Supplement 1: Laboratory Methods for diagnosis of anthrax and for immunological study of the infection in humans

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- **Cultures and *B. anthracis* identification**: Blood samples of the four cows were cultured in 5% horse sheep blood agar [17]. After overnight incubation at 37°C, typical *B. anthracis* colonies (grey, non-hemolytic, with a ground glass surface) were detected from the samples of all four animals.

- **PCR methods**: suspect isolates of *B. anthracis* were identified according to International Standards, targeting DNA sequences of pOX1 and pOX2 [17], and chromosome specific sequences [18, 19]. DNA was extracted with QiaCube Platform using QIAamp® DNA Mini Kit (QIAGEN) with an additional pre-incubation step with lysozyme buffer at 37° for 1 h, proteinase K and AL buffer at 56° for 45 minute. DNA was eluted in 50 ml AE buffer. A *Bacillus* species-specific sequence (*B. anthracis* Pasteur #2H) of chromosomal region (DNA-gyrase subunit b) was also amplified with a PCR assay based on the method of Yamada et al [20]. Bacterial primers used to amplify a 245 bp fragment of the gyrB gene were: BA1 – AATCGTAATATTAAACTGACG; and BA2r – CCTTCATACGTGTGATTG. All PCR reactions were performed in a total volume of 50 µl containing 5 µl of template DNA, 0.25 U Taq DNA Polymerase (Invitrogen by Thermo Fisher Scientific.), 200 mM of each dNTP (SIGMA.), 200 nM of each primer and 1 mM MgCl2. To verify the presence of plasmids, the gene encoding the edemigenous factor was identified: plasmid marker pXO1 and the coding gene for unit A of the capsular protein complex CAP: pXO2 plasmid marker [18].

- **Flow cytometry**: T-cell specific immunity was evaluated by flow cytometry at different time points: at 12 days (T1), 18 days (T2), 24 days (T3) and 73 days (T4) from exposure. Briefly, Peripheral Blood Mononuclear Cells (PBMC) were isolated by Ficoll procedure, counted and stored at −80. Cryopreserved PBMC were rapidly thawed, washed with culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, penicillin 50 IU/ml, streptomycin 50 µg/ml), cultured 1x10⁶ cells/ml, stimulated with *B. anthracis* secretome at 5µg/ml and PMA/ionomycin (PMA 50 nM and Ionomycin 1μM, Sigma Aldrich) for 24 hours. Brefeldin A (10 µg/ml, Serva) was added after one hour of stimulation. Cells were stained and analyzed by flow cytometry using the following anti-human monoclonal antibodies to assess T-cell subsets and T-cell cytokines production: CD4 V450, CD8 PeCy7, CD3 PerCp Cy 5.5, TNF-α FITC, IFN-y-PE (BD Pharmigen). Briefly, PBMC were incubated for 20 min at 4 °C with anti-CD3, anti-CD4 and anti-CD8 mAbs, washed with buffer (PBS/1%BSA/0.1% sodium azide), and fixed with 1% paraformaldehyde 5 minutes at RT. After washing, cells were stained with anti-TNFα/anti-IFNy in buffer (PBS/1%BSA/0.1% sodium azide, 0.5% Saponin) 20 minutes at RT, washed and acquired using
a FACSCanto II flow cytometer (Becton Dickinson). Data analysis were performed with DIVA software (Becton Dickinson).

- **Elispot assay**: T-cell functionality during *B. anthracis* infection was assessed by detecting interferon-gamma (IFNγ) production using an enzyme-linked immunosorbent spot-forming cell assay (ELISpot) after stimulation. PBMCs were thawed in culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, penicillin 50 IU/ml, streptomycin 50 µg/ml) and assessed for vitality by Trypan Blue exclusion, counted, and plated at 3 × 10^5 cells/well in ELISpot plates (AID GmbH, Strabberg, Germany). PBMCs were then stimulated with *B. anthracis* secretome at 5 µg/ml and PHA, included in the Elispot kit, for 24 hours with 5% of CO₂. At the end of incubation, the ELISpot assay was developed according to manufacturer's instructions. Spontaneous cytokine production (background) was assessed by incubating PBMC with 1 µg/ml αCD28 and αCD49d (IgG1, clones CD28.2 and 9f10, respectively; Becton Dickinson). Results are expressed as spot forming cells (SFC)/10^6 PBMCs in stimulating cultures after subtracting spontaneous background.

- **Complement fixation test**: serum samples of the two patients were tested with the Sterne-based CFT which utilizes an inactivated suspension of *B. anthracis* strain 34F2 as antigen, as previously described by Adone et al., 2016 [21]. Sera were diluted 1:2 in Veronal buffer and incubated for 30 min at 56°C to inactivate the native complement. Then, in 96-well round-bottom plates 25 µl of each serum was serially diluted from 1:2 to 1:128 prior to adding 25 µl of antigen and 25 µl of complement at working dilution. Plates were incubated at 37°C for 30 min and then 25 µl of sensitized erythrocytes were added to each well. After incubation, plates were centrifuged at 2000 g for 5 min to allow any unlysed cells to deposit and the reaction was read over a diffused white light: 100% hemolysis was considered as negative reaction, while all reactions showing complete absence of hemolysis (0%) or partial hemolysis 75, 50, or 25% were considered as positive. The titer of each serum was the highest dilution showing a positive reaction; the serum dilution 1:2 showing 50% of hemolysis was taken as the reactivity threshold of the reaction. A bovine, hyper immune serum containing anti-anthrax antibodies was used as positive control and a pool of human sera from healthy people as negative control.