The scale bar represents an evolutionary distance of 0.1 nucleotide per site. The nodes with bootstrap values greater than 70% are significantly supported with >95% confidence (robustness) The tree was rooted by using complete genome sequence of woolly monkey hepatitis virus (AY226578).

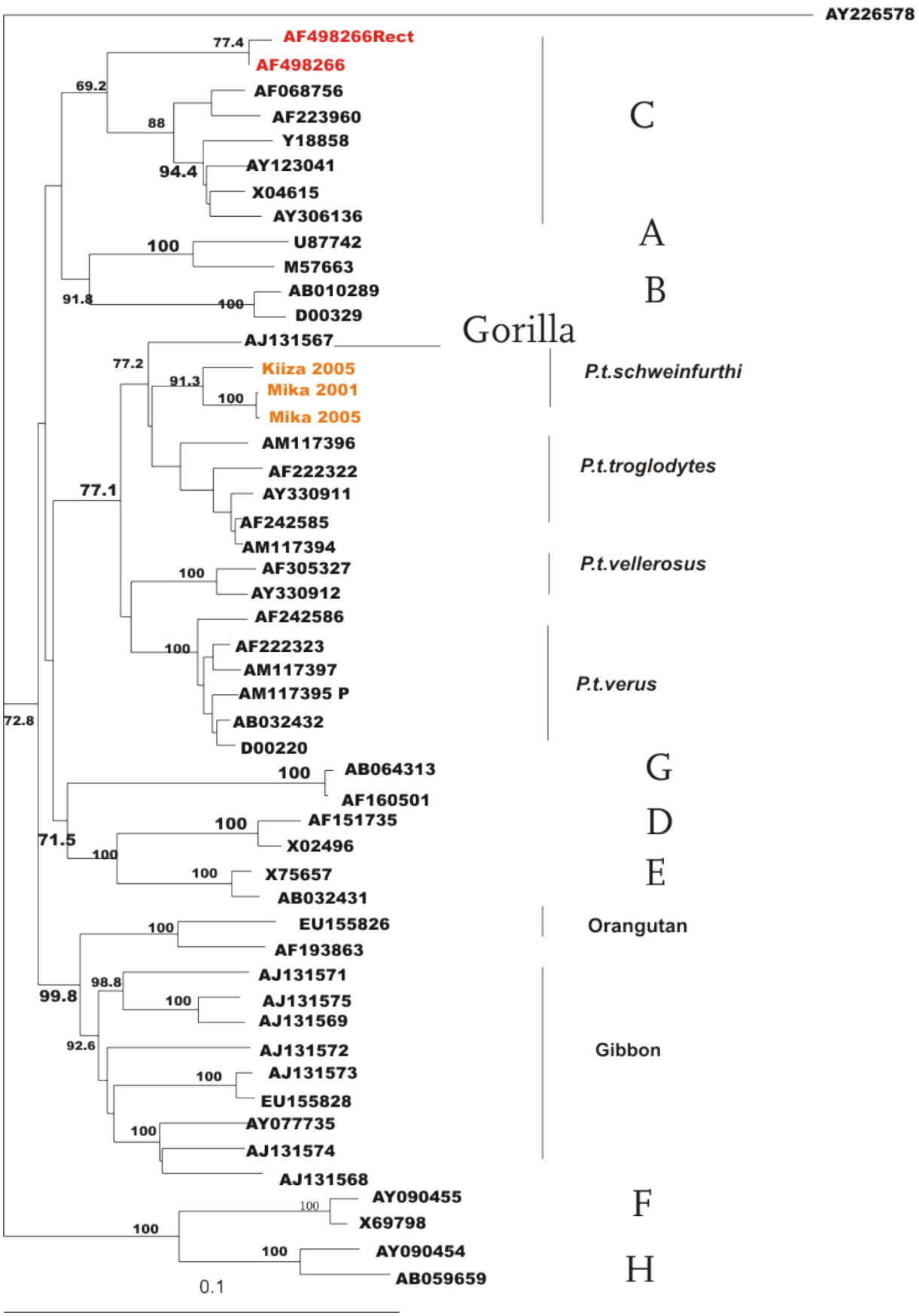
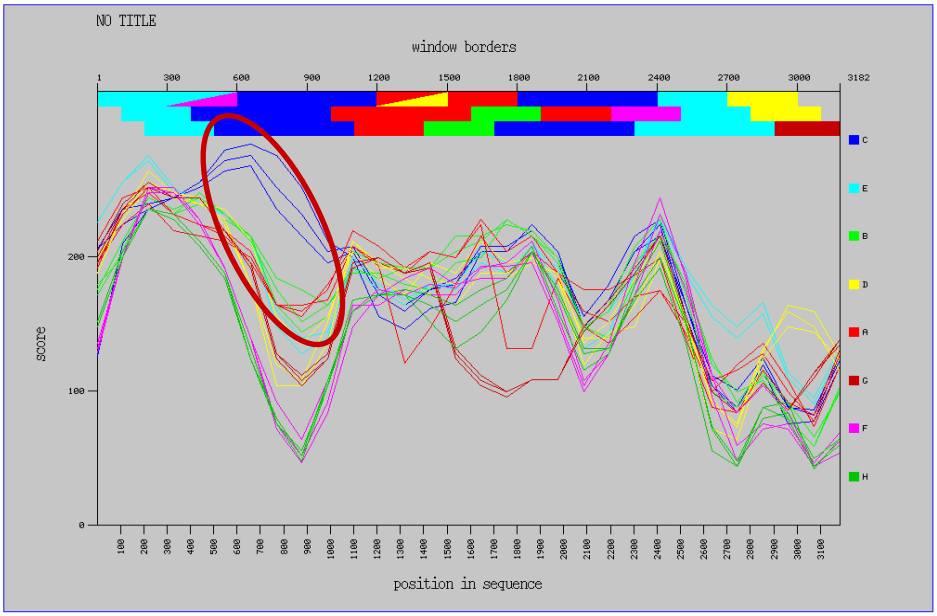

AY226578
AF498266Rect
AF498266
AF068756
AF223960
Y18858
AY123041
X04615
AY306136
U87742
M57663
AB010289
D00329
AJ131567
Kiiza 2005
Mika 2001
Mika 2005
AM117396
AF222322
AY330911
AF242585
AM117394
AF305327
AY330912
AF242586
AF222323
AM117397
AM117395 P
AB032432
D00220
AB064313
AF160501
AF151735
X02496
X75657
AB032431
EU155826
AF193863
AJ131571
AJ131575
AJ131569
AJ131572
AJ131573
EU155828
AY077735
AJ131574
AJ131568
AY090455
X69798
AY090454
AB059659
77.4
69.2
88
94.4
100
91.8
100
77.2
91.3
100
77.1
100
100
72.8
100
100
71.5
100
100
100
98.8
100
99.8
92.6
100
100
100
100
100
0.1
C
A
B
Gorilla
P.t.schweinfurthi
P.t.troglodytes
P.t.vellerosus
P.t.verus
G
D
E
Orangutan
Gibbon
F
H

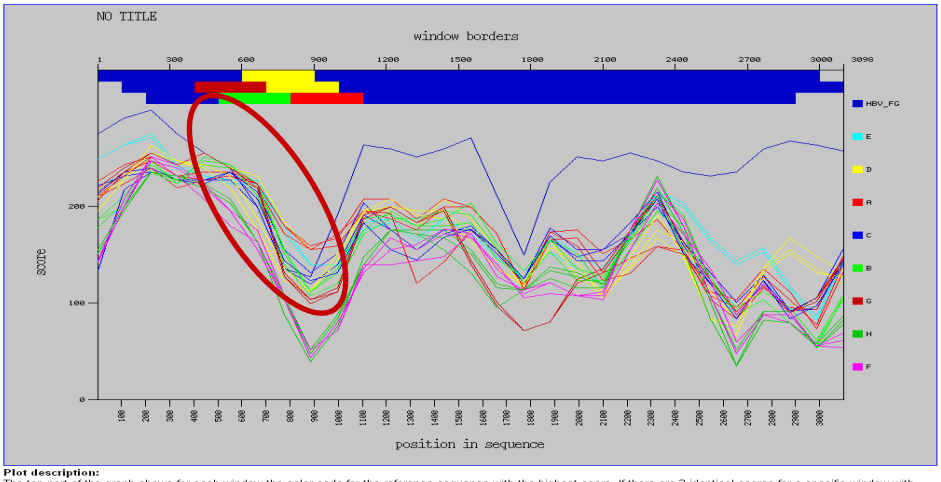
Figure 5.4 (a and b): HBV_FG (from database; *P.t.schweinfurthii*) against Reference sequences of HBV A-H showing recombinant position 500-1000nt similar to HBV C. b) Mika 2001 (HBV isolated from *P.t.schweinfurthii*) against Reference sequences of HBV A-H and HBV_FG. Only position 500-1000 seems not to be similar to HBV_FG → recombination in HBV_FG

a)



Plot description:
The top part of the graph shows for each window the color code for the reference sequence with the highest score. If there are 2 identical scores for a specific window with different subtypes the window bar is divided diagonally.

(b)



Plot description:
The top part of the graph shows for each window the color code for the reference sequence with the highest score. If there are 2 identical scores for a specific window with different subtypes the window bar is divided diagonally.

CHAPTER SIX

EVALUATION OF POLIOVIRUS ANTIBODY TITERS IN ORALLY VACCINATED SEMI-CAPTIVE CHIMPANZEES IN UGANDA

Running Head: Oral polio vaccination in captive chimpanzees

Evaluation of poliovirus antibody titers in orally vaccinated semi-captive chimpanzees in Uganda[5]

Keywords: Poliomyelitis, neutralizing antibodies, transmission, chimpanzees, great apes

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6.0. Abstract

To understand immunological responses in chimpanzees vaccinated with live-attenuated vaccine (Oral Polio Vaccine; OPV), serum neutralizing antibodies against poliovirus types 1, 2 and 3 were investigated over time. The neutralizing antibody titers against poliovirus types 1, 2 and 3 were determined by microneutralization test using 100 ID_{50} of poliovirus types 1, 2 and 3 (Sabin strains). Neutralizing antibodies against poliovirus types 1, 2 and 3 were detected in 85.7%, 71.4% and 65% of the serum from 42 chimpanzees tested 9 years post vaccination. The neutralizing antibody titers in chimpanzees were similar to the documented levels in human studies as an indicator of vaccine efficacy. Therefore, this study reveals persistence of neutralizing antibodies in chimpanzees for at least 9 years after vaccination with OPV. This first study in chimpanzees provides useful information for the evaluation of the success of vaccination with OPV in other captive apes.

6.1. Introduction

Poliomyelitis is a life-threatening acute paralytic disease caused by poliovirus (PV) (Bodian et al., 1949; Bodian, 1972; Hovi et al., 2005; Moriniere et al., 1993; Nathanson and Martin, 1979; Wood et al., 2000). Poliovirus is highly infectious belonging to the genus *Enterovirus* in the family *Picornaviridae* with three distinct serotypes (type 1, 2 and 3) (Bodian et al., 1949). Poliovirus is mainly transmitted through the feacal-oral route as the virus replicates efficiently in the intestinal tract. In humans it is typically shed with the stool for two to four weeks. Feaces can serve as a source of contamination of water, milk and food. Houseflies can passively transfer poliovirus from feaces to food (Gear, 1952). Virus transmission is facilitated by poor sanitation and factors such as crowding, low levels of hygiene, water quality and sewage handling facilities. Humans are considered as the sole reservoir for poliovirus. The three serotypes are able to cause human infection with incubation periods of 5-35 days. Poliomyelitis is an acute viral infection which ranges in severity from a non-specific illness to paralysis with permanent disability and death. Historically poliovirus paralyzed and killed high numbers of people before the licensing of inactivated poliovirus vaccine (IPV) in 1955 and OPV in 1962 and introduction of vaccination worldwide (Nathanson and Martin, 1979). The IPV is prepared by growing the poliovirus in monkey kidney tissue culture (vero cell line) and inactivated with formaldehyde (CDC, 2001). It contains 2-phenoxyethanol, neomycin, streptomycin, polymyxin

B (used to prevent bacterial and fungal growth) and all three serotypes of poliovirus (CDC, 2002a). The vaccine is effective in inducing circulating antibodies in blood thus preventing polio virus in the gut from entering and replicating in the central nervous system (Wood et al., 2000). Whereas trivalent OPV is a live attenuated vaccine containing all three serotypes of poliovirus in a ratio of 10:1:6 (CDC, 2002a; Kew et al., 2004). These attenuated PV strains replicate in the human gut inducing mucosal immunity that inhibits replication of the virus in the gastrointestinal tract (CDC, 2002a; Kew et al., 2004). The OPV produces long-lasting mucosal immunity by stimulating the formation of IgA antibodies in the intestine and also serum antibodies in the bloodstream (Pelczar et al., 1993).

Although humans are the only known natural reservoirs of poliovirus, non-human primates, especially apes (chimpanzees and gorillas), are susceptible (Dowdle and Birmingham, 1997; Douglas et al., 1970). Macaques, African green monkeys and *Cebus* spp. can be experimentally infected with poliovirus but do not generally develop a paralytic disease when infected by the peripheral route (Samuel et al., 1993). However, paralytic disease due to poliovirus infections via human contacts has been reported in captive chimpanzees, orangutans, gorillas and colobus monkeys (Allmond et al., 1967; Bodian et al., 1949; Douglas et al., 1970; Guiloud et al., 1969; Howe and Bodian, 1944; Howe and Bodian, 1948). Antibodies and shedding of virus have been found in imported animals (Douglas et al., 1970), and some chimpanzees may act as symptomless carriers (Brack, 1987). A suspected poliovirus outbreak in wild apes was reported in Kasakela Community of the Gombe chimpanzee population research group in Tanzania in 1966 where six chimpanzees died from the disease and at least six others were paralyzed lifelong (Goodall and Van, 1968; Goodall, 1983; Goodall, 1986). A similar outbreak among the free-ranging chimpanzees was reported in Beni, Zaire, with at least 7 out of 48 chimpanzees in the study group handicapped by limb paresis (Kortlandt, 1996). Analysis of chimpanzee skeletons of two adult females with long-term, partial paralysis and a group of unaffected adult Gombe chimpanzees revealed that poliovirus caused considerable asymmetries in the skeleton (Morbeck, 1991). It was not clarified whether the outbreak was part of a natural cycle within the great ape population or a result of poliovirus transmission from humans. Chimpanzees in Gombe National Park (25 km^2) inhabit a potentially contaminated environment from human communities living close to the park in the north and south sharing the same resources such

as water (Goodall, 1986; Teleki et al., 1976). Poliovirus was widespread in the local human population during the 1960s and therefore, transmission from humans was discussed as the most likely route when the chimpanzee community was habituated for research (Huskster, 1975; Teleki et al., 1976). Chimpanzees are susceptible to almost all human pathogens, so close proximity increases the risk of disease transmission (Leendertz et al. 2006). Hence, it was recommended that chimpanzees and other apes in captive facilities should be vaccinated against poliovirus to avoid the risk of direct or indirect transmission from humans (Junge, 1995; Wallis and Lee, 1999; Woodford, 2000).

Therefore, primates in sanctuaries are generally vaccinated with the human poliovirus vaccine. However, as yet no long-term studies have been undertaken to evaluate immunological responses in vaccinated apes and all recommendations on vaccination strategies are based on human studies. The American Zoo and Aquarium Association Species Survival Plan (SSP) recommend vaccination of captive great apes with IPV at 12-13 months of age with a booster once at 1-2 years (Junge, 1995; Wallis and Lee, 1999). Captive facilities and sanctuaries in Africa are using OPV in apes following human vaccination schedules, but various regimes are used. To complement the Global Polio Eradication Program vaccination programs of non-human primates in sanctuaries have to be adapted to the current state of art.

In this study, we show that OPV induces a long lasting immunity against poliovirus in sanctuary chimpanzees in Uganda and we discuss the future use of IPV for the vaccination of non-human primates in captivity.

6.2. Materials and Methods

6.2.1. Study site

Ngamba Island Chimpanzee Sanctuary which started in 1998, is located on Ngamba Island (S 000 06/E 32°39, 0.46 km^2, 1160 m above sea level) and is part of the Koome group of islands in Mukono District, Uganda, lies 23 km off Entebbe in the north-west of Lake Victoria. Ngamba Island Chimpanzee Sanctuary currently cares for 44 rescued orphan chimpanzees on 100 hectares of secondary rain forest in a semi-captive management system with dietary supplements every day.

6.2.2. Study species

The study was conducted on 42 orphan chimpanzees that live in a semi-captive management system on Ngamba Island Chimpanzee Sanctuary. The group consisted of 23 females and 19 males with age group categorized as follows: 4 infants (1 to 5 years), 6 juveniles (6 to 8 years), 12 sub-adults (9 to 11 years) and 20 adults (12 years and more). They have lived on the island for varying lengths of time after being rescued from illegal traders and poachers since 1998. Rescued individuals are held in quarantine for 90 days during which period they are vaccinated against polioviruses using Oral Polio Virus Vaccine (OPV, 0.1 ml of Sabin Polio, SB Biologicals 52904, 4A). Routine management of these rescued chimpanzees on the island exposes them to direct human contact by caregivers and veterinarians and indirectly to tourists, school groups, local community members and researchers. They are fed on locally grown fruits and vegetables purchased from local markets.

6.2.3. Blood Collection

Blood was collected from all chimpanzees during our annual medical checks in February, 2007. Individual animal welfare is of paramount importance and as part of our standard operating procedures; hand-held intramuscular injection of anesthetic drugs using a combination of Ketamine (3 mg/kg) and Meditomidine (0.03 mg/kg) is administered to minimize stress. Blood (7.5 ml) was taken from the femoral vein using EDTA Vacutainer tubes. Plasma/serum was extracted by centrifugation and stored at –80°C at the Uganda Virus Research Institute until transported on dry ice to Robert Koch-Institut, Berlin, Germany, for analysis. Retrospective serum collected in 2001 and 2005 and stored at –40°C were included in the assessment of antibody titers. This study was approved by Chimpanzee Sanctuary &Wildlife Conservation Trust as part of the veterinary preventive healthcare management, and research and export permits were issued by Uganda Wildlife Authority, Uganda National Council of Science and Technology, CITES Office in Uganda and Germany.

6.2.4. Serology

The methods for determination of the neutralizing antibody titers against poliovirus types 1, 2 and 3 have been described elsewhere (WHO, 2004). Briefly, the microneutralization test using $100ID_{50}$ poliovirus (Sabin strains) was performed according to WHO guidelines (WHO,

2004). A positive control using an In-House Reference serum (IHR) of known neutralizing activity was included in each test to control reproducibility of results. The International Standard Serum (ISS, a preparation of pooled human serum) containing 25 IU, 50 IU and 5 IU for polioviruses 1, 2 and 3, respectively, was used to calibrate the potency of the IHR (Diedrich et al. 2002). Before laboratory testing, serum was inactivated for 30 minutes at 56°C in a water bath. Afterwards, twofold dilutions of serum starting from 1:4 to 1:512 were incubated for 3 hr at 36°C in duplicate with 100 $TCID_{50}$ of the corresponding Sabin-type poliovirus. Human rhabdomyosarcoma (RD) cell suspensions were added and results were scored after 7 days of culture. A serum dilution of ≥1:4 giving protection against 100 $TCID_{50}$ of poliovirus was considered to be positive.

6.3. Results

The chimpanzees vaccinated with OPV under this study were found with neutralizing antibodies against poliovirus types 1, 2 and 3 detected in 85.7%, 71.4% and 65%, respectively of the tested serum 9 years post vaccination. Of the studied chimpanzees, five had no neutralizing antibodies (<1:4) against poliovirus types 1, 2 and 3, including the captive-born infant that had not been vaccinated. Percentages for neutralizing antibodies from 2001 and 2005 serum was not computed due to high levels of bacterial contamination in the samples. For non-contaminated serum samples (results not shown here), neutralizing antibody titers were comparable with those obtained from 2007. The three recent orphan arrivals (Kityo, Rutoto and Rambo) had multiple vaccinations (more than three with OPV) while in quarantine as shown in table 6.1, but the neutralizing antibodies showed the same trend as those vaccinated once or twice.

6.4. Discussion

We present for the first time a vaccination success of captive chimpanzees with OPV. The neutralizing antibody titers found in chimpanzees are similar to those reported in human studies (Diedrich et al. 2002) indicating a high level of protection in the OPV vaccinated chimpanzees. This study also reveals persistence of neutralizing antibodies for at least 9 years post vaccination with OPV. The percentage of neutralizing antibodies detected against poliovirus types 2 and 3 (71.4% and 65%) were generally lower than those for poliovirus 1 (85.7%). This is in agreement with serological studies in humans, showing lower antibody titers for poliovirus

types 2 and 3 (Conyn-van Spaendonck, et al. 2003; Diedrich et al. 2002). Four chimpanzees had no neutralizing antibodies (<1:4) against poliovirus types 1, 2 and 3 despite records showing they had been vaccinated at least once. This may be due to other viral or helminth intestinal infections prohibiting uptake of OPV as reported in developing countries where OPV has been shown to be less potent in inducing humoral immunity. Repeated vaccinations up 5 to 10 times are hence required to protect all children (John and Jayabal, 1972; Melnick, 1996; Moriniere et al. 1993; Oduntan et al. 1978).

It is known that immunity to poliomyelitis is dependent on humoral neutralizing antibodies, both after natural infection and after vaccination. Hence, presence of antibodies beyond a threshold antibody level is an indication of protective immunity in case of poliomyelitis. It has been shown that enterovirus infections also induce T-cell immunity (Juhela et al. 1999), but little information is available about the cellular immunity in great apes. It is not even clear whether these individual chimpanzees with low titers or no detectable antibodies are susceptible to infection as it is with humans.

Trivalent OPV is recommended by Expanded Programme on Immunisation(EPI) as a vaccine of choice for eradication of poliomyelitis because of its low cost, ease of administration, superiority in conferring intestinal immunity, and the potential to infect household and community contacts secondarily (EPI, 1991). However, it has been reported that OPV is less potent in inducing humoral immunity in developing countries where the infection of the intestines by other viruses may prohibit the intake of OPV. Further, the continuous use of oral polio vaccine may result in increase vaccine-associated paralytic polio (VAPP), circulating vaccine-derived polioviruses (cVDPV) and those originating from immune deficient patients (iVDPV) (Kew et al. 2004; Martin, 2006). Therefore, the inactivated polio vaccine (IPV), which is already widely used in developed countries, should play the major role during the endgame of polio eradication and thereafter.

Hence, poliovirus vaccination of non-human primates, especially apes in captive facilities (zoos and sanctuaries), should be evaluated along with the global eradication program and follow the established WHO global action for laboratory containment of wild polioviruses (WHO, 1998; WHO, 2003). This study shows that OPV induces high levels of protective

immunity in chimpanzees, but future use of OPV in captive apes should be assessed. Since vaccine-derived strains (VDPV) may be excreted in feaces for several weeks and even years (in the case of immunodeficient patients), it serves as a source of dissemination of polioviruses and the cause of poliomyelitis (Hovi et al. 2005; Kew et al. 2005; Martin, 2006). Sequence drift has been shown in VDPV as an indication of prolonged replication of the vaccine strain either in one individual or in the community (Cherkasova et al. 2003; Kew et al. 2005; Martin, 2006). Although, it is not known for how long the polioviruses are excreted in feaces of vaccinated apes, it may be risky to continue to use OPV in apes, and IPV may be safer to use at this point.

The results presented in this study will serve as a baseline data for more studies to be undertaken in primates to estimate risks and advantages of OPV versus IPV vaccinations for great apes in sanctuaries.

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Table 6.1. Neutralizing poliovirus antibodies in chimpanzee serum collected in 2007.

Chimpanzee name	Sex	Vaccination dates (Day.Month.Year)	Neutralizing antibodies, 2007		
			Poliovirus 1	Poliovirus 2	Poliovirus 3
Masiko	§C.male	21.11.01	1:4	>1:32	<1:4
Robbie	Male	27.11.01	>1:32	1:4	>1:32
Tumbo	Male	21.11.01	>1:32	>1:32	>1:32
Eddy	Male	21.11.01	>1:32	1:16	1:8
Sunday	§C.Male	27.11.01	>1:32	>1:32	1:16
Mika	Male	20.11.01	>1:32	1:16	>1:32
Megan	Female	03.10.01	>1:32	>1:32	>1:32
Kidogo	Female	26.09.01	>1:32	>1:32	>1:32
Peace	Female	21.11.01	>1:32	>1:32	1:16
Sophie	Female	26.09.11	>1:32	>1:32	1:8
Nagoti	Female	03.10.01	>1:32	>1:32	<1:4
Katie	Female	26.09.01	>1:32	1:16	>1:32
Connie	Female	26.09.01	>1:32	>1:32	<1:4
Bahati	Female	03.10.01	>1:32	>1:32	<1:4
Natasha	Female	21.11.01	>1:32	1:8	1:8
Becky	Female	27.11.01	>1:32	1:16	>1:32
Sally	Female	27.11.01	>1:32	1:4	>1:32
Cindy	Female	11.10.01	>1:32	1:4	1:4
Ikuru	Female	11.10.01	>1:32	1:4	<1.4
Nkumwa	Female	11.10.01	<1:4	1:4	<1:4
Billi	Female	20.11.01	>1:32	1:8	1:4
Yoyo	Female	20.11.01	1:4	<1:4	<1:4
Namukisa	female	20.11.01	>1:32	>1:32	1:4
Pasa	Female	20.11.01	Ct	1.8	ct
Ndyakira	Female	23.05.02/30.07.02	>1:32	1:8	1:16
Kazahukire	Female	20.08.02	1:4	1:4	<1:4
Nakuu	Female	31.05.02/30.07.02	>1:32	ct	Ct
Nani	Female	2002	1:8	ct	Ct
Mawa	Male	10.11.01	>1:32	>1:32	>1:32
Kalema	Male	10.11.01	1:16	1:4	>1:32
Umutama	Male	20.11.01	>1:32	1:4	1:8
Umugezi	Male	20.11.01	>1:32	<1.4	>1:32
Baluku	Male	20.11.01	>1:32	<1.4	>1:32
Asega	Male	10.11.01	>1:32	>1:32	<1.4
Kisembo	Male	11.10.01	<1.4	<1.4	<1.4
Indi	Male	20.11.01	>1:32	>1:32	<1:4
Okech	Male	19.04.02/21.05.02	>1:32	1:8	<1.4
Bwambale	Male	09.03.02	1:16	>1:32	<1:4
Kyewunyo	Female	Not vaccinated	<1.4	<1.4	<1.4
Kityo	Male	9/06; 11/06; 02/07	>1:32	1:16	<1:4
Rutoto	Male	06/06; 08/06; 02/07	>1:32	1:8	1:8
Rambo	Male	09/06; 10/06; 12/06; 02/07	>1:32	>1:32	<1:4

Cut-off at dilution of 1:4 in serum

§: Castrated male; Ct: results could not be read due to bacterial contamination

CHAPTER 7

GENERAL DISCUSSION

7.1 General Discussion and Recommendations

Pathogen transmission between non-human primates (NHP) and humans is a major concern especially between workers in primate sanctuaries, zoos, research centers and laboratories who are occupationally exposed. This study has provided evidence that chimpanzees in sanctuary harbour multiple viral pathogens with zoonotic potential. Specific Polymerase Chain Reactions (PCR) and enzyme-linked immunoassays employed in this study revealed that chimpanzees on Ngamba Island Chimpanzee Sanctuary (NICS) are infected with simian foamy virus, herpesviruses, adenoviruses and hepatitis B virus. Some chimpanzees were infected with more than one virus.

7.1.1. Retrovirus infections in chimpanzees

Phylogenetic analysis of SFV sequences obtained in this study formed four sub clusters within the specific SFV *P. t. schweinfurthii* clade with significant variability among the new SFVs strains. This finding gives evidence for on-going transmission of SFV among chimpanzees within the sanctuary mostly likely through horizontal routes leading to co-infection of individuals with more than one strain. This transmission pattern and the fact that the chimpanzees in sanctuaries are housed closely together for a long-time may attribute to co-infection with strains from different regions leading to likely recombination of viral genomes. Chimpanzees and other NHPs are usually confined in limited space and in large groups that induce high levels of aggression leading to bite wounds ranging from scratches to deep bite wounds. SFV transmission in sanctuaries is mainly through bite wounds and scratches as already described by others (Murry *et al*., 2006). Comparable transmission routes seem to play an important role also for trans-species transmission to humans (Calattini et al. 2008; Jones-Engel et al., 2006). This study could not amplify faecal RNA and proviral DNA for SFV from fresh extracts of faeces but it is known the SFV is shed in faeces and transmitted predominately by horizontal routes and prone to super infection and recombination (Lui et al., 2008). This increases the risks of SFV transmission to persons working daily with NHPs as in sanctuaries. SFV and other retroviruses especially Simian Immunodeficiency Virus (SIV) have attracted public health attention after discovery that HIV types 1 and HIV-2, respectively originated from cross-species transmission of SIV from infected chimpanzee (*Pan troglodytes*) and Sooty mangabeys (*Cercocebus atys*) in Central and West Africa, respectively (Hahn et al., 2000; Keele et al., 2006; Lemey et al., 2003;

Sharp et al., 2005). Furthermore, it was shown that SIVcpz, the immediate precursor of HIV-1, is pathogenic in infected free-ranging chimpanzees (Keele et al., 2009).

Humans are susceptible to cross-species infections by SFV from a variety of wild living primate species as already described for persons living near non-human habitats in rural Congo (Calittini et al., 2008) or hunters living in Cameroon (Wolfe et al., 2004) and persons with frequent contacts with NHPs in several South and Southeast Asian countries (Engel et al., 2006; Murphy et al., 2006; Wolfe et al. 2004). Though this study was not extended to search in staff working with chimpanzees for all possible viral markers, the risks for them having or potentially acquiring SFV infection is high and future studies should include animal care takers and all other persons coming into direct contact with chimpanzees.

Although SFV is currently considered as non-pathogenic in natural hosts, SFV could alter the course of SIV and HIV infections following documentation of dual SFV/HIV infections in both sex worker and blood donor cohorts in Africa (Murry et al., 2006; Switzer et al., 2008). It has also been suggested that the pathogenic properties of retroviruses can be altered by recombination of two different virus strains. This has for example been shown for SIVcpz, which is most likely a recombinant of SIVrcm, from red-capped mangabeys (Cercocebus torquatus), and SIVgsn, from greater spot-nosed monkeys (Cercopithecus nictitans) (Bailes et al., 2003). This SIVcpz has later been transmitted to humans and resulted in the HIV-1 epidemic (Gao et al., 1999).

Until now, SFV could not be associated with a clinical disease in NHPs However, there are humans known to carry the infection since many years and it should be kept in mind that a) the number of infected humans is low and may not reflect all possible outcomes of a SFV infection b) there is a variety of SFV strains which may have different properties after trans-species transmission and trans-species transmission may change pathogenic properties, c) recombination of strains may alter pathogenicity and infectivity d) also other retroviruses such as SIV and STLV do not (or rarely) cause symptoms in the NHP host and would be considered a “harmless virus”. Only relatively few SIV strains have developed pathogenic properties in the human host.

7.1.2 Herpesvirus infections in Chimpanzees

Chimpanzees at NICS were infected with herpesviruses of subfamily *Gammaherpesvirinae* including the novel virus that fell into the clade of primate rhadinoviruses and the Kaposi sarcoma herpesvirus (human herpesvirus 8). This provides more evidence that chimpanzees and other primates harbour a variety of until now unknown viruses. These viruses may have zoonotic potential and their human counterparts have been shown to pose significant health risks including induction of cancer and lymphoproliferative diseases especially in immune-compromised individuals. This again calls for comprehensive staff health monitoring and development of measures for prevention of transmission.

7.1.3 Hepatitis B virus infection in chimpanzees

In this study antibodies against hepatitis B (core) antigen (ant-HBc) were determined in chimpanzees on NICS. Furthermore, chHBV DNA was detected by PCR in one captive wild born (Mika) and one wild chimpanzee (Kiiza). The study provides evidence of persistent infection with high viral load in the HBV infected chimpanzees and shedding of virus particles in faeces. HBV infection in humans is responsible for approximately 1 million deaths from chronic liver disease and hepatocellular carcinoma every year. To date, few cases of recombination of chimpanzee HVB strain (hHBV genotype C and chHBV-FG isolate) and recombination among chimpanzee subspecies (*P.t.ellioti* and *P.t.troglodytes* in Cameroon) have been reported (Njouom et al., 2010). This gives evidence of cross-species transmission which might be followed by recombination of the strains whose biological significance is not known. Hence, HBV cases in sanctuaries need to be monitored to avoid cross-species transmission and all staff members should be vaccinated against HBV.

7.1.4 Adenovirus infection in chimpanzees

In this study, we found high prevalence of adenovirus infection in wild captive chimpanzees from faecal samples using universal Adv-PCR. Our findings are similar with recent findings that healthy populations of great apes (chimpanzees, bonobos, gorillas and orangutans) shed substantial quantities of infectious adenoviruses in stool (Roy et al., 2009). Their study also revealed evidence for intraspecies recombination between adenoviruses, high degree of phylogenetic relatedness across their primate hosts providing evidence of

cross species transmission. The shedding of live adenoviruses in stool presents high risks for zoonotic transmission to caregivers in sanctuaries coming into close contact with primate feacal material during cleaning of housing facilities. This study draws attention to the required need for improvement of cleaning, disinfection and disposal faecal waste procedures in ape sanctuaries.

7.1.5 Application of non-invasive methods

This study also shows the applicability of using non-invasive methods for search and detection of some of the viral pathogens as evidenced in this study by detection of DNA particles for adenoviruses, herpesviruses and hepatitis B virus in faeces of chimpanzees by PCR. Non-invasive methods have recently been employed in disease investigations in the wild apes and provided useful information on natural reservoirs of HIV-1 groups M and N in distinct populations of chimpanzees (Keele et al., 2006; Van Heuverswyn et al., 2007) by detecting antibodies and viral RNA in ape feacal samples. Western gorillas (*Gorilla gorilla*) were found to be endemically infected with SIV designated SIVgor by analyzing the faecal samples (Van Heuverswyn et al., 2006). Non-invasive methods were employed in the identification and characterization of SFVcpz infections in wild living chimpanzees (Lui et al., 2008) and establishing the origin of the human malaria parasite *Plasmodium falciparum* in gorillas (Hahn et al., 2010). Hence, non-invasive methods provide important tool for health monitoring of wild apes plus understanding of the epidemiology and dynamics of naturally circulating viral pathogens in the wild and their transmissions. Sanctuaries provide special opportunities for developing and validating non-invasive methods and their subsequent use in disease monitoring in sanctuaries and in wild living apes.

7.1.6. Multiple infections and risk of recombination

In the sanctuary setting described in these studies, a high prevalence of simian foamy virus, herpesviruses, adenoviruses and hepatitis B virus in chimpanzees was determined. This may have direct implications to the management of the sanctuary in a variety of ways: i) in sanctuaries, ape hand raising of recently rescued baby apes plus long term interactions accompanied with bites and scratches presents a high risk of the transmission of such agents to employees. These findings sound a serious warning to the sanctuary operators of the risks

exposed to workers and high lighten a need for extra precautions, ii) sanctuaries receive apes at different times of the year and hence all in coming apes should be screened for known pathogens before being integrated in the groups to reduce risks of intra-species transmission and iii) most sanctuaries aim at reintroduction of great apes into the wild as already being implemented. These results show that potentially a mixing of viruses is more likely in sanctuary great apes. The possible impact of those on wild primates, mixing possibly again with other regional strains, is unknown. With current recognition of the public health significance due to retrovirus cross species transmission in persons occupationally exposed to non-human primates and those exposed to other social economic factors like through bushmeat and pet trade, calls proactive monitoring and assessment of all incoming primates to sanctuaries and during the course of management of these colonies. At the same time, measures to reduce the risks of acquiring occupationally related diseases should be intensified.

7.1.7 Vaccination strategies in sanctuaries and health monitoring of employees and visitors

Proactive preventive measures through vaccination as recommended in the management of sanctuaries may prove valuable but also requires careful assessment. This study shows that chimpanzees vaccinated against poliomyelitis using oral polio vaccine had neutralizing antibodies against polio virus type 1, 2 and 3 even nine years post vaccination. Conversely only a few chimpanzees vaccinated against measles had detectable protective titers. Hence vaccination of chimpanzees and other primates requires monitoring of immunological responses to ensure protection is provided. Where it is not possible to carryout vaccination in NHP, all persons working in close contact with NHPs should be vaccinated against major diseases of concern. Most sanctuaries are being open now to tourists as a way of raising awareness for the endangered apes and funds for sanctuary management. Regarding tourists, it has been noted that a large proportion of travelers to tropical regions are not protected against vaccine preventable diseases and the majority of these travelers demonstrate poor recall of their actual vaccination status (Muehlenbein et al., 2008). The described behaviours plus the health status of tourists visiting primate sanctuaries and participating in some rehabilitation programs poses risks of anthropozoonotic transmission of infectious diseases with un-predetermined threat to survival of apes and other wildlife.

Hence various preventive measures employed by sanctuaries need to be evaluated and Ngamba Island Chimpanzee sanctuary veterinary preventive healthcare program for the chimpanzees and employees health program provides some insights. NIC requires for all staff members an up to date vaccination against Hepatitis A and B, Measles, Polio, Meningiococal meningitis, yellow fever, and a recent TB every year. The health program also covers complete physical examination, full blood count, liver and kidney function tests, urine analysis, HIV and malaria screening, common helminthes and protozoa examination at a selected medical service provider. Full medical coverage through an insurance company is provided to all staff plus few members of their household. Treatment and counseling for chronic diseases is offered to individual staff members according to their health status. This approach, if well designed and monitored along with other occupational health and safety protocols, can minimize the risks of disease transmission. As a requirement all tourists and volunteers participating in special programs of rehabilitation bringing them into close contact with chimpanzees must present proof of up to date vaccinations (Hepatitis A and B, Measles, Polio, Meningiococal meningitis, yellow fever, and a recent negative TB test) which is cross checked by the veterinarian before and on their arrival at the island. But only 65% of all tourists (day and night visitors) to the island per year have their vaccinations update (unpublished data). The relatively low percentage of vaccinated tourists is due to the high number of day tourists that are not obliged to have their vaccination up dated and are not checked by the veterinarian.

Experience in managing this program has shown that tourists visiting primate sanctuaries and other great apes areas are willing to undertake extra precautions including updating their vaccinations so long they are given information well ahead of time and there is evidence that these policies are enforced. In this respect, tour operators can play a significant role in disseminating information to their potential clients and system set up for paper verification. Visitors to all sanctuaries and wild great ape sites should fax their medical documents to the designated responsible person for review before they leave their country and feedback is given to them immediately.

If this approach is adopted for all NHP sanctuaries and sites for ecotourism in all great ape habitats could significantly reduce the risks of disease transmission. However, these precautions should not be seen as true measures on their own but have to be enforced by additional regulations. Direct interactions of tourists with great apes should be forbidden. Precautions to minimize levels of interactions with apes in sanctuaries should be implemented as more risks exist beyond vaccination preventable diseases as evidenced in this study. Programs like forest walks and baby integration allowing tourists to interact directly with chimpanzees should be completely discouraged. This close interaction presents risks of pathogen transmission to both chimpanzees and tourists. From the list of vaccinations required, it is recognized that it is based on long-term recognizable preventable illnesses and is not up to date with current global disease situation. Hence, this practice should be re-evaluated and when necessary new recommendations developed.

7.1.8 Specific recommendations for the sanctuaries

The current evidence provided in this study, that chimpanzees on Ngamba Island Chimpanzee Sanctuary are infected with multiple viral pathogens with zoonotic potential, requires proactive review of management protocols to address the levels of interactions and minimize the risks of disease transmission between NHPs and humans. The specific recommendations are therefore suggested for the sanctuaries as follows:

1. Rescues and acquisitions of NHPs should be handled by trained personel with the knowledge of potential hazards associated with handling of NHPs and equipped with preventive measures including personal protective equipments.

2. Proper quarantine infrastructure that minimizes close interactions with caregivers should be put in place. The quarantine protocols should ensure the NHPs are screened for the viral and bacterial pathogens of zoonotic potential like Simian immunodeficiency virus, simian foamy virus, hepatitis viruses, herpes viruses, adenoviruses among others. The caregivers should also be monitored for any signs of ill health especially the respiratory symptoms during the quarantine period. Blood samples should be collected from both NHPs and caregivers at the beginning and end of quarantine, analysed for baseline and aliquots bio-banked for future reference.

3. All staff working with NHPs should be vaccinated against most of the communicable diseases and efforts undertaken to evaluate their immunological status especially for hepatitis B virus. Vaccination of tourists against major communicable diseases visiting great ape areas including sanctuaries should be enforced as a mechanism to reduce risks of disease transmission.

4. NHPs in sanctuaries should be vaccinated against poliovirus using inactivated poliovirus vaccine (IPV) instead of live attenuated oral poliovirus vaccine (OPV) in line with the global efforts for eradication of poliomyelitis. Other vaccines used against measles and tetanus should be evaluated for their effectiveness by analyzing antibody titers overtime.

5. Standard operating procedures and employee health programs should be reviewed and updated to emphasize minimization of direct interactions between NHPs and humans especially caregivers, volunteers and researchers. Employees should have full knowledge of the occupational health and hazard risks associated with working with NHPs. Interactions of NHPs with tourists through programs like forest walk and integration as part of "rehabilitation process" should be **stopped** as it poses a risk of disease transmission during interaction process.

6. NHPs in sanctuaries should be managed based on their health status and disease risk analysis performed if meaningful intervention and decisions are to be undertaken regarding routine care, re-introduction and other conservation and management approaches.

7. Health monitoring of chimpanzees and other NHPs in sanctuaries should be based on extensive health data (clinical and underlying viral and bacterial potential pathogens) on all individuals with emphasis on zoonotic pathogens and perfomed periodically at an interval of 3-5years.

8. Chronic infections and some cases of normal colonization my play critical role in progression of illnesses during acute infections and in immunocompromised individuals and hence important for monitor the body systems and organs of affected individuals like for the documented cases of hepatitis B virus infection.

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APPENDIX: Research approval and permits

UGANDA WILDLIFE AUTHORITY
HEADQUARTERS, PLOT 7 KIRA ROAD KAMWOKYA
P O Box 3530, Kampala Uganda

Your Ref:
Our Ref: UWA/TBDP/RES/50 3rd February 2006

Dr. Lawrence Mugisha
Faculty of Veterinary Medicine
Makerere University
P. O. Box 7062
Kampala
UGANDA

RE: RESEARCH APPLICATION APPROVAL

I am in receipt of your application dated 19th January 2006 seeking to carry out a study on Ngamba Island Chimpanzee Sanctuary addressing **" Seroepidemiological and Molecular Study of Immunological responses to vaccination against Measles, Polio and Tetanus and other Naturally Acquired viruses in Captive Chimpanzees in Uganda."**

I am glad to inform you that your research application has been approved for you to carry out research from 30th April 2006 to 30th April 2009. You will be expected to submit a progressive report by October 2007 and a final report of your findings by October 2009 to the Monitoring and Research Unit of the Uganda Wildlife Authority.

Should you be unable to work within these dates, please notify me in writing. Please note that, any researcher failing to submit the final report, will not be allowed to come back to wildlife protected areas to do further research.

You will be required to pay an application fee of 10,000/= (Ten thousand Uganda shillings) to Uganda Wildlife Authority.

Since your research involves specimen collection and export, you will be required to fill a Material Transfer Agreement (MTA) with UWA and apply to the Uganda National Council for Science and Technology for an export permit before exporting the specimen.

By copy of this letter, UNCST is dully informed that your research has been approved by UWA.

Sincerely,

Aggrey Rwetsiba
For: EXECUTIVE DIRECTOR

c.c. Executive Secretary, UNCST
c.c. Executive Director, CSWCT

Uganda National Council For Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Your Ref: .....

Our Ref: NS 71

Date: 12-Feb -07

Dr. Lawrence Mugisha
Department of Wildlife Health and Management
Faculty of Veterinary Medicine
Makerere University
Kampala

Dear Dr. Mugisha,

The Uganda National Council for Science and Technology (UNCST) has granted your request for approval to continue with the study entitled, **"Seroepidemiological responses and molecular study of immunological responses to vaccination against measles, polio and tetanus and other naturally acquired viruses in captive chimpanzees in Uganda ".** The approval will expire on March 01, 2008. If, however, it is necessary to continue with the research beyond this expiry date, a request for continuation should be made to the Executive Secretary, UNCST.

The Resident District Commissioner of Mukono District in which the study will be conducted are informed by copy of this letter, and are kindly requested to give you the necessary assistance to accomplish the study.

Yours sincerely,

Jane Nabbuto
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

LOCATION/CORRESPONDENCE
Plot 3/5/7 Nasser Road
P.O Box 6884
KAMPALA, UGANDA.

COMMUNICATION
TEL: (256) 41-250499, (256) 41-705500
FAX: (256) 41-234579
E-MAIL: uncst@starcom.co.ug
WEBSITE: http://www.uncst.go.ug

Uganda National Council For Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Your Ref:.....

Our Ref:.....NS 71 *Date:*.....20/02/08.....

Dr. Lawrence Mugisha
Department of Wildlife Health and Management
Faculty of Veterinary Medicine
Makerere University
P.O Box 7062
Kampala

Dear Dr. Mugisha,

The Uganda National Council for Science and Technology (UNCST) has granted your request for approval to continue with the study entitled, **"Sero-epidemiological study of immunological responses to vaccination against measles, polio and other naturally acquired viral pathogens in captive chimpanzees in Uganda".** The approval will expire on March 01, 2009. If, however, it is necessary to continue with the research beyond this expiry date, a request for continuation should be made to the Executive Secretary, UNCST.

The Resident District Commissioner of Wakiso District in which the study will be conducted is informed by copy of this letter, and is kindly requested to give you the necessary assistance to accomplish the study.

Yours sincerely,

Leah Nawegulo
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

LOCATION/CORRESPONDENCE
Plot 3/5/7, Nasser Road
P.O. Box 6884
KAMPALA, UGANDA.

COMMUNICATION
TEL: (256) 414-250499, (256) 414-705500
FAX: (256) 414-234579
E-MAIL: uncst@starcom.co.ug
WEBSITE: http://www.uncst.go.ug

Uganda National Council For Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Your Ref:.....

Our Ref: NS 71 *Date:* 20/02/08

Dr. Lawrence Mugisha
Department of Wildlife Health and Management
Faculty of Veterinary Medicine
Makerere University
P.O Box 7062
Kampala

RE: TRANSFER OF BIOLOGICAL MATERIAL

We refer to your application to transfer 200 vials of 2 mls each of serum/plasma, 300 vials of 2 mls each of feaces, 300 vials of 2 mls each of urine, 45 vials of 2 mls each of oral swabs, 90 vials of 2 mls each of biopsy samples and 40 vials of 2 mls each of tissue samples in RNA from Chimpanzees of Ngamba Island Sanctuary, Uganda under the approved research project entitled, "Sero-epidemiological study of immunological responses to vaccination against measles, polio and other naturally acquired viral pathogens in captive chimpanzees in Uganda", to Robert Koch Institute, Berlin, Germany through Entebbe International Airport, Uganda.

The Council has approved your application to transfer the above specimen within the framework of the agreed terms and conditions of the Material Transfer Agreement between yourself on behalf of Uganda Wildlife Authority and the Robert Koch Institute, Berlin, Germany. We, however, request that you submit to Council progress reports of studies done on the specimen.

The Commissioner Customs, Uganda Revenue Authority is duly informed by copy of this letter and is kindly requested to give you the necessary assistance to facilitate the transfer of the specimen.

Yours sincerely,

Leah Nawegulo
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

cc The Secretary, Office of the President
cc The Commissioner Customs, Uganda Revenue Authority
cc Executive Director, Uganda Wildlife Authority
cc CITES Management Authority
cc Prof. Georg Pauli, Robert Koch Institute, Berlin, Germany

LOCATION/CORRESPONDENCE
Plot 3/5/7, Nasser Road
P.O. Box 6884
KAMPALA, UGANDA.

COMMUNICATION
TEL: (256) 414-250499, (256) 414-705500
FAX: (256) 414-234579
E-MAIL: uncst@starcom.co.ug
WEBSITE: http://www.uncst.go.ug

ÄISCHE GEMEINSCHAFT / *EUROPEAN COMMUNITY*

ORIGINAL / *ORIGINAL*

1

1. Ausführer/Wiederausführer / *Exporter/re-exporter*
Makerere University-Faculty of Vet. Medicine
P.O. Box 7062
Kampala
UGANDA

GENEHMIGUNG/BESCHEINIGUNG *PERMIT/CERTIFICATE*
- [X] **EINFUHR** / *IMPORT*
- [ ] **AUSFUHR** / *EXPORT*
- [ ] **WIEDERAUSFUHR** / *RE-EXPORT*
- [ ] **SONSTIGES** / *OTHER:*

Nr. / No
E - 0335/07

2. Letzter Gültigkeitstag: *Last day of validity:*
06.08.2007

Übereinkommen über den internationalen Handel mit gefährdeten Arten frei lebender Tiere und Pflanzen
Convention on International Trade in Endangered Species of Wild Fauna and Flora

3. Einführer / *Importer*
Robert-Koch-Institut
Nordufer 20
13353 Berlin
GERMANY

4. (Wieder-)Ausfuhrland / *Country of (re)-export*
UGANDA

5. Einfuhrland / *Country of import*
GERMANY

6. Ort, an dem lebende, der freien Wildbahn entnommene Exemplare der in Anhang A aufgeführten Arten gehalten werden dürfen / *Authorised location for live specimens of Annex A species*

7. Ausstellende Vollzugsbehörde / *Issuing management authority*
BUNDESAMT FÜR NATURSCHUTZ
Konstantinstraße 110
D-53179 BONN

8. Beschreibung der Exemplare (einschl. Kennzeichen, Geschlecht/Geburtsdatum von lebenden Tieren) / *Description of specimens (incl. marks, sex/date of birth for live animals)*
SPE scientific specimen
280 PC serum/plasma
200 PC feaces
200 PC urine
40 PC vaginal swabs
40 PC oral swabs
60 PC blood filter papers
40 PC feaces in RNAlater

9. Nettomasse (kg) / *Net mass (kg)*

10. Menge / *Quantity*
860 PC

11. CITES-Anhang / *CITES Appendix*	12. EG-Anhang / *EC Annex*	13. Herkunft / *Source*	14. Zweck / *Purpose*
I	A	W	S

15. Ursprungsland / *Country of origin*
UGANDA

16. Genehmigungs-Nr. / *Permit No*
001856

17. Ausstellungsdatum / *Date of issue*
04.04.2007

18. Letztes Wiederausfuhrland / *Country of last re-export*

19. Bescheinigungs-Nr. / *Certificate No*

20. Ausstellungsdatum / *Date of issue*

21. Wissenschaftlicher Artname / *Scientific name of species*
PAN TROGLODYTES

22. Üblicher Artname / *Common name of species*
SCHIMPANSE

23. Besondere Bedingungen / *Special conditions*
Gemäß Artikel 8 Abs. 1 der Verordnung (EG) Nr. 338/97 ist es verboten, Exemplare der in Anhang A der Verordnung aufgeführten Arten zu vermarkten. Die mit der Genehmigung verbundenen Auflagen (s. Anlage) sind zu beachten.

Diese Genehmigung/Bescheinigung ist nur gültig, wenn lebende Tiere unter Einhaltung der CITES-Leitlinien für den Transport und die Vorbereitung des Transports von lebenden Wildtieren oder, im Falle eines Lufttransports, der Vorschriften des Internationalen Luftverkehrsverbandes (IATA) für den Transport lebender Tiere befördert werden. / *This permit/certificate is only valid if live animals are transported in compliance with the CITES Guidelines for the Transport and Preparation for Shipment of Live Wild Animals or, in the case of air transport, the Live Animals Regulations published by the International Air Transport Association (IATA).*

24. Die (Wieder-)Ausfuhrunterlagen des (Wieder-)Ausfuhrlandes / *The (re)-export documentation from the country of (re)-export*
- [ ] wurden der ausstellenden Behörde vorgelegt / *has been surrendered to the issuing authority*
- [X] müssen der Grenzzollstelle bei der Einfuhr vorgelegt werden / *has to be surrendered to the border customs office of introduction*

25. Die / *The* [X] Einfuhr / *importation* [ ] Ausfuhr / *exportation* [ ] Wiederausfuhr / *re-exportation*
der oben beschriebenen Ware wird genehmigt. / *of the goods described above is hereby permitted.*

Unterschrift und Stempel der Behörde / *Signature and official stamp:*

BUNDESAMT FÜR NATURSCHUTZ 1

Name des ausstellenden Beamten / *Name of issuing official*
Im Auftrag Raths

Ort und Datum der Ausstellung / *Place and date of issue:*
Bonn, den 07.02.2007

26. Frachtbrief/Luftfrachtbrief Nr. / *Bill of lading / Air waybill No:*

27. Nur von der Zollbehörde auszufüllen / *For customs use only*

Tatsächlich eingeführte oder (wieder-) ausgeführte Menge/Nettomasse (kg) / *Quantity/net mass (kg) actually imported or (re)-exported*	Anzahl der bei der Ankunft toten Tiere / *Number of animals dead on arrival*

Zolldokument / *Customs document*
Typ / *Type:*
Nummer / *Number:*
Datum / *Date:*

Unterschrift und amtlicher Stempel / *Signature and official stamp:*

WILHELM KÖHLER VERLAG Bestell-Nr. 221
32372 Minden, Postfach 1261, Telefon 0571/82823-0, Telefax 0571/8282323
60323 Frankfurt/M., Telemannstr. 13, Telefon 069/972025-97+98, Telefax 069/727296
20095 Hamburg, Mönckebergstr. 11, Telefon 040/303805-33+34, Telefax 040/337723
53113 Bonn, Kaiserstr. 15, Telefon 0228/224050, Telefax 0228/261640
04317 Leipzig, Kippenbergstr. 12, Telefon 0341/26145-10+11, Telefax 0341/2619407
(2006)

AISCHE GEMEINSCHAFT / *EUROPEAN COMMUNITY*

KOPIE zur Rücksendung an die ausstellende Vollzugsbehörde / *COPY for return by customs to the issuing authority**

3

1. Ausführer/Wiederausführer / *Exporter/re-exporter*

Chimpanzee Sanctuary & Wildlife Conservation Trust
P.O. Box 884
Entebbe
UGANDA

GENEHMIGUNG / BESCHEINIGUNG / *PERMIT/CERTIFICATE*

[X] **EINFUHR** / *IMPORT*
[ ] **AUSFUHR** / *EXPORT*
[ ] **WIEDERAUSFUHR** / *RE-EXPORT*
[ ] **SONSTIGES** / *OTHER:*

Nr. / ***No***

E - 0437/08

2. Letzter Gültigkeitstag: / *Last day of validity:*

28.07.2008

3. Einführer / *Importer*

Robert-Koch-Institut
Nordufer 20
13353 Berlin
GERMANY

Übereinkommen über den internationalen Handel mit gefährdeten Arten frei lebender Tiere und Pflanzen
Convention on International Trade in Endangered Species of Wild Fauna and Flora

4. (Wieder-)Ausfuhrland / *Country of (re)-export*

UGANDA

5. Einfuhrland / *Country of import*

GERMANY

6. Ort, an dem lebende, der freien Wildbahn entnommene Exemplare der in Anhang A aufgeführten Arten gehalten werden dürfen / *Authorised location for live specimens of Annex A species*

7. Ausstellende Vollzugsbehörde / *Issuing management authority*

BUNDESAMT FÜR NATURSCHUTZ
Konstantinstraße 110
D-53179 BONN

8. Beschreibung der Exemplare (einschl. Kennzeichen, Geschlecht/Geburtsdatum von lebenden Tieren) / *Description of specimens (incl. marks, sex/date of birth for live animals)*

SPE scientific specimen
200 x 2 ml Serum
300 Kotproben
300 Urinproben
45 Rachenabstriche
45 Biopsieproben
20 Gewebeproben

9. Nettomasse (kg) / *Net mass (kg)*

10. Menge / *Quantity*: 910 PC

11. CITES-Anhang / *CITES Appendix*	**12.** EG-Anhang / *EC Annex*	**13.** Herkunft / *Source*	**14.** Zweck / *Purpose*
I	A	U	S

15. Ursprungsland / *Country of origin*: UGANDA

16. Genehmigungs-Nr. / *Permit No*

17. Ausstellungsdatum / *Date of issue*

18. Letztes Wiederausfuhrland / *Country of last re-export*

19. Bescheinigungs-Nr. / *Certificate No*

20. Ausstellungsdatum / *Date of issue*

21. Wissenschaftlicher Artname / *Scientific name of species*

PAN TROGLODYTES

22. Üblicher Artname / *Common name of species*

SCHIMPANSE

23. Besondere Bedingungen / *Special conditions*

Gemäß Artikel 8 Abs. 1 der Verordnung (EG) Nr. 338/97 ist es verboten, Exemplare der in Anhang A der Verordnung aufgeführten Arten zu vermarkten. Die mit der Genehmigung verbundenen Auflagen (s. Anlage) sind zu beachten.

Diese Genehmigung/Bescheinigung ist nur gültig, wenn lebende Tiere unter Einhaltung der CITES-Leitlinien für den Transport und die Vorbereitung des Transports von lebenden Wildtieren oder, im Falle eines Lufttransports, der Vorschriften des Internationalen Luftverkehrsverbandes (IATA) für den Transport lebender Tiere befördert werden. / *This permit/certificate is only valid if live animals are transported in compliance with the CITES Guidelines for the Transport and Preparation for Shipment of Live Wild Animals or, in the case of air transport, the Live Animals Regulations published by the International Air Transport Association (IATA).*

24. Die (Wieder-)Ausfuhrunterlagen des (Wieder-)Ausfuhrlandes / *The (re)-export documentation*

[ ] *from the country of (re)-export* wurden der ausstellenden Behörde vorgelegt / *has been surrendered to the issuing authority*
[X] müssen der Grenzzollstelle bei der Einfuhr vorgelegt werden / *has to be surrendered to the border customs office of introduction*

1 annex attached

25. Die / *The* [X] Einfuhr / *importation* [ ] Ausfuhr / *exportation* [ ] Wiederausfuhr / *re-exportation*

der oben beschriebenen Ware wird genehmigt. / *of the goods described above is hereby permitted.*

Unterschrift und Stempel der Behörde / *Signature and official stamp:*

BUNDESAMT FÜR NATURSCHUTZ

Name des ausstellenden Beamten / *Name of issuing official:*
Im Auftrag Raths

Ort und Datum der Ausstellung / *Place and date of issue:*
Bonn, den 29.01.2008

26. Frachtbrief/Luftfrachtbrief Nr. / *Bill of lading / Air waybill No:*

27. Nur von der Zollbehörde auszufüllen / *For customs use only*

Tatsächlich eingeführte oder (wieder-) ausgeführte Menge/Nettomasse (kg) / *Quantity/net mass (kg) actually imported or (re)-exported*	Anzahl der bei der Ankunft toten Tiere / *Number of animals dead on arrival*

Unterschrift und amtlicher Stempel / *Signature and official stamp:*

Zolldokument / *Customs document*
Typ / *Type:*
Nummer / *Number:*
Datum / *Date:*

*) Im Fall einer Einfuhrgenehmigung für Exemplare der in Anhang I von CITES aufgeführten Arten kann diese Kopie dem Antragsteller zur Vorlage bei der Vollzugsbehörde des (Wieder-)Ausfuhrlandes zugesandt werden.
*) *In the case of an Import permit for specimens of CITES Appendix I species, this copy may be returned to the applicant for submission to the MA of the (re-)exporting country.*

WILHELM KÖHLER VERLAG Bestell-Nr. 221 (2006)
32373 Minden, Postfach 1761, Telefon 05 71 / 9 38 93-0, Telefax 05 71 / 9 38 93 33
60323 Frankfurt/M., Telefon 0 69 / 72 20 25-27 +28, Telefax 0 69 / 72 72 96
20095 Hamburg, Mönckebergstr. 11, Telefon 0 40 / 30 38 05-33 +34, Telefax 0 40 / 33 77 23
53119 Bonn, Kaiserstr. 15, Telefon 02 28 / 22 40 50, Telefax 02 28 / 26 16 40
04317 Leipzig, Kippenbergstr. 12, Telefon 03 41 / 26 14 5-10 +11, Telefax 03 41 / 2 61 94 07

CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES OF WILD FAUNA AND FLORA

☑ EXPORT ☐ IMPORT ☐ RE-EXPORT

1. **PERMIT** Original
Sn. UG 001944

2. Valid until 13.03.2008

3. Consignee (name and full address including Telephone, country)
Robert-Koch-Institut
Nordufer 20 13353 Berlin
Germany

4. Permittee (name and full address including Telephone, country)
Dr. Lawrance Mugisha, C/O Department of Wildlife Health and Management, Faculty of Veterinary Medicine P.O.Box 7062 Kampala, Uganda Tel. +256 772566551

5. Special conditions for the Export/Re-export/Import
- Inspected by Uganda Wildlife Authority, Customs and Veterinary Department on exit
- Samples for Scientific purposes, and research update reports required after analysis
- Copy of certified permit returned to this office within 7 days of export

6. Name, full address including Telephone, and national seal of Management Authority
Justus Tindigankayo-Kashagire, Commissioner Wildlife Conservation, Ministry of Tourism, Trade and Industry P.O.Box 4241 Kampala Uganda
Tel. +256 414 251294

THE REPUBLIC OF UGANDA
WILDLIFE DIVISION

	7./8. COMMON NAME AND SCIENTIFIC NAME (Genus and Species) OF ANIMAL OR PLANT	9. Description of part or derivative, including identification marks or numbers (age/sex if live)	10. Appendix No. and source (W.C.A. or O)	11. Quantity: number of specimen and/or net weight (kg)
A	Chimpanzee Pan troglodytes schweinfurthii	Serum/Plasma	I W Country of Origin*: Uganda	Two hundred (200) vials x 2 ml Licence/Permit No. and Date: NS 71 20.02.2008
B	Chimpanzee Pan troglodytes schweinfurthii	Feaces	I W Country of Origin*: Uganda	Three hundred (300) vials x 2ml Licence/Permit No. and Date: NS 71 20.02.2008
C	Chimpanzee Pan troglodytes schweinfurthii	Urine	I W Country of Origin*: Uganda	Three hundred (300) vials x 2ml Licence/Permit No. and Date: NS 71 20.02.2008
D	Chimpanzee Pan troglodytes schweinfurthii	Oral Swabs	I W Country of Origin*: Uganda	Forty-five (45) vials x 2 ml Licence/Permit No. and Date: NS 71 20.02.2008
E	Chimpanzee Pan troglodytes schweinfurthii	Biopsy samples	I W Country of Origin*: Uganda	Fourty-five (45) vials x 2 ml Licence/Permit No. and Date: NS 71 20.02.2008
F	Chimpanzee Pan troglodytes schweinfurthii	Tissue samples in RNA Later	I W Country of Origin*: Uganda	Twenty (20) vials x 2 ml Licence/Permit No. and Date: NS 71 20.02.2008
G			Country of Origin*	Licence/Permit No. and Date

12. THIS PERMIT IS ISSUED BY: Justus Tindigankayo-Kashagire

Kampala (Place) 14.03.2008 (Date) (Signature) (Official Stamp and Title)

13. Endorsement by Authorised Wildlife Enforcement Officer at port of exit/entry

14. EXPORT ENDORSEMENT (By Customs Officials at Port of Entry/Exit)

15. Bill of Lading/Air Way Bill Number

See block/field 7/8	Quantity Exported
A	
B	
C	
D	
E	
F	
G	

Signature, Official Stamp and Title | Port of Exportation | Date | Signature, Official Stamp and Title

CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES OF WILD FAUNA AND FLORA

☑ EXPORT ☐ IMPORT ☐ RE-EXPORT

1. **PERMIT** Original
Sn. UG 001856

2. Valid until
04.10.2007

3. Consignee (name and full address including Telephone, country)
Robert-Koch-Institut Nordufer 20 13353 Berlin, Germany. Attn. Prof. Dr. Georg Pauli

4. Permittee (name and full address including Telephone, country)
Dr. Lawrence Mugisha, C/O Department of Wildlife Health and Management, Makerere University, P.O.Box 7062 Kampala, Uganda Tel. 256 772 366551

5. Special conditions for the Export/Re-export/Import
- Examined by Uganda Wildlife Authority, Customs and Veterinary Department on exit
- Samples packed safely and used strictly for Scientific purposes
- Copy of endorsed permit returned to this office within 7 days by export

6. Name, full address including Telephone, and national seal of Management Authority
Justus Tindigarukayo-Kashagire
Commissioner Wildlife, Ministry of Tourism, Trade and Industry
P.O.Box 7103 Kampala
Uganda
Tel. 256 414 251294

THE REPUBLIC OF UGANDA
WILDLIFE DIVISION

	7./8. COMMON NAME AND SCIENTIFIC NAME (Genus and Species) OF ANIMAL OR PLANT	9. Description of part or derivative, including identification marks or numbers (age/sex if live)	10. Appendix No. and source (W.C.A. or O)	11. Quantity: number of specimen and/or net weight (kg)
A	Chimpanzee Pan troglodytes	Serum / Plasma	IW Country of Origin*: Uganda	Two hundred and eighty (280) vials x 2mls Licence/Permit No. and Date: MTA 0079 14.02.2007
B	Chimpanzee Pan troglodytes	Feacal samples (frozen)	IW Country of Origin*: Uganda	Two hundred (200) vials x 2mls Licence/Permit No. and Date: MTA 0079 14.02.2007
C	Chimpanzee Pan troglodytes	Urine	IW Country of Origin*: Uganda	Two hundred (200) vials x 2mls Licence/Permit No. and Date: MTA 0079 14.02.2007
D	Chimpanzee Pan troglodytes	Vaginal swabs in RNALater	IW Country of Origin*: Uganda	Forty (40) vials x 2mls Licence/Permit No. and Date: MTA 0079 14.02.2007
E	Chimpanzee Pan troglodytes	Oral swabs in RNALater	IW Country of Origin*: Uganda	Forty (40) vials x 2mls Licence/Permit No. and Date: MTA 0079 14.02.2007
F	Chimpanzee Pan troglodytes	Blood Filter paper	IW Country of Origin*: Uganda	Sixty (60) Licence/Permit No. and Date: MTA 0079 14.02.2007
G	Chimpanzee Pan troglodytes	Faecal samples in RNALater	IW Country of Origin*: Uganda	Forty (40) vials x 2mls Licence/Permit No. and Date: MTA 0079 14.02.07

12. THIS PERMIT IS ISSUED BY: Justus Tindigarukayo-Kashagire

04.04.2007 Kampala
Place Date

Signature

Official Stamp and Title

13. Endorsement by Authorised Wildlife Enforcement Officer at port of exit/entry

14. EXPORT ENDORSEMENT (By Customs Officials at Port of Entry/Exit)

15. Bill of Lading/Air Way Bill Number

See block/field 7/8	Quantity Exported
A	
B	
C	
D	
E	
F	
G	

Signature, Official Stamp and Title | Port of Exportation | Date | Signature, Official Stamp and Title

*Country in which the specimens were taken from the wild, bred in captivity or artificially propagated

[P.T.O.]