Outbreak of Anthrax among the Hippopotamus population of the Queen Elizabeth National Park, Uganda

by

Fabian H. Leendertz, ZBS-1 / Robert Koch-Institute

SERVICE CONTRACT financed under the 8th European Development Fund - Project Nr 8/ACP/TPS 32

PAN-AFRICAN PROGRAMME FOR THE CONTROL OF EPIZOOTICS (PACE)

International Services

NOVEMBER 2004

Outbreak of Anthrax among the Hippopotamus population of the Queen Elizabeth National Park, Uganda

1. Summary

A severe epidemic affecting hippopotami was reported in Queen Elizabeth National Park (QENP), Uganda. At least 215 individuals had died of an acute infection with *B. anthracis*, which is known to be lethal for hippopotami. In the course of this consultancy further samples from dead hippopotami were collected and *B. anthracis* was confirmed using a Real Time PCR. Results from analyses on water samples collected at different places of the area did not reveal the presence of the pathogen in the waterways. However, the carcass density was very low at the time of sample collection and the concentration of contaminating spores may have undergone significant changes depending on time and environmental factors.
The main recommendation is to enhance the diagnostic capacity of the MAAIF laboratory, in order to allow reliable diagnosis which, in combination with an "emergency plan", will assure fast and effective outbreak response and will prevent outbreaks of this degree in the future.

2. Background:

2.1. History of the outbreak

The Uganda Wildlife Authority (UWA) informed the Ministry of Agriculture Animal Industry and Fisheries (MAAIF) of a massive death of hippopotami in Queen Elizabeth National Park (QENP) on 19th August 2004.
A team of scientists from UWA, MAAIF and Makerere University Kampala was constituted to carry out the first investigation between the 23rd and 28th August 2004. This team came up with findings suggestive of an Anthrax or Rinderpest outbreak. Another team, composed of UWA, MAAIF and PACE Technical Advisor carried out a second investigation between 14th and 17th September 2004.
In the course of this investigation a male hippopotamus, apparently clinically ill, was sacrificed and a necropsy performed. Gross pathological findings and first laboratory examinations suggested infection with *B. anthracis* as the cause of the illness. Samples where subsequently sent to the Robert Koch-Institute Berlin, where the tentative diagnosis was confirmed definitively by PCR (Ellerbrok et al. 2002, Leendertz et al. 2004) to be B. anthracis infection.
The MAAIF/Uganda PACE team, and the UWA agreed to mobilize a consultant through the PACE technical support program contracted to GTZ. The aim of this consultancy was to collect further relevant data on the current outbreak and to assess the possibilities for enhancement of laboratory capacities at the MAAIF Diagnostic and Epidemiology Center.

2.2. Anthrax in Hippopotami

Anthrax is a spore forming bacillus and is known to virtually infect all mammals, including humans. Three forms of anthrax are described, a gastrointestinal, a respiratory and a cutaneous form. In hippopotami acute B. anthracis infections have been described for various regions of Africa, including the QENP. Very little data are available on the ways an infection is contracted and on the mode of transmission of B. anthracis among hippopotami; however, the main hypothesis is that the initial case(s) get infected via grazing in anthrax contaminated areas as described for ruminants. Spores of *B. anthracis* may be found in contaminated soil for centuries. Possible chains of infection may be explained by ingestion of spores or vegetative bacilli that may be found near carcasses in water or soil. In the literature few reports are found stating, that no sporulation occurs in water, if dilution levels of 1:640 are reached since the vegetative bacilli die once the protective blood is left and before sporulation takes place (Lindeque et al. 1994). Dilution depends on water movements and quantity of water and takes place quickly (Lindeque et al. 1994).
Carnivorous mammals (including humans) usually get infected via the consumption of meat of infected animals, whereby scavengers are generally resistant to high concentrations of anthrax bacilli (Jäger et al. 1990). Carnivorous behavior among hippopotami has been observed in the recent past, (Dudley 1996, 1998a, 1998b) leading to the suggestion that they may also get infected by consuming parts of cadavers (see also discussion in ProMedmail)..

3 Approach and methods used

3.1. Assessment of the present situation

An overview on the activities carried out in the course of the consultancy is given in annex I.

3.1.1 Field investigation

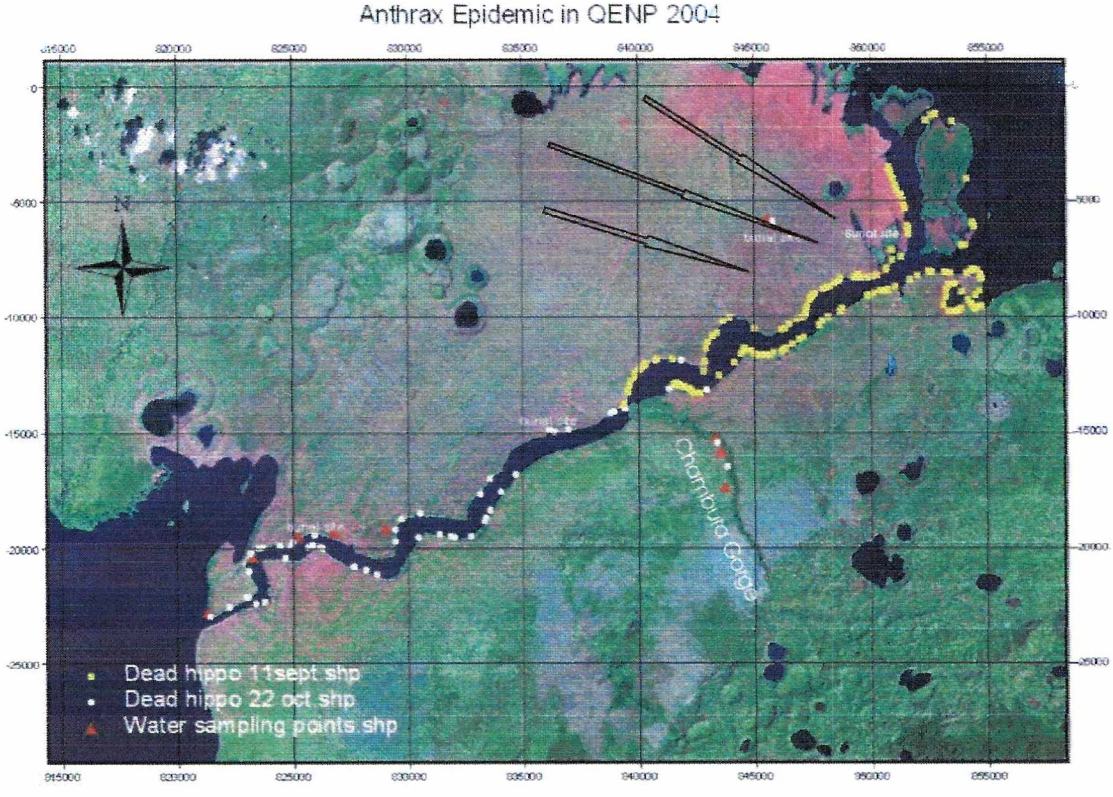
Carcass burial was continuing during the field mission. The carcasses were buried in 4 different sites, at least 7 meters off from the shore.
During the first investigation by boat one dead hippopotamus was found located floating in the Kazinga Channel, driven by the current, and another near the shore, surrounded by a school of hippopotami. Both were dead since about 2 days. One older carcass (dead since 5-7 days) was found entangled in papyrus close to the shore.
Removal of carcasses was performed the next day. A fourth carcass was found on the 07th of November, probably dead since 2 days.
All four dead hippopotami were found near the Mweya Safari Lodge in the Kazinga Channel. Samples (a little piece of the ear) were collected from 2 hippopotami (H1 and H2) and stored in 5% Formaldehyde and 10% Glycerol saline for later analyses. Little streams of blood were running out of the natural openings, such as eyes and mouth. Interestingly, life hippopotami were observed frequently around the carcasses, especially in those cases where the intestines of the dead hippopotamus were exposed . The life hippopotami refused to leave the area and showed an aggressive behavior toward us approaching the carcass.
During investigation in the Chambura Gorge also a dead hippopotamus was found. Since the body was not accessible no detailed investigation of this carcass could be performed. However, the parts of the body visible showed no signs of fighting.
The cadaver of one about 1 year old buffalo (B1) was found. The cadaver did not show external damages except that both eyes were missing (which had been removed by vultures seen on the cadaver when we arrived). Samples from the prescapulary lymph nodes were taken and conserved following the procedure described above. No signs of external bleedings were observed, the lymph nodes examined were slightly enlarged.
From one "mud fish" (eating the intestines of dead hippopotamus according to the wardens) the stomach was sampled and preserved (sample F1).

At the time leaving the QENP about 215 hippopotami had died, furthermore 16 buffalos and one (young) elephant were found dead.

3.2. Distribution of carcasses

From the reports given by the QENP staff and experts working on the ground in QENP it was not clear, whether the first cases occurred in the Chambura Gorge or at the Lake George. No matter where the epidemic started, it is obvious, that the cases of death progressed slowly from east to west along the Kazinga Channel (direction Lake George to lake Edward). This may be correlated to the water flow between lake George and Lake Edward, however, the opinion on the direction in which the water flows is controversial (Is this really controversial? George gets its water from Rwenzori Mountains and emties to Edward, which flows via Semliki to Albert and finally to Nile). At the time of the investigation most carcasses where found in the Kazinga Channel near Lake Edward and there are also reports on dead hippopotami in the Lake Edward.

Map 1 shows the locations of dead hippopotami as detected between 11th of September and 22nd of October 2004, further data were not made available. On the 11th of September 155 dead hippopotami were counted, on the 22nd of October 55. The estimated total number of hippopotami for the Park is ~ 2000 individuals.



Map 1: Distribution of hippopotami carcasses 11th of September and 22nd of October 2004

3.3. Investigation on the Diagnostic and Epidemiology Center of MAAIF

The laboratory of the MAAIF in Entebbe is the national reference laboratory of the Ugandan Veterinary Services. Examinations carried out include ELISA, bacteriological culturing, and helminthology. Suspicion of *B. anthracis* was made in this laboratory based on microscopical examination..

The laboratory has available sufficient working space and facilities that could be used for the introduction of further diagnostic tests, such as PCR (see recommendations).

3.4. Collection of environmental samples

In the course of discussions the question of water safety was one of the major concerns.

3.4.1. Collection of water samples

In order to determine a potential contamination of the water of the Kazinga channel samples were collected at different sites:

Water directly near the carcass (1-2 cm next to the skin)
Water near shore at "hot spots" of high density of live and dead hippopotamus (sites, where hippopotami go on land, also other animals come here to drink water)
Water about 20 metres off these bays
Water at different points of the Chambura Gorge
Water at different points of the water system of the Mweya Lodge and village (first tank, after 1st filtration, after 2nd filtration)

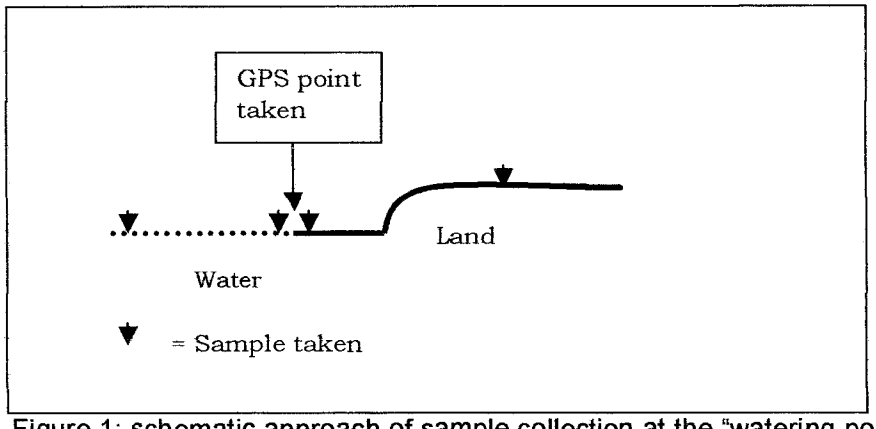


Figure 1: schematic approach of sample collection at the "watering points"

All sample all sample sites were recorded using a GPS and a digital video camera, an overview is given in annexe II. The water and soil sample at "drinking places" were collected according to the figure 1.

From each of the sites in the Chambura Gorge, the hippopotamus pool and the Kazinga channel 250 ml of water were collected and 500 ml from the different points of the water system. The samples were stored on ice upon arrival in the MAAIF laboratory in Entebbe. Here the samples were centrifuged at 500 revolutions per minute and the pellet in approximately 2 ml supernatant (potentially containing spores and bacteria) stored in 2 ml cryotubes as concentrated samples.

3.4.2. Collection of soil samples

Soil samples were collected from the following sites:

Soil near shore at "hot spots" of high density of live and dead hippopotamus (bays where hippopotami go on land, also other animals come here to drink water)
Soil about 50 metres off these bays
Soil at different points of the Chambura Gorge

The samples were stored in 50 ml tubes and stored on ice during transportation and later stored at 4°C. The samples will serve for further analyses, an overview is given in annexe III.

3.5. Analyses

3.5.1. DNA extraction

a) Tissue samples:

DNA was extracted from the tissue samples taken from the hippopotami, the buffalo and the fish following the standard protocol of the DNeasy tissue kit (Qiagen®) for DNA extraction from tissues conserved in formalin. The amount of DNA extracted was measured at 640 nm in a photometer and is shown in annex IV.

b) Water samples:

An aliquot of 1 ml of the (previously concentrated) water samples was centrifuged at 14000 revolutions/minute and the pellet resuspended in RLT buffer, which is the first buffer used for the DNeasy Tissue Kit. From this point on all steps of DNA extraction followed the standard protocol for DNA extraction as described by the manufacturer.

3.5.2. PCR Exclusion of false negative PCR results

In order to verify the absence of so called inhibitors that may interfere with the PCR and produce false negative results, all samples extracted were first tested using the following system:
For testing DNA from tissue samples a final concentration of 500 ng was used for the PCR, for environmental samples the maximum volume of 33 µl was used. An aliquot of the extracted DNA was spiked with an artificial control sequence ("Rokoko"), integrated into a plasmid. This mixture was then analysed in a Real Time PCR assay, with primers designed for the detection of these artificial sequences. Positive results are confirmation for amplifyable DNA, negative ones indicate the presence of inhibitors in the sample. Since in the first Real Time PCR no positive signal could be observed, representative samples were chosen and diluted 1:10 and 1:100 in order to dilute potential inhibitors and in an additional aliquot of these dilutions bovine serum albumin (BSA) was added since BSA is known to overcome inhibitory effects. Real Time PCR was performed on the samples in order to determine conditions that minimize the effect of the inhibitors. All reactions were performed in duplicate in order to exclude false results.

PCR Mix:	Universal Master Mix (Perkin Elmer®)	12,5 µl
	Sens primer	1,5 µl (10 mMol)
	Antisens primer	1,5 µl (10 mMol)
	Taq Man probe	0,5 µl (10 nMol)
	ad 50 µl	sample plus water

Conditions:	1x	2'	50°C
		10'	95°C
	45 x	15"	95°C
		1'	60°C

3.5.3. Taq Man PCR for *B. anthracis*

Real Time PCR was carried out following the standard protocol as published by Ellerbrok et al. (2002).
Samples from "clean DNA" were tested for the presence of *B. anthracis* specific *pag* sequences. For the water samples a dilution of 1:10 plus BSA was used. For examination of the effect of inhibitors again control PCR was performed adding Rokoko.
The Taq Man PCR for the *pag* virulence genes of *B. anthracis* has a sensitivity of 10 copies of *pag* genes per test.

3.5.4. Enrichment of *B. anthracis*

In order to confirm the absence of *B. anthracis* in water samples for 4 representative samples an enrichment was performed, using LB-medium. This medium allows spores and vegetative bacilli, potentially contained in a sample, to multiply so the total number of bacilli will be high enough for detection. After incubation for one day the samples were tested using the *B. anthracis* specific Real Time PCR.
To date sample W01, W02, W03 and W24 are tested.

4. Results

4.1. Exclusion of false negative results:

The results are shown in annex V. Environmental sample frequently contain inhibitors that may interfere with the PCR reaction. The results shown here indicate, that it is possible to avoid inhibition by dilution and addition of BSA. However, extremely "muddy" samples such as the sample "W 25" still were not amplifyable as seen after spiking with the artificial control sequence "Rokoko".

4.2. Real Time PCR for B. anthracis:

Even though inhibition could be excluded, all water samples tested negative for *B. anthracis.* Considering the different steps of concentration and dilution it can be stated that in the water samples tested less than 10 spores can be expected per 3,1 ml sampled water for those where 250 ml were initially collected and less than 10 copies per 6,2 ml for samples from the water system of the Mweya lodge, where 500 ml were sampled.

4.3. Enrichment, Real Time PCR and classical bacteriology

However, also enrichment of samples using LB medium did not result in a positive reaction. This indicates, that none of the most likely spore containing samples (W01, W02 = water from next to a dead hippopotamus) and the other samples W03 (water 20 m off shore) and W24 (water from Mweya Safari Lodge) contained infectious spores. For these samples it can be stated, that no spore was found in 125 ml of water and for W24 (water from Mweya Safari Lodge) no spore could be detected on 250 ml of water. Further classical bacteriological tests were performed on these samples but no B. anthracis was detected.

5. Discussion

5.1. Estimation of areas of high risk to wildlife, livestock and humans

The areas of high risk may vary according to the dynamic of the outbreak. Sites with many dead carcasses in water with low movement should present a higher risk than areas with water movement and lower number of carcasses.
High concentrations of spores and vegetative bacteria in water can probably be the case when carcasses are opened to direct contact of the vegetative bacilli with air.

Studies have shown that the concentration of spores around carcasses in water is usually very low and the effect of dilution quickly results in non detectable levels. Since as well in the direct Real Time PCR as after enrichment of spores no *B. anthracis* could be detected in water next to any cadaver, this seems to apply to the outbreak in QENP.. However for a certain time frame and at specific places under certain conditions (like calm water, high density of carcasses) a significantly higher level of contamination could be expected.

Experiences gathered in other outbreaks have shown that spores can work their way up from the buried cadavers to the surface and high concentrations may be found in the top soil. Also animals have been observed to dig holes and use existing caves (mainly thoraxes) as housings in burial sites since here the manipulated soil remains easy to dig. Even insects have been described to prefer these sites; especially termites seem to be important vehicle for transportation of spores from depth to the surface.
It should therefore be made sure that the burial sites are fenced off sufficiently well and the areas should be monitored routinely for invasion by any digging animals. Superficial decontamination should be conducted using 10% Formaldehyde solution. This may be repeated when necessary.

5.2. Development of a hypothesis regarding the origin of the outbreak

Anthrax is a pathogen, distributed worldwide and spores of the bacillus may survive in soil for probably hundreds of years. "Hot spots" of contaminated areas are known since domestication of livestock. Expressions such as "dammed fields" describe areas contaminated with *B. anthracis* spores, indicated through mortalities among grazing domesticated ruminants. *B. anthracis* is endemic in sub-Saharan Africa.

It will be difficult to determine the origin of the present infection.Two reasonable hypotheses regarding the origin of the epidemic and the spread among the hippopotami of the QENP should be mentioned:

a) The pathogen is endemic

Outbreaks of *B. anthracis* among the hippopotami of the QENP probably already occurred at least 4 times in the history of the park (1959, 1962, 1992, 1999). Due to drought the grassland near the water was overgrazed, so the animals had to walk longer distances and graze the grass close to the ground. This may have resulted in unusually high intake of soil and dust which may have led to intake of *B. anthracis* spores. Furthermore the general body condition of the hippopotami may have declined due to longer travel distances and lower food intake. This may affect the immune system directly and cause "stress" dropping the level of resistance.
Regarding the place where hippopotami may have first acquired the pathogen, one hypothesis would be that in geographically lower areas, like the swamps indicated by the flashes on map 1, the concentration of spores is higher compared to other areas since the spores may have been washed in cause of time downstream and accumulated in the lowlands near Lake Gorge.

b) The pathogen was imported from outside the park:

One opinion is that first cases of death occurred relatively high upstream in the Chambura Gorge. If this is the case it may be possible that the first hippopotamus got infected via a cadaver of a domestic animal, which was floating down the river. Since the river is used by humans and domestic animals outside QENP boundary, one could imagine a scenario where a dead contaminated animal has floated along the river down to the schools of hippopotami.

5.3. Why did it become an epidemic?

Almost as important as the question of origin of the epidemic is how it could spread from one hippopotamus to another and why – if any - buffalo died from *B. anthracis*.

Herbivores normally get infected by the consumption of spores, acquired from contaminated soil. Most of the known cases are linked to special circumstances like drought, where animals are dependent on small water holes and limited grass, or also creation of artificial water holes and other changes of the environment.

In theory it may be possible that all the hippopotami that died of *B. anthracis* got independently infected with the pathogen from one or several environmental sources. However, the temporal and spatial data related to located carcasses indicate, that the epidemic was progressing along the Kazinga Channel continuously, mainly (or even exclusively) affecting hippopotami. So the main question is: How the pathogen was transmitted from one hippopotamus to another?

Unfortunately, no data are available on the amount of spores or vegetative bacilli necessary to infect a hippopotamus. One hypothesis would be that the hippopotami got infected via contaminated water. This cannot be excluded at a given time point, as locally the concentration of B. anthracis may have been high enough to infect a hippopotamus. Also the (non verified) cases of dead buffalo may have been due to consumption of contaminated water, especially on sites where many dead hippopotamus where found.
,It is well established however, that *B. anthracis* can be transmitted via the consummation of meat of infected animals. Thus the second hypothesis is that the hippopotami got infected via the consumption of meat and intestines of hippopotami that previously died of anthrax. Recent data have shown that hippopotami indeed consume parts of other hippopotami that died (Dudley 1996, 1998a, 1998b). My own observation at the Kazinga channel was, that especially near cadavers of hippopotami whose intestines where ejected (due to aerosis and consecutive high pressure in the body) other, live hippopotami would gather around these cadavers and show an aggressive behavior when we approached the cadaver. One assumption could be that the hippopotami defended a good source of protein, especially important in time of extreme drought. Consumption of ejected intestines would have been easily, and even without making a connection to the body which may be guarded (see below). This could have taken place without leaving obvious marks of scavenging on the cadavers. According to the personal burying the carcasses ejected intestines were seen frequently. However, it may also be possible that this behavior was due to territoriality and continued defend of (dead) family members over a certain period of time as observed for some other species

6. Conclusions

An outbreak of B. anthracis is responsible for the massive death among the hippo of the QENP. Detection of B. anthracis (spores or vegetative bacilli) was not possible at the time of this investigation, because the concentration is highly depended on dilution and the fate of bacilli in the water. The ways of transmission from individual to individual remains unclear. Two main hypothesis are presented as infection via contaminated water or consumption of B. anthracis containing organic material.
Since no human cases were proved to be connected to the outbreak and also the results from the analyses of the water did not show contamination, the risk to people using water from the Kazinga Channel is to be considered low at the time of investigation.
The consultancy was of very short duration (10 days within Uganda), only data about what had happened and the present situation could be gather. Consequently MAAIF and UWA should remain present in the outbreak area, be alert and undertake additional sampling + data collection as required.

7. Recommendations

Since the area (especially the burrila sites) around the Kazinga Channel will remain a contaminated area for many years, outbreaks of *B. anthracis* have to be expected also in the future.
In order to avoid epidemics of this extent an emergency plan should be put in place and immediate actions should be taken as soon as unusual cases of death are observed.

7.1 Immediate recommendation during the field investigation

In the course of the field investigation a number of recommendations were made in order to avoid further spread of the disease:

- Since the carcasses of some of the disposed animals were not burned completely it is important to pile up again all remains and burn them completely.
- At burial sites teeth of hippopotami were found hidden in the bushes. It should be made absolutely clear, that these may be contaminated and dangerous to human life.
- Since the amount of soil covering the carcasses buried varies according to the number of hippopotami per hole and also on the open ground remains of the hippopotami where found (claws, blood, intestines), the area should be fenced off.
- The burial sites should be monitored for scavengers and digging animals that may use the cadavers as potential caves/homes.
- Decontamination of the burial sites using a 10% formaldehyde solution, applied on the ground.
- when fresh dead hippopotami are found the carcasses should not be left near the shore where there is low water movement and a high density of other hippopotami, buffalos and other animals. The carcasses should be pulled by boat into the middle of the channel or lake until burial. This will avoid high concentrations of spores along the shoreline at sites where other animals are drinking.
- Inform staff sufficiently about the dangers of the infection and ways of transmission of *B. anthracis*. (This was done on the 07 November together with Dr. R. Ademun (MAAIF), Dr. P. Atimnedi (UWA) and Mr. N. J. Bosco.)

7.2. Draft for an emergency plan

An overview on a possible emergency plan is given in figure 2. It will be crucial, that this plan remains as simply structured as possible since from suspicion of an anthrax case to action should not take more than 4 days. The emergency plan should strictly focus on the outbreak and emergency measures. Permanent presence of a veterinarian would be advisable, at least involvement of local veterinarians working near the park. For those a special training regarding wildlife diseases should be offered.
Further analyses and additional data and/or sample collections should be part of consecutively planned and conducted "field research projects". These investigations will provide over time important reference information and should be supported wherever possible.
For "emergency cases" there should be an "emergency fund" available, which can be immediately released to covers costs of necessary field investigations including sample dispatch and laboratory analysis.

Important points of the plan are:

- Monitoring for dead animals
- Sample collection from dead animals: just a little piece (minimal sample) of the ear is enough to make a blood smear and preserve the rest in a special liquid for sample conservation, for example "RNA-later" (Qiagen)
- Immediate diagnosis using microscopy (in QENP) and the "minimal sample" for PCR diagnostic by a laboratory , immediate communication of the results
- If the sample is negative for B. anthracis and further (fresh) carcasses are found: complete necropsies, pathological evaluation and sample collection by a veterinarian in order to trace other potential pathogens responsible for the death.

immediate carcass removal: In the case of a few dead hippopotami we recommend to just pull the carcass in the middle of the water and drown it without opening the carcass (for example attach it to a sack with stones). *B. anthracis* vegetative forms quickly die in water and no spores can be formed. Spores are only formed when it comes into contact with air. Immediate drowning will minimize the degree of pollution and keep scavengers from opening the carcass exposing it

to air. These measures do not consider the danger of eutrophication of Lake Edward or Lake George, this has to be evaluated by specialists for water ecology.

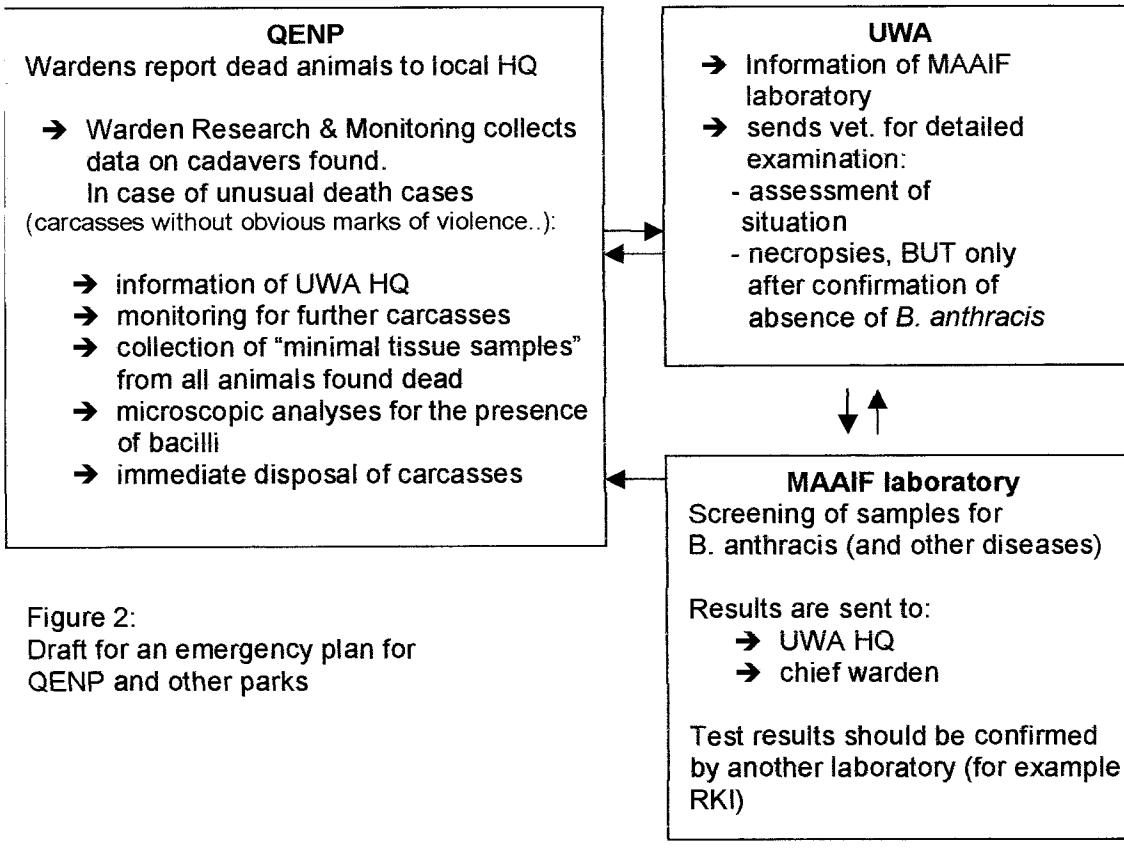


Figure 2:
Draft for an emergency plan for QENP and other parks

7.3. Enhancement of capacities of the MAAIF laboratory

A central point for the efficient control of outbreaks is fast and reliable diagnosis. The experience of the present outbreak has shown that time was lost due to discussions on the possible pathogens responsible for this outbreak and samples had to be send abroad. Robert Koch-Institute was then able to make a definitive diagnosis of the *B. anthracis* within 5 hours upon arrival of the samples applying nucleic acid sequencing techniques. This kind of diagnosis should also be made possible at a laboratory in Uganda. Since the MAAIF laboratory is the national laboratory for animal diseases, we strongly recommend to enhance the capacities of this laboratory to perform modern techniques, which are reliable, sensitive and simple to apply .

We recommend to provide new equipment and to train personnel in the application of standard PCR methods. The advantage would be that culturing of the bacteria will not be necessary and the work can be performed in a standard laboratory. Applying European standards for culturing of *B. anthracis* would require specific safety of BSL3 level.

In the course of this consultancy the laboratory was assessed and the requirements for PCR analyses are given (sufficient separate space in order to avoid contaminations). The Robert Koch Institute Berlin would be willing to provide further know how in order to ensure rapid set up of the methods in question and to ensure quality control by confirming diagnoses and sequencing of PCR products in the future.

Furthermore a simple diagnostic within QENP should be made possible in order to perform a first microscopic examination for Bacilli. It should just be clear, that a confirmatory diagnosis of B. anthracis CANNOT be made using simple microscopy. The confirmation has to be done in a laboratory applying methods mentioned above.

7.4. Research project on B. anthracis in the region

In order to gain knowledge about the ecology and role of B. anthracis in the region a research project should be implemented. Analysing bigger amounts of environmental samples was out of the scope of a consultancy as presented here, but further data on distribution of B. anthracis in and around the QENP would contribute to prevent further outbreaks. This research project could ideally be linked to capacity building and involvement of Ugandan veterinarians.

8. Literature

Dudley, J.P. (1996) Record of carnivory, scavenging and predation for Hippopotamus amphibius_ in Hwange National Park, Zimbabwe. Mammalia 60(3): 486-490.

Dudley, J.P. (1998a) Reports of carnivory by the common hippo Hippopotamus amphibius_. South African Journal of Wildlife Research 28(2): 58-59.

Dudley, J.P. (1998b) Strange but true: first records of carnivory for hippos in southern and eastern Africa. African Wildlife 52(5): 12-13.

Ellerbrok, H., H. Nattermann, M. Ozel, L. Beutin, B. Appel, and G. Pauli. 2002. Rapid and sensitive identification of pathogenic and apathogenic Bacillus anthracis by real-time PCR. FEMS Microbiol Lett 214:51-59.

Leendertz F. H., Boesch C., Mätz-Rensing K., Couacy Hymann E., Formenty P., Abaza P., Junglen S., Pauli G., Ellerbrok H. 2004. Anthrax kills wild chimpanzees in a tropical rainforest. Nature, 430:451-452.

Lindeque, P.M. and P.C. Turnbull. 1994. Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. Onderstepoort J Vet Res 61:71-83.

Annex I: Activities carried out

Day	Date	Activity
1	31 / 10 / 2004	Flight from Berlin, Germany to Entebbe, Uganda
2	01/ 11 / 2004	Introductory discussion with: (change order acc. to rank!) • Dr. W. Olaho-Mukani, Director Animal Resources, MAAIF • Dr. C.S. Rutebarika, Assistant Commissioner Disease Control, MAAIF, Entebbe • Dr. R. Ademun, Head of Diagnostics, MAAIF, Entebbe • Mr. Mapesa, Director Field Operations, UWA, Kampala Dr. P. Atimnedi, Veterinary Coordinator, Field Operations, UWA, Kampala • Dorothee Hutter, GTZ Country Director,, Kampala
3	02 / 11 / 2004	Estimation of diagnostic possibilities at MAAIF, Entebbe Arrangements for field trip Discussion with: • Dr. N. K. Kauta, Commissioner, MAAIF Meeting with: • Dr. N. K. Kauta, Commissioner, MAAIF • Dr. R. Ademun, Head of Diagnostics, MAAIF, Entebbe • Mr. Mapesa, Director Field Operations, UWA • Dr. P. Atimnedi, Veterinarian, Field Operations, UWA • Mr. N. J. Bosco, Chief warden, Queen Elizabeth National Park
4	03 / 11 / 2004	Preparation of the field mission Discussion with: • Dr. P. Atimnedi, Veterinarian, Field Operations, UWA
5	04 / 11 / 2004	Departure to Katonga Game Reserve In the car: Dr. Joseph Odimim – MAAIF Mr. Esau Martin – Technician Mr. John Okello –Driver Sample collection at Katonga Game Reserve Continuation to Fort Portale, over night stay.
6	05 / 11 /2004	Arrival Queen Elizabeth Nationalpark (QENP) Discussion with: • Mr. N. J. Bosco, Chief warden, Queen Elizabeth National Park Investigation of the situation at Kazinga Channel by boat. Sample collection from dead hippopotamus and water.
7	06 / 11 / 2004	Sample collection (water and soil) at hot spots in Kazinga Channel by boat. Arrival of second vehicle with: Dr. R. Ademun, Head of Diagnostics, MAAIF, Entebbe Dr. P. Atimnedi, Veterinarian, Field Operations, UWA Mr. Joseph Turyamureeba - Driver Sample collection at: • hippopotamus pool (Point XY)

		• sites where carcasses where burned (point xx)
8	07 / 11 / 2004	Sample collection and evaluation in "Chambura Gorge"
9	08 / 11 / 2004	Immobilisation of 5 buffaloes ford collection of blood samples sample collection from one juvenile buffalo found dead
10	09 / 11 / 2004	Samples collection from dead hippopotamus Evaluation of burial site Travel to Lake Mburo National Park (see annexe 3) Collection of soil samples from GPS recorded sites, places where zebra had died in September 2003 Discussion with Edward Asalu, Senior warden Lake Mburo Conservation Area
11	10 / 11 / 2004	Travel to Entebbe
12	11 / 11 / 2004	Preparation of samples for transportation Discussion with: • Mr. Damian B. Akankwasa, Director Tourism, Business Development & Planning • Ms. Eunice N. Duli, Deputy Director, Field Operations • Mrs. Edigold Monday, Director Finance and Administration, UWA • Dr. N. K. Kauta, Commissioner, MAAIF • Dr. C.S. Rutebarika, Assistant Commissioner Disease Control, MAAIF, Entebbe • Dr. P. Atimnedi, Veterinarian, Field Operations, UWA
13	12 / 11 / 2004	Preparation of samples for transportation Return flight to Berlin, Germany
14	13 / 11 / 2004	Arrival Berlin, Germany Unpacking of samples, storage
15	15 / 11 / 2004	Discussion with RKI Staff about analysis methodology DNA extraction from tissue samples
16	16 / 11 / 2004	DNA extraction from water samples Report writing
17	17 / 11 / 2004	Real Time PCR for *B. anthracis* Report writing
18	18 / 11 / 2004	Gel electrophoresis, search for inhibitors responsible for failure of PCR Report writing
19	19 / 11 / 2004	Real Time PCR Report writing
20	20 / 11 / 2004	Real Time PCR Report writing
21	22 / 11 / 2004	Real Time PCR Report writing
22	23 / 11 / 2004	Report writing
23	24 / 11 / 2004	Report writing
24	25 / 11 / 2004	Report writing
	26 / 11 – 03 / 12 / 2004	Report writing, preparation of GIS presentation

Annexe II: Water samples

Sample	Amount sampled (ml)	Place	Location Latitude	Location Longitude	Comment
W01	250	next to dead Hippopotamus	-0.10458	29.56141	Hippopotamus was dead since about 2 days
W02	250	next to dead hippopotamus	-0.10371	29.57332	Hippopotamus was dead since about 1 week
W03	250	20 m off shore	-0.12348	29.53193	watering place, no dead hippopotami reported here
W04	250	directly at the shore			
W05	250	20 m off shore	-0.10549	29.55313	watering place, here several hippopotami had died
W06	250	directly at the shore			
W07	250	20 m off shore	-0.11050	29.54233	watering place, here several hippopotami had died
W08	250	directly at the shore			
W09	250	hippo pool	-0.03121	30.06255	one dead hippopotamus and several live hippopotami were in the pool
W13	250	Chambura gorge	-0.09402	30.05260	here 2 dead hippopotami were reported, no more carcasses visible
W15	250	Chambura Gorge	-0.08569	30.05195	about 16 died here, skeletons visible in the water
W16	250	Chambura Gorge	-0.08577	30.05178	carcass of hippopotamus at the other side of river visible
W17	250	Chambura Gorge	-0.08218	30.05057	about 25 died here, no more carcasses visible
W18	500	concrete tank of Mweya			water is pumped from the channel into this tank at water level
W19	500	tank of Mweya			water is pumped up into another tank
W20	500	water at treatment 1			here the water is treated with aluminium SO4
W21	500	water at treatment 2			here the water is treated with chlor
W22	500	water from main tank			storage of water, distribution to hotel, where it is treated again. Others use this water directly from the tank
W23	500	water used at canteen			water from W22
W24	500	water from Mweya Safari Lodge			Water from W21 but treated again before used by Tourist Lodge
W 25	50	Water from boat			Boat was used for pulling dead hippopotami to burial site,

					potential high risk of contamination

Annex III: Soil samples

Sample	Place	Location Latitude	Location Longitude	Comment
E01	20 m off shore	-0.12348	29.53193	watering place, no dead hippopotami reported here
E02	directly at the shore			
E03	20 m off shore	-0.10549	29.55313	watering place, here several hippopotami had died
E04	directly at the shore			
E07	20 m off shore	-0.11050	29.54233	watering place, here several hippopotami had died
E08	directly at the shore			
E15	ashes from burned buffalo	-0.05210	30.02554	buffalo was burned, partly meat was still seen, especially on bones carried away by scavengers
E16	Chambura gorge	-0.09402	30.05260	here 2 dead hippopotami were reported, no more carcasses visible
E15	Chambura gorge	-0.08569	30.05195	about 16 died here, skeletons visible in the water
E16	Chambura gorge	-0.08577	30.05178	carcass of hippopotamus at the other side of river visible
E17	Chambura gorge	-0.08218	30.05057	about 25 died here, no more carcass visible

Annex IV: Results from DNA isolation

Sample	DNA concentration (ng/µl)	Comment
Tissue		
H 1	34	
H 2	148	
B 1	92	
F 1	30,8	contents of the stomach of a "mud fish"
Z 1	11,6	from lake Mburo Nationalpark
water		
W01	51	
W01 (supernatant)	14	control for spores in supernatant after centrifugation
W02	7	
W03	10	
W04	16	
W05	10	
W06	n.d.	DNA visibly coloured
W07	27	
W08	25	
W10	n.d.	DNA visibly coloured
W12	n.d.	DNA visibly coloured
W13	n.d.	DNA visibly coloured
W17	n.d.	DNA visibly coloured
W18	20	
W23	7	
W24	2	
W25	n.d.	DNA visibly coloured

Table 3: DNA extracted from tissue and water samples. n.d. not determined because of visible colouration of extracted DNA.

Annexe V: Results of Real Time PCR

Tissue samples	µl DNA tested (dilution factor)	Result from control (Rokoko)	Results *B. anthracis* (pag)
H1	15	**pos**	**pos**
H2	3	**pos**	
H2	20	**pos**	**pos**
B1	16,5	**pos**	
B1	20	**pos**	neg
B1	10 (1:10)	**pos**	neg
B1	10 (1:10) + BSA	**pos**	neg
B1	10 (1:100)	**pos**	neg
B1	10 (1:100) + BSA	**pos**	neg
F1	16,5	**pos**	neg
Z1	33	**?**	neg
Z1	10 (1:10)	**pos**	neg
Z1	10 (1:10) + BSA	**pos**	neg
Z1	10 (1:100)	**pos**	neg
Z1	10 (1:100) + BSA	**pos**	neg
Water samples			
W01	33	neg	neg
W01	10 (1:10) + BSA	**pos**	neg
W01 (supernatant)	10 (1:10) + BSA	**pos**	neg
W02	33	neg	neg
W02	10 (1:10)	**pos**	
W02	10 (1:10) + BSA	**pos**	
W02	10 (1:100)	**pos**	neg
W02	10 (1:100) +BSA	**pos**	
W03	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W04	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W05	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W06	33	neg	neg
W07	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W08	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W10	33	neg	neg
W12	33	neg	neg
W13	33	neg	neg
W17	33	neg	neg
W18	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W23	33	neg	neg
	10 (1:10) + BSA	**pos**	neg

W24	33	neg	neg
	10 (1:10) + BSA	**pos**	neg
W 25	33	neg	neg
W 25	10 (1:10)	neg	
W 25	10 (1:10) + BSA	neg	
W 25	10 (1:100)	neg	
W 25	10 (1:100) +BSA	neg	

Annex VI: Abbreviations

AU-IBAR	African Union - International Bureau for Animal Resources
B. anthracis	*Bacillus anthracis*
BSA	Bovine Serum Albumin
BSL-3	Bio Safety Level 3
DNA	Deoxyribonucleic Acid
GTZ	*Gesellschaft für technische Zusammenarbeit*
HQ	Head Quarter
PACE	Pan African Program fort he Control of Epizootics
MAAIF	Ministry of Agriculture, Animal Production and Fisheries
PCR	Polymerase chain reaction
QENP	Queen Elizabeth National Park
RKI	Robert Koch-Institute
UWA	Uganda Wildlife Authority
ZBS-1	*Zentrum für Biologische Sicherheit-1*
B...	Buffalo sample
F...	Fish sample
H...	Hippopotamus sample
S...	Soil sample
W...	Water sample
Z...	Zebra sample (Lake Mburo national park)

Annexe IV

Samples from Lake Mburo / Zebra

Also the blood sample from the 2003 outbreak among the Zebra of Lake Mburo Nationalpark were available and were tested for B. anthracis as described above. All samples were negative.

Acknowledgements

This consultancy would have not been possible without the support of Dr. R. Ademun, Dr. C.S. Rutebarika, Dr. P. Atimnedi and Mr. J.S. Bosco and many other people from the MAAIF laboratory, UWA and wardens of the QENP.
Information on the topology of the QENP was provided by Claudia Jaksic, enrichment methods for *B. anthracis* and consecutive testing as well as further classical bacteriological methods were performed by Dr. Nattermann, ZBS-2/RKI. Hjalmar Kuehl, Max Planck Institute for evolutionary Anthropology, Leipzig has created the satellite image and integration of GPS data. The report was revised by Dr. Ellerbrok, ZBS-1/RKI and the procedure of sample collection and analyses discussed with Prof. Pauli, ZBS/RKI. Dr. Heinonen was a key person in organizing in advance the consultancy and sample transportation to Germany.
Their support is gratefully acknowledged. Further persons involved and contributing to this consultancy are mentioned in the main text.