Final Report

1. General Information

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Topics of projects:

BE2157/3-1: Immunoprophylaxis and molecular epidemiology of anthrax and the fate of *Bacillus anthracis* in living vectors and the environment of Namibia and South Africa

BE2157/4-1: Revision of WP3 to the original project application entitled: “Immunoprophylaxis and molecular epidemiology of anthrax and the fate of *Bacillus anthracis* in living vectors and the environment of Namibia, South Africa, Kenya, and Uganda”.

Reporting-/funding period: 29.04.2009 – June 2014

Publications related to the projects:

Peer reviewed publications

1. Koehler SM, Baillie LW, Beyer W (2015): BclA and toxin antigens augment each other to protect NMRI mice from lethal Bacillus anthracis challenge. Vaccine (accepted: JVAC-D-14-01483R2, manuscript in A.II.1.11).
2. Klumpp J., Schmuki M., Shanmuga S., Beyer W., Fouts D.E., Bernbach V., Calendar R., Loessner M.J. (2014): The odd one out: Bacillus ACT bacteriophage CP-51exhibits unusual properties compared to related Spounavirinae W.Ph.and Bastille. Virology 462-463, 299–308.
3. Ganz H.H., Law C., Schmuki M., Eichenseher F., Calendar R., Loessner M.J., Wayne M.G., Korlach J., Beyer W., Klumpp J. (2014): Novel Giant Siphovirus from Bacillus anthracis Features Unusual Genome Characteristics. PLoS ONE 9(1): e85972.
4. Ndumnego, O.C., Crafford, J., Beyer W., van Heerden H. (2014): Quantitative anti-PA IgG ELISA; assessment and comparability with the anthrax toxin neutralization assay in goats. BMC Veterinary Research 2013, 9:265
5. von Terzi B., Turnbull P.C.B, Bellan S.E., Beyer W. (2014): Failure of Sterne- and Pasteur-Like Strains of Bacillus anthracis to Replicate and Survive in the Urban Bluebottle Blow Fly Calliphora vicina under Laboratory Conditions. PLoS ONE 9(1): e83860.
6. Beyer, W. and Turnbull, P.C.B. (2013): Co-infection of an animal with more than one genotype can occur in anthrax. Letters in Applied Microbiology 57:380-4.
7. Bellan, S.E., Turnbull, P.C.B., Beyer, W., Getz, W. M. (2013): Effects of experimental exclusion of scavengers from carcasses of anthrax-infected herbivores on Bacillus anthracis sporulation, survival, and distribution. Appl. Environ. Microbiol., 79(12):3756.
8. Owen, M.P., Schauwers, W., Hugh-Jones, M.E., Kiernan, J.A., Turnbull P.C.B., Beyer, W. (2013): A simple, reliable M'Fadyean stain for visualizing the Bacillus anthracis capsule. J. Microbio. Meth., 92: 264-269.
9. Milhomme, O., Koehler, S.M., Ropartz, D., Lesur, D., Pilard, S., Djedaïni-Pilard, F., Beyer, W., Grandjean, C. (2012): Synthesis and immunochemical evaluation of a non-methylated disaccharide analogue of the anthrax tetrasaccharide. Org. Biomol. Chem., 10, 8524.
10. Beyer W., Bellan S., Eberle G., Ganz H.H., Getz W.M., Haumacher R, Hilss K.A., Kilian W., Lazak J., Turner W.C., Turnbull P.C.B. (2012): Distribution and Molecular Evolution of Bacillus anthracis Genotypes in Namibia. PLoS Negl Trop Dis 6(3): e1534.
11. Beyer, W., P.C.B. Turnbull (2009): Review – Anthrax in animals. JMAM, 30, p. 481-489, ISSN: 0098-2997.

Selected Poster- and oral presentations

12. Ndumnego O., Koehler S., Crafford J., Beyer W. , van Heerden H. (2013): Immunogenicity and protective efficacy of the Sterne 34F2 live spore anthrax vaccine in goats. The International Conference on B. anthracis, B. cereus, and B. thuringiensis, Sept. 1-5, 2013, Victoria, Canada, BC, abstract book 90C, p. 93.
13. Koehler S.M., Huwar T., Baillie L., Beyer W. (2013): Vaccination of NMRI mice against Bacillus anthracis using DNA-vectors and novel adjuvants as an approach to a new acellular vaccine-combination. The International Conference on B. anthracis, B. cereus, and B. thuringiensis, Sept. 1-5, 2013, Victoria, Canada, BC, abstract book S9:4, p. 46.
14. Von Terzi B., Turnbull P.C.B., Beyer W. (2013): Bacillus anthracis does not replicate and survive in the blowfly Calliphora vicina. The International Conference on B. anthracis, B. cereus, and B. thuringiensis, Sept. 1-5, 2013, Victoria, Canada, BC, abstract book 42C, p. 70.
15. Ndumnego O., Koehler S., Crafford J., Beyer W. , van Heerden H. (2013): Quantitative anti-anthrax IgG ELISA correlates with the anthrax toxin neutralization assay in goats. The International Conference on B. anthracis, B. cereus, and B. thuringiensis, Sept. 1-5, 2013, Victoria, Canada, abstract book 95B, p. 95.
16. Koehler S.M., Sahin M., Otlu S., Doganay M., Ndumnego O., van Heerden H., Beyer W. (2013): Recombinant acellular vaccines tested in goats for immunogenicity and protectivity. The International Conference on B. anthracis, B. cereus, and B. thuringiensis, Sept. 1-5, 2013, Victoria, Canada, BC, abstract book 79A, p. 87.
17. Koehler, S., Huwar, T., Baillie, L., Beyer, W. (2011): Vaccination of NMRI mice against Bacillus anthracis using DNA and protein based acellular vaccine candidates and novel adjuvants. International Conference on B. anthracis, B. cereus and B. thuringiensis**.** Bruges, Belgium, abstract book p. 185.
18. Haumacher, R., Hilss, K., Lazak, J., Eberle, G., Kilian, W., Ganz, H., Turner, W., Bellan, S., Getz, W. M., Turnbull, P.C.B., Beyer, W. (2011): Distribution and Evolution of genotypes of Bacillus anthracis in the wildlife of the Etosha National Park and their correlation to outbreaks in farm animals. International Conference on B. anthracis, B. cereus and B. thuringiensis**.** Bruges, Belgium, abstract book p. 148.

2. Final progress report (see also Annex III for a tabularly overview)

Projects' initial objectives

The projects encompassed the investigation of the molecular and environmental epidemiology of anthrax in Namibia and South Africa, studies on the fate of *Bacillus (B.) anthracis* in environmental habitats and within insect vectors. Work packages (WPs) 1 and 2 addressed the genetic diversity of *B. anthracis,* circulating in livestock, wildlife and in environmental habitats and the relationship between genotype and spatial, temporal and host distribution, using modern molecular fingerprinting techniques. The roles of perennial and seasonal water holes and of living vectors like amoeba and flies in the epidemiology of anthrax were investigated.

The second main topic of the projects (WP3 and O1-O5) comprised studies in laboratory rodents and goats to compare the immunogenicity, protective efficacy and safety of recombinant peptide and DNA vaccine candidates with those of the live spore vaccine licensed in these countries.

Underpinning the science was the aim to support the academic and professional careers of young Namibian, South African and German scientists through exchange of personnel and technological skills within the framework of a multi-national academic network (WP4).

Projects' structures

According to WPs 1-3 and O1-O5 the work was structured at investigating

- the correlation between genotypic diversity and spatial and temporal distribution of outbreak strains of *B. anthracis* and their possible host specificity,
- the epidemiological correlation between anthrax outbreaks in wildlife and livestock in endemic areas,
- the role of carcasses for generating new sites of infection,
- the role of living vectors (e.g. vultures, insects, amoeba) in the epidemiology of anthrax,
- the role as "site of infection" of natural habitats like soil and water holes under the different seasonal conditions,
- the immunogenicity, protective efficacy and safety of antigenic components of the toxins, spore and vegetative cell applied as either recombinant proteins or as DNA vaccines encoding the appropriate antigens, alone and in combination, in comparison to the Sterne live spore vaccine in laboratory animals and goats,
- the feasibility of a simultaneous antibiotic treatment and immunization with non-living vaccines in comparison to immunization with the living spore vaccine.

BE2157/3-1 - WP1: Genotyping of *B. anthracis* and epidemiology of anthrax

Over the past two decades the development of genetic methods has enabled discrimination between pathogen genotypes (17, 18, 22, 33). These methods are widely used to determine whether cases of disease are related, to identify the most probable source of infection, and to establish by what means the causative agent is spreading.

In our studies a newly established 31-marker Multi-Locus-VNTR-Analysis (MLVA) together with analysis of Single Nucleotide Polymorphisms (SNP) and Single Nucleotide Repeats (SNR) were used. Until May 2015, in total more than 880 isolates from African countries ((South Africa (297), Namibia (570), Zambia (15), and Zimbabwe (4)) were genotyped by the 31-marker MLVA and in part by SNP and SNR analysis. By MLVA only, about 90 genotypes were identified. These isolates were related to another 194 European and Asian isolates. In total, the currently available strain collection established during the funding period comprises nearly 1200 genotyped isolates of *B. anthracis*, representing about 190 MLVA-genotypes. So far the Namibian strain database was made available to the public via the MLVA database, see http://mlva.u-psud.fr/mlvav4/genotyping/view.php. Further extensions of this database are currently hindered by the lack of funding for the database.

In the case of the Namibian collection, methods were used to analyse interrelationships between isolates from domestic and wild animals and to identify the possible epidemiological correlations of anthrax outbreaks within and outside the Etosha National Park (ENP). The MLVA data were further

used to establish a model of the probable evolution of GTs within the endemic region of the ENP (3). The isolates came from 20 animal species and from the environment, collected between 1983 and 2010. From the analyses it is apparent that some genotypes (GT) persistently cause deaths for long periods of time. There were also many examples of outbreaks occurring at the same time in the same region but caused by different GTs. A total of 38 GTs were identified by MLVA; of these 13 were not found in the ENP, 19 were found only within the ENP and 6 were found both inside and outside the ENP. All GTs found so far in Namibia belong to cluster A (17). From the 25 GTs found within the ENP, 23 belong to SNP group A.Br.Aust94 and one to A.Br.008/009. Two other SNP groups, A.Br.005/006 and A.Br.001/002, were only found outside the ENP. The majority of the GTs from the ENP were from sporadic occurring cases with just one or a very few representative isolates. A number of these were found just in early years (1987 to 1994) and others only since 2002, indicating a continuous evolution of the genetic background of *B. anthracis* in endemic regions. In contrast, two GTs were found to be dominant in terms of their temporal occurrence since 1983 and the number of cases they caused. A minimum spanning tree (MST) analysis points to one of those GTs being the probable ancestor of all other GTs found in the ENP (3).

Of the 210 isolates of the South African strain collection from the National Institute of Communicable Diseases (NICD), 23 GTs were identified. The data are being combined with data generated from new isolates at the University of Pretoria (UP). The work under the work plan for the NICD in SA had been delayed by original problems to hire students. This issue had been solved since 2012 by a closer cooperation between the UP and the NICD, whereby students of the former can get access to the facilities of the latter.

The SNR analysis provides additional genetic resolution among *B. anthracis* isolates of the same MLVA genotype and is therefore a useful tool for molecular-epidemiological analysis of outbreak strains. We described an improved methodical approach of the method published by Kenefic et al. (19) by first amplifying a larger DNA fragment, each including the original PCR-fragment, that could then be sequenced. The SNR analysis was helpful in correlating an isolate with its source but did not further elucidate epidemiological relationships.

One of the challenging pre-requisites for the successful application of any genotyping method is the stability of the markers during laboratory processing of samples. Culture media supplemented with several antibiotics, lysozyme, EDTA, and thallous acetate may exert a mutagenic selective pressure, leading to higher than normal rates of mutations. Moreover, the infection of mice for diagnostic purposes may resemble natural conditions which are thought to be the source of the mutations recognized as new genotypes in typing systems. The influence of routine laboratory procedures like repeated sub-culturing and passage through small laboratory animals on the results of the three molecular typing schemes has been studied. None of three strains (Sterne, Ames, and a wild type isolate) tested after at least 6 subcultures on TSPBA or PLET-agar showed any variation among the 31 VNTR-, 13 SNP- and 4 SNR-markers examined. Re-isolates from 25 A/J-mice, infected s. c. with Sterne vaccine spores or 68 NMRI-mice and 3 rabbits infected s. c. with Ames spores in vaccine challenge studies also exhibited no traceable mutations in the 31 VNTR-markers whereas the SNR-analysis showed mutations in three of the examined re-isolates. The excellent stability of VNTRs and SNPs support their suitability to differentiate related isolates and to reveal phylogenetic relationships, respectively, and renders epidemiological interpretations highly reliable. The rapidly evolving SNR-markers may rather confuse epidemiological relations but are useful to define the origin of an isolate.

The international exchange of genotyping data based on MLVA requires a common protocol for the normalization of raw data, meaning a “coding system”, which should be based on the real number of repeat units contained in a given allele of each marker. In cooperation with leading European laboratories in this field a coding system was established and agreed upon between the Army Medical Research Center of Rome, Italy, the Microbiological Institute of the Bundeswehr (München) and the laboratory at Hohenheim University. The coding system was published in Beyer et al. (3) and is part of the MLVA e-database.

During the routine fingerprinting of outbreak strains of *B. anthracis* of European and African origin by means of a 31-marker MLVA, cultures from the ENP, Namibia, and from an outbreak in the Pyrenees, France, in 1997 were found to harbour different genotypes (GTs). To investigate this further, isolates from 10 samples of blood-soaked soil from beneath anthrax carcasses and 18 clinical swabs taken from carcasses in the ENP were re-examined. While only a single GT was found in any one of the 10 soil samples, four of the 18 swabs (22%) yielded different GTs. Two GTs were isolated from each of a zebra and a springbok and three GTs from each of a second zebra and an elephant. The results confirm the indications noted previously that co-infection with more than one GT is probably not especially uncommon. The results, as published in Beyer and Turnbull (4) show that, for the purpose of analyzing genotypes involved in an outbreak, it is important to examine more than a single colony from a clinical sample.

Serological studies

The aim of an additional epidemiological sub-project on the type of naturally acquired antibodies against anthrax in predators and scavengers was investigating whether the humoral immune response against *B. anthracis* in different species of predators and scavengers is the result of a subclinical infection or simply elicited by the consumption of high amounts of the anthrax toxins, present in fresh carcasses of animals which have died from anthrax. Results from vultures in the ENP support the hypothesis that vultures do not acquire a productive infection, but instead are immunized against the lethal action of the anthrax toxins by uptake of the native toxins from the carcasses they feed on. On the other hand, it is suspected that antibodies in mammalian scavengers result from infection. To test this hypothesis, 175 sera from various scavenging and/or predating species that have become available from other projects running in the ENP or elsewhere in Namibia were tested by M.Sc. students of both the University of Namibia (UNAM) and the UHO (see A.II.3.5) for the presence of antibodies against either the toxins of *B. anthracis*, the spores or the replicating cells or all of them. Preliminary results show a clear correlation of sero-conversions with regions endemic for anthrax. Relations between antibody-titres against the different antigenic compounds remain to be elucidated further. The natural acquisition of specific anthrax antibodies in scavengers/predators is a valuable adjunct to the study of the incidence and spread, i.e. the epidemiology of the disease, in an area.

BE2157/3-1 - WP2: **Fate of *B. anthracis* in natural environmental habitats and the influence of this on the epidemiology of anthrax**

The current understanding of the fate of *B. anthracis* (germination, replication, sporulation) in different environments is fragmentary. One of the important unknowns in this regard is the fate of *B. anthracis* in natural environmental habitats, such as soil or waterholes, and in living vectors.
The traditional belief is that spores remain dormant in the soil until taken up again by a mammalian host. However, this is not universally accepted and some observations are not easily explained in terms of this theory (16, 21). Recently, Saile and Koehler (25) demonstrated in a microcosm study that the rhizosphere can stimulate the germination of spores of *B. anthracis*, although replication of the vegetative cells and re-sporulation remained low. Subsequently Schuch et al. (26) demonstrated that lysogenic bacteriophages can change the physiology of a bacillus by the interference of phage regulatory molecules with bacterial gene promoters, thereby altering the life cycle of the phage infected *B. anthracis*. Lysogenic infection with certain phages results in an inability to sporulate. These cells, however, where shown to survive as vegetative cells in laboratory media and the guts of invertebrates by forming biofilms. It is not known where else this form of the pathogen can live. The topic of the fate of the organism in non-host organisms, infected or not by temperate phages, became another focus in the work of UHO and UP.

Soil and waterholes

During the project >500 samples from permanent and seasonal waterholes in the ENP were collected over a period of four seasons, starting from the dry season in 2009 through to the end of the rainy season in 2011. These were tested semi-quantitatively by colony counting and real time PCR to

determine the ratio of spores to vegetative bacilli and the total number of *B. anthracis*. The results are part of the doctoral thesis of L. Lazak at UHO (see A.II.3.7) and will be published in a paper with combined African, German and US co-authorship. The results revealed nil to very low numbers of spores in perennial water holes and low numbers of spores in seasonal water holes (gravel pits). The concentrations determined via semi-quantitative PCR argue against a meaningful role of such sites as infectious sources in the epidemiology of anthrax under the conditions of a highly endemic region.

Another >200 soil samples around a carcass site in the ENP were quantitatively evaluated for the number of spores of *B. anthracis* remaining over a 6-month period. In line with recent publications on the role of carcass sites as the true infectious sites under natural conditions (13, 31) our data indicate that carcass sites may be heavily contaminated irrespective of whether or not the carcass is opened by scavengers (2). This recognition revises the traditional view as stated in former standard publications.

In preparation for further studies, we produced strains of different bacilli by infecting them with lysogenic phages known to interfere with sporulation (27). In addition, to enable us to monitor the fate of the organisms *in situ* by microscopy, various derivatives of *Bacillus* spp. were genetically labeled with GFP. These modified laboratory strains were used for investigating the fate of phage infected bacilli in various environments.

Amoeba studies

In a diploma thesis at UHO (see A.II.3.3) the topic of whether or not free living amoeba can play a role either as a host, protecting *B. anthracis* from environmental influences, or as a vector in oral infections has been addressed. The fate of the organisms after uptake of spores by the amoeba was traced by culture to determine total numbers and the ratio of spores to vegetative cells. The investigations in *Acanthamoeba (A.) castellanii* revealed differences in the survival of *B. anthracis* dependent on the presence of its virulence plasmids. However, survival or even replication did not occur in *A. castellanii, Naegleria clarkii* or *Willertia magna*, infected in vitro with spores of either the Sterne vaccine strain 34F2 or a Pasteur like strain. These results were not in line with those of Glomski et al. (14) who reported that 34F2 can germinate and replicate in *A. castellanii* if harboured in natural river water. To further investigate the role of amoebae under natural conditions an M.Sc. thesis was initiated at the NICD in cooperation with the UP in 2014. Results of this study are not yet available.

Fly studies

Three studies examined the fate of either different derivatives of *B. anthracis* in artificially infected flies (Dipl. thesis at UHO, see A.II.3.4) or of *B. anthracis* in naturally occurring flies in the ENP in Namibia (M.Sc. thesis at UNAM, see A.II.3.6). As reviewed elsewhere (1) biting and non-biting flies were long considered important as vectors of the pathogen. Non-biting blowflies (*Chrysomya* spp.) have been incriminated as the principal vector of anthrax in browsing wild herbivores in the Krueger National Park (KNP), South Africa (10). The ability of the biting flies (*Stomoxys* spp.), mosquitoes (*Aedes* spp.) and non-biting houseflies (*Musca domestica*) to transmit anthrax has been demonstrated experimentally (28, 30, 12).

One study (see A.II.3.4) was aimed to elucidate the bacteriological events occurring within the gut of *Calliphora (C.) vicina*, selected as the European representative of blow flies held responsible for the spread of anthrax during epidemics in certain parts of the world. Green-fluorescent-protein (GFP) carrying derivatives of *B. anthracis* were used either infected or not with a worm intestine phage (Wip4) published to influence the phenotype and survival of the pathogen (26, 27). After feeding the flies on blood meals containing fully developed vegetative cells flies were tested for the internal presence of vegetative cells or spores of *B. anthracis* for up to 10 days. In line with earlier findings our results demonstrated that *B. anthracis* does not multiply and fails to survive for any length of time in the guts of *C. vicina* (35). It also supports the contention of De Vos (11) that vegetative *B. anthracis* disappear from the digestive tracts of blowflies (*C. albiceps* and *C. marginalis*) within two weeks of feeding on a carcass.

In the second study fly samples were collected sporadically from around 10 animals found dead within a 2-weeks window in March 2012. The results, presented in the M.Sc. thesis of M. Nalisa (UNAM, see A.II.3.6), conflicted with the common view that spots left on surrounding vegetation by flies feeding on a carcass pose a risk for infection to the browsing wild life. *B. anthracis* could not be cultured from leaves carrying fly excretions collected along 10 m transect lines, even where flies caught from the carcass tested positive for *B. anthracis*. Moreover, in line with results from Blackburn et al. (4) not every fly collected from a carcass tested positive, at least in the gut content.

In a field study following up the trials performed during the M.Sc. study of M. Nalisa flies were caught directly off a caged carcass. The carriage of flies was investigated in comparison to samples taken from vegetation around the carcass and the carcass itself over a period of 5 consecutive days. Data were recorded and analyzed to determine the abundance of fly species (genus) involved, the number of flies positive and the number and status of living *B. anthracis* within the gut of flies in relation to the time after death and condition of the carcass. The average number and type of cells in fly spots and on leaves was determined in relation to the time after death and condition of the carcass and the pathogen carriage of flies collected off the carcass as well as temperature and humidity.

From the results of all these experiments the conclusion was drawn that a time dependent spread of vegetative cells versus spores, taken up by a fly from a carcass and deposited on the vegetation, leads to variable viability of *B. anthracis* in the environment. Whether they survive as infectious spores depends on their physiological status during deposition and, in case of vegetative cells being deposited, on the environmental conditions allowing or preventing sporulation (23).

The preliminary studies of the M.Sc. student in Namibia also focused on determining the presence and diversity of biting flies attracted to anthrax carcasses and their carriage of the pathogen. The trapping method used was successful and yielded an abundant number of Tabanids. The average numbers caught in and around gravel pits in the ENP reached 400 individuals during the peak rainfall period between December 2011 and February 2012. None of these flies tested positive for *B. anthracis*.

Soil worm study

An additional short laboratory study was conducted to investigate the fate of phage infected *B. anthracis* in soil and soil worms (see A.I.1). The worms (*Eisenia foetida*) were introduced into soil-microcosms inoculated with spores of different strains for 2 weeks to check how the worm gut might affect the microbes' lifecycle. None of the strains persisted in the worm gut after the transfer of the worms to clean soil. Whether phage infected or not, all showed high germination rates after 1 day while the vegetative and total cell count decayed over 2 weeks in inoculated soil suggesting that germinated cells likely didn't proliferate. Differences were monitored according to whether the plasmid pXO1 was present in the strain or not, somewhat in line of what was seen in the laboratory fly studies. Further studies would need to include higher sampling frequency, different soils to investigate the role of soil quality on *B. anthracis* germination, the use of fully virulent field strains and a control soil microcosm containing spores but no worms.

BE2157/3-1 - WP3: Comparative studies on the immunogenicity, protective efficacy and safety of the live spore animal vaccine versus recombinant peptide and DNA vaccine candidates in goats

Despite proving effective in controlling anthrax worldwide over the last 75 years, the Sterne live spore vaccine (LSV) used as a veterinary vaccine has a number of problems, such as handling of live *B. anthracis* during vaccine production as well as in the field, the standardisation of vaccine production and avoidance of batch to batch variation in content and immunogenicity, and the residual virulence known from several laboratory species and livestock. Even more important for the use in livestock and endangered wild species is the inefficacy of the live vaccine(s), when administered concomitantly with

an antibiotic, preventing an appropriate simultaneous treatment of animals and vaccination to avoid the re-infection of animals at risk after the activity of an antibiotic has ceased. These limitations may be overcome with the advent of recombinant vaccines, based on the experiences made with both the licensed acellular human vaccines AVA and AVP.

The antigenicity and protectiveness of multi-component non-living vaccines were investigated in laboratory animals (outbred NMRI mice and rabbits) and finally in goats.

First we sought to develop a non-living vaccine based on the protective antigen (PA83), the spore surface glycoprotein (BclA), a capsule conjugate, formalin-inactivated spores (FIS) and a lipopeptide adjuvant (Pam_3Cys) in various combinations.
The DNA-vaccines consisted of vector-backbones comprising signal sequences able to direct the integrated antigens (rPA83, PAD4LFD1 and BclAD1D3) to the MHCI, MHCII and the secretory pathway. A separate vector encoding for a positive MHCII-regulator (CIITA) and a vector internal sequence for the Interferon-ß promotor stimulator (mIPS1) served as adjuvants for the DNA-vaccines.

We also investigated whether the non-living vaccines can induce a protective immune response with comparable efficacy to the Sterne 34F2 living spore vaccine.

Vaccination of NMRI mice against B. anthracis using DNA and protein based non-living vaccine candidates and novel adjuvants (20 and A.II.3.10)
Mice were immunized 2-3 times by s. c. injection or in case of DNA vaccines, by gene gun. Three weeks after the final immunization animals were subjected to s. c. challenge with 25 or 50 LD_{50} spores of a fully virulent Ames strain and were monitored for a further 3 weeks to assess protection. Blood taken before immunization and before challenge was tested by ELISA and toxin-neutralizing-assays.

DNA-vectors encoding rPA and rBclA that contain signal sequences for targeted expression of the antigens to stimulate either humoral or cell mediated immune responses were used in combination with regulatory adjuvants like IPS1 and CIITA. Most interestingly, rBclA encoding vectors protected 50% of the animals when challenged with fully virulent Ames strain spores. The serological analysis of survivors indicated a possible sterile immunity as inferred from the lack of PA antibodies. The combination of plasmids encoding epitope specific sequences (LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1) protected 90% of the animals against a lethal challenge of the Ames strain of *B. anthracis*. Single applications of either antigen component showed significantly lower protection rates, indicating the beneficial interaction between anti-spore and anti-toxin components for an acellular vaccine formulation in mice.

Mice immunized with a combination of spore and toxoid antigens in the presence of a lipopeptide adjuvant survived significantly better than the negative control and groups having received the single antigens only. The addition of spore antigen to the toxoid component drastically improved the survival rate (60-70%), in line with data reported previously from our group in the A/J mouse model (15). This former study concluded that BclA enhanced the protectiveness by inducing opsonisation of spores by antibodies. While the addition of a capsule conjugate did not add to the overall survival rate, the addition of FIS to a mixture of the single antigens led to 100% survival against a lethal challenge with a fully virulent *B. anthracis*. These results were later confirmed in rabbits (see A.I.2).

Immunization and challenge studies in goats were launched by the University of Pretoria (UP) partners. Experiments in African Boer goats were performed under the guidance of Dr. Henriette van Heerden of the UP, with the support of the Onderstepoort Biological Products (OBP) company, the Veterinary Service of the Department of Agriculture, Forestry and Fisheries (DAFF), and the management of SANParks. A repetition of trials in goats was guided by the Kars University in Turkey. In these experiments, performed between 2011-2013, goats were immunized with either the living spore vaccine 34F2 (LSV), a combination of toxoid and spore proteins with or without FIS or a

combination of DNA vaccine vectors to prime the immune response plus the protein-component vaccine as a final boost. Groups of goats achieving an appropriate immune response, toxin neutralizing antibodies and cell proliferation after the last immunization, were selected for a lethal challenge with spores of *B. anthracis*.

Ethical approval processes were started in 2008 (in advance of funding in June 2009) by Dr. J. Picard to permit the goat experiments to be carried out at the BSL3 Trans-boundary Animal Diseases Program (TADP) facility of the Onderstepoort Veterinary Institute (OVI). Animal ethics approval was finally obtained in August 2009. For Dr. Picard left the UP in January 2010 Dr. v. Heerden (UP) took over the responsibility. The ARC-OVI TADP then indicated that the BSL3 facility needed to be approved by the DAFF, in line with Section 20 of the South African Animal Diseases Act 35 (1984). The facility was evaluated by the DAFF and a section 20 application was submitted in April 2010. The DAFF then required a recommendation from a newly established Anthrax Advisory Committee (AAC). In August 2010, the DAFF gave out the judgement that the BSL3 facility had non-compliances that need to be addressed by OVI. The AAC indicated that experiments should instead be carried out within the endemic region of the KNP. In January 2011 the project partners were told that permission to carry out the trials in the TADP facility would not be granted for “undisclosed reasons”. After negotiations with SANParks, the controlling body for the KNP, permission was obtained in May 2011 to use an old military facility within the endemic region of KNP until December 2012. Section 20 approval for the vaccination part of the experiment was then obtained and the trials took place in November and December 2012.

Immunizations with the live spore vaccine Sterne, 34F2, (LSV)(see A.I.3)
Occasional adverse reactions and deaths, particularly in goats, associated with the common veterinary LSV and lack of data on its immunogenicity in domestic herbivores have long needed addressing. Therefore, we sought to evaluate the immunogenicity of this vaccine in Boer goats during the course of a year and its protection against challenge with a fully virulent wild-type strain of *B. anthracis*. After determining the minimum infective dose (MID) of an s. c. infection goats were vaccinated and held for, respectively, 6 weeks and 62 weeks before challenge. One group of goats was re-vaccinated at 58 weeks and challenged at 62 weeks. Survival was monitored for 14 days after challenge. Serum samples were collected at monthly intervals and analyzed for anti-PA and anti-FIS antibodies. Pooled serum samples were assessed for toxin neutralizing antibodies using an *in vitro* macrophage culture assay.

The MID of the challenge strain was found to be <36 spores and naive animals died within 3 days with little or no change in normal rectal temperature (<40℃). There was no evidence of blood extravasation from body orifices. Capsulated bacilli were visible in blood smears up to 2.8 hours before death. Interestingly, goats immunized with the LSV were only fully protected after re-vaccination one year after the first vaccination. The evaluation of the immune responses of those groups lead to the conclusion that the current vaccination schedule should be revised to a double vaccination with a 3-4 months interval instead of a single vaccination with annual boosts. This study was the first providing insight in the correlation of immunogenicity and protectiveness of the LSV in livestock.

The study also indicates that goats are protected more than a year after a single vaccination and supply insight into the development of antibodies to protective antigen, toxin and spore antigens during the course of a year. None of the goats reported in the tests suffered any significant adverse reactions to the LSV.

Recombinant peptides and FIS tested as vaccine candidates for immunogenicity and protectiveness in farm goats (see A.I.3)
Goats were immunized with peptide antigens (rPA83, rBclA) and the lipopeptide adjuvant, plus the addition of 10^8 FIS in one group, or with 1 mg of the DNA-vaccine vectors successfully tested in mice, before. Since the titres of the DNA-vaccine group measured after 3 immunizations were not sufficient

the animals were boostered once with the proteinaceous vaccine formulation. For even then the humoral immune responses did not meet the criteria, this group was excluded from the challenge. The other non-living vaccines induced equivalent anti-rPA83 titres but very low to undetectable toxin neutralization titres, as had also been seen for one of the living spore vaccinated groups which later showed 60% survival. Generally the anti-FIS-IgG titres were much higher than those measured for rBclA and finally matched in the FIS receiving group those of groups having received a booster vaccination with the living spore vaccine and later surviving the challenge by 100%. Surprisingly, except 1/5 goats in the group vaccinated with recombinant proteins and FIS all other goats succumbed to the challenge. The re-analysis of the whole immunization procedures revealed a mistake in the administration of the vaccine components, where every component had been applied with a separate syringe. While the quantitative immune responses were not questionable, either the qualitative composition of antibodies or cellular factors not tested for may have caused the lack of protectiveness. However, one also needs to take into account the whole environment of these trials, where the immunized animals had to be transported from Pretoria (OBP) to the place for challenge in the KNP, leading to animal losses caused by other infections like heart water and pseudotuberculosis.

Because of the draw backs encountered during the goat experiments in South Africa, trials with the proteinaceous vaccines, with and without FIS, were repeated at the Kafkas University of Kars, Turkey, including a new negative control group (see A.I.4). Goats of both genders, different races and sizes and from different farms were immunized 3 times s. c. at 3 weeks intervals and lethally challenged s. c. with spores of a fully virulent *B. anthracis* field strain 5 weeks after the last immunization. As before, blood taken after each vaccination, before challenge and from survivors was analyzed in ELISA for IgG titres against rPA83, rBclA, FIS and vegetative antigen as well as neutralization activity in the TNA.
Goats immunized with the addition of FIS yielded 80% (8/10) survival and 50% (4/8) survived without FIS, though this difference is not significant. The ELISA showed a moderate titre against rPA83 and high titres against FIS when given, while almost no titres were observed for rBclA. Generally, titres against all reactive antigens were higher if FIS was part of the vaccine indicating some adjuvanticity of FIS. The evaluation of the vaccine regimen showed the highest titres after the 2nd immunization and rather a decline in titres after the 3rd vaccination, indicating a probably low value of the last vaccination, at least in the schedule used here. In total, immune responses in goats after vaccination with *B. anthracis* antigens differ from what is generally known from laboratory animals, especially from mice experiments, and need further investigations.

BclA has been included in the immunizations as the exosporium of *B. anthracis* has been in the focus of numerous novel vaccination approaches where BclA has been shown to be an immunodominant factor. However, in contrast to the mouse experiments goats injected with a protein formulation of rPA83, rBclA and lipopeptide showed little to no increase in IgG titres against rBclA or FIS. The addition of FIS to the vaccine also showed little to no increase in titres against BclA but significantly increased the anti-FIS titres, which might have contributed to the survival ratio of 80%. Even animals vaccinated with the commercial Sterne spore vaccine did not show anti-rBclA titres at any time, while anti-FIS titres increased strongly after each immunization.

Together these results indicate that the antibodies generated against FIS in goats are not comprised of antibodies against BclA and that BclA seems to be poorly or not immunogenic in goats. Interestingly, while previous reports showed the enhancement of protection by BclA, Vergis et al. (34) observed an even better survival rate in mice immunized with a ΔBclA strain of the anthracis-like *B. cereus* G9241. BclA may partially masks recognition of antigens beneath the exosporium (6, 9). Recent articles show that several proteinaceous antigens, physiologically located beneath the exosporium, can contribute to a protective immune response (8, 34), like the S-layer protein EA1 (29, 32) and the cortex protein SoaA (7). It would, therefore, be worth extending the mouse trials of Vergis et al. (34) into field trials.

BE2157/4-1 - O5.3-O5.5 Goat immunization and passive mouse protection tests (see A.I.5)

In this test series a total number of 35 goats were immunised with the LSV or the proteinaceous vaccine combination (PVC) with and without FIS as described earlier. Additionally, various prime-boost schedules using the DNA-vaccines plus FIS or the peptides were tested. Moreover, the LSV and the PVC were serologically compared when administered simultaneously with a Penicillin antibiotic. The potential protectiveness of induced antibodies was tested in A/J-mice (5 per goat serum) by i. p. administration of 0.5 ml serum followed by a lethal s. c. challenge with $2x10^5$ spores of the LSV.
The sera from goats immunized with the complete peptide vaccine plus FIS gave a survival rate in mice of 72,7% followed by sera of the appropriate goat group without FIS (68,4%) and the combination of PA and BclA encoding plasmids followed by two boosters with rPA, rBclA and lipopeptide (prime-boost model) with 42,8% survival. No mouse tested with sera from the non-immunized goat group and all mice tested with sera from goats double immunized with LSV survived. Hence, the passive mouse protection assay (PMPA) showed a good correlation of serological data with survival in the PMPA and of the latter with the outcome of the real challenge in goats. Spearman's rank correlation of the time to death (TTD) of the A/J-mice to the pre-challenge titres in the goat sera showed high correlation between survival and anti-rPA, anti-rBclA, TNA-titres and cell proliferation data. The PMPA proved to be reliable as a correlate for protection in the target animal and can help to reduce the number of higher species in future direct challenge experiments.

Simultaneous treatment with Penicillin (Pen.) and immunization
For the first time, antibody titres raised against rPA, rBcla and FIS were compared after simultaneous immunization with LSV or NLV and antibiotic treatment. While goats receiving PVC+Pen. developed a full humoral response indistinguishable from an immunization without Pen., in the LSV+Pen. group the humoral response was completely blocked.

Quantitative anti-rPA ELISA for goat sera (24)
Presently, few data exist on the level and duration of anti-protective antigen (PA) IgG in vaccinated livestock. Various adaptations of ELISAs have been developed in studies to assess the humoral immune response following vaccination, albeit mostly in laboratory rodent models. We developed a quantitative anti-anthrax IgG ELISA to determine the concentration of anti-PA specific IgG present in sera of immunized goats, with the aid of an affinity-purified caprine polyclonal anti-anthrax PA-83 IgG standard. This was compared with the anthrax toxin neutralization assay (TNA). The spearman's rank correlation of log-transformed IgG concentrations and TNA titres showed strong positive correlation ($r_s = 0.942$; $p = 0.01$). The study provides evidence that an indirect ELISA can be used for the quantification of anti-anthrax PA IgG in goats with the added advantage of using single dilutions to save time and resources. The use of such related immunoassays can serve as potential adjuncts to potency tests for the living spore and other vaccine types under development in ruminant species.

Compared to one another the LSV immunized animals showed equal or higher antibody titres against the measured antigens, with FIS and rPA83 being the most immunogenic antigens. Utilizing a dose of 75 µg the non-living vaccines (PVC) protected equivalently to the LSV (60 – 100%) yielding 50% protectiveness without FIS and 80% if FIS was included. The DNA-vaccines showed little to no immunogenicity in goats, thus no challenge was performed on these animals. The humoral reaction against BclA was generally poor in goats, which has not been noted before and could be a basis for further improvements concerning the LSV and PVC alike. The immunizations with the LSV revealed a broad range for its efficacy of the first vaccination as well as a notable difference in the antibody spectrum between first and re-vaccination. Together with the recorded data of the antibody titre development throughout a year a more optimal protocol for immunization with the LSV, possibly in combination with a PVC can be postulated.

BE2157/3-0 and 3-1 - WP4: German-African academic network

The first project (BE2157/3-0) was launched with a meeting in Johannesburg in 2009, bringing together the principal personnel from the Central Veterinary Laboratory (CVL) and Etosha Ecological Institute (EEI) in Namibia, the National Institute of Communicable Diseases (NICD) and the Veterinary Faculty of the University of Pretoria (UP), South Africa, and representatives of different Ministries of South Africa. This meeting was held to discuss the goals, pre-requisites and logistics of cooperative research on anthrax, and also to discuss the exchange of sample materials, and data and reporting of anthrax outbreaks in both livestock and wildlife. Associated partners brought into the project network are the Onderstepoort Veterinary Institute (OVI) and the Onderstepoort Biological Products Company (OVP), both historically involved in the planning of vaccine studies in South Africa, and the SANParks organisation, responsible for research in the KNP. At a second meeting of all partners in Namibia, 2010, the University of Namibia (UNAM) was also added to this network. The third meeting with all project partners, held in South Africa, in April 2012, focused on the major achievements and open questions from the first funding period and was aimed at strengthening the cooperation between different national institutions (UP, NICD, and the State Veterinary Service of the Department of Agriculture Forestry and Fisheries) within South Africa. A fourth meeting brought together all partners involved so far and was held in Pretoria from 18-20 July 2013. It was mainly aimed at providing the young scientists a platform to present their work. A final meeting was held in Pretoria, December 2014, to sum up the results achieved so far and discuss possibilities for further co-operations.

Achievements resulting from these collaborations have been visits by 7 African researchers (5 from South Africa and 2 from Namibia) for training at the UHO laboratories and of two German doctorands to carry out, assist or supervise the studies in Namibia and South Africa. The following theses and studies have been completed:

- Investigation of biting and non-biting flies as vectors for B*acillus anthracis* in Etosha National Park, Namibia. Mwangala Nalisa, M.Sc., UNAM, 2013.
- Nachweis und Charakterisierung der humoralen Immunität von Wildtieren gegen *Bacillus anthracis* Rahel Hartmann, M.Sc., UHO, 2013.
- Untersuchungen zum Verhalten GFP-markierter phageninfizierter und nicht phageninfizierter *Bacillus anthracis* Stämme in verschiedenen Amöbenspezies. Annika Benner, Diploma, UHO, 2013.
- Untersuchung zum Verhalten phageninfizierter Stamme von *Bacillus anthracis* in Fliegen (*Calliphora vicina*). Britta von Terzi, Diploma, UHO, 2013.
- Immunogenicity and protectivity of a live spore *Bacillus anthracis* vaccine in goats. Okechukwu Ndumnego, M.Sc., UP, 2013.
- Entwicklung und Testung neuer DNA- und Protein-basierer Multikomponentenvakzinen sowie regulatorischer Adjuvanzien gegen eine Infektion mit *Bacillus anthracis* in Auszucht-äusen und Ziegen. Susanne Köhler, Doctoral thesis, Dr. rer. nat., submitted in March 2015.
- GIS-gestützte Analysen zur Verbreitung von *Bacillus anthracis* im Etosha Nationalpark sowie auf Wild- und Nutztierfarmen in Namibia unter Verwendung molekularepidemiologischer Methoden. Judith Lazak, Doctoral thesis, Dr. med. vet., 2013.

Additionally, 1 M.Sc. thesis at the NICD in Johannesburg and 2 PhD works at the University of Pretoria were started in 2013 under the umbrella of this project and are under way.

The following dissertations were not directly funded from the project but had a great benefit from being part of the working group in this project:

- Klonierung und Charakterisierung eines Phagenlysins und sein Einsatz zur diagnostischen Erkennung von *Bacillus anthracis*. Doctoral thesis, Dr. med. vet., Kerstin Boll, 1012.
- Herstellung monoklonaler Antikörper gegen thermostabile Antigene von *Bacillus anthracis* zur Anwendung in der Anthraxdiagnostik. Doctoral thesis, Dr. rer. nat., Karen Anja Hilss, 2012.
- Entwicklung eines neuen Therapieansatzes gegen Infektion mit *Bacillus anthracis.* Doctoral thesis, Dr. rer. nat., Olga Rudolf, 2012.

Summary

The projects encompassed investigations of the genetic diversity and fate of *B. anthracis* circulating in livestock, wildlife and in environmental habitats in Namibia and South Africa. The roles of perennial and seasonal water holes and of living vectors like flies, amoeba and others were investigated. Another main part comprised studies in laboratory rodents and goats to compare the immunogenicity, protective efficacy and safety of recombinant peptide and DNA vaccines with those of the live spore vaccine (LSV) 34F2 (Sterne). Underpinning the science was the aim to support the academic and professional careers of young Namibian, South African and German scientists within the framework of a multi-national academic network.

A newly established 31-marker MLVA together with analysis of SNPs and SNRs was used for epidemiological investigations. The MLVA database established comprises nearly 1200 isolates of *B. anthracis*, representing about 190 MLVA-genotypes. Data from the Namibian database became part of the MLVA database under http://mlva.u-psud.fr/mlvav4/genotyping/view.php. Efforts were successfully undertaken to harmonize a common MLVA coding system between German, Italian and South African institutions.
During the project >500 environmental samples were collected over a period of four seasons and tested to determine the ratio of spores to vegetative bacilli and the total number of *B. anthracis*. The results revealed nil to very low numbers of spores in perennial water holes and low numbers of spores in seasonal water holes (gravel pits). The concentrations determined via semi-quantitative PCR argue against a meaningful role of such sites as infectious sources in the epidemiology of anthrax under the conditions of a highly endemic region.
Three studies examined the fate of *B. anthracis* in either artificially infected or naturally occurring flies in the ENP in Namibia. From the results of these experiments the conclusion was drawn that a time dependent spread of vegetative cells or spores, taken up by a fly from a carcass and deposited on the vegetation, leads to variable viability of *B. anthracis* in the environment. Whether and to what extend they survive as infectious spores would then depend on their physiological status during deposition and, in case of vegetative cells being deposited, on the environmental conditions allowing or preventing sporulation. In any case, the survival in the gut of flies is very limited.

For comparative studies on the immunogenicity, protective efficacy and safety of the LSV versus non-living vaccine (NLV) candidates recombinant peptide vaccines based on components of the toxins, spore, and capsule in combination with a lipopeptide adjuvant, with and without formalin-inactivated spores (FIS), were tested in various combinations in mice, rabbits and finally goats. Moreover, appropriate DNA-vaccines designed for directed presentation of expressed antigens were tested in combination with vectors coding for regulatory adjuvants.
In field experiments with goats the protein based NLVs showed up to 80% protection from a lethal challenge if FIS was part of the vaccine. This was equivalent to the LSV (60 – 100%). In contrast, the DNA-vaccines showed little to no immunogenicity in goats, though 90% protection from a lethal challenge was seen in mice.
Surprisingly, our results indicate that antibodies generated against FIS in goats are not comprised of antibodies against BclA and that BclA seems to be poorly or not immunogenic in goats, in strong contrast to the mouse model. Recent publications have shown that antigens, physiologically located beneath the exosporium, can contribute to a protective immune response and should be considered for further veterinary vaccine tests.
Immunological data from the study with the LSV indicate that the current veterinary immunization protocol, one dose with annual boosters, is suboptimal at best and can be improved.
A passive mouse protection assay based on testing the protective capacity of animal sera in A/J mice challenged with the LSV proved to be reliable as a correlate for protection in the target animal.

Achievements resulting from collaborations have been the training of seven African researchers at the UHO laboratories and of two German doctorands in Namibia and South Africa. Two doctorates and six MSc theses have been finished, two PhD studies and one MSc thesis are nearly completed.

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