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# Proteomics of diphtheria toxoid vaccines reveals multiple proteins that are immunogenic and may contribute to protection of humans against *Corynebacterium diphtheriae*

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ABSTRACT

Introduced for mass immunization in the 1920s, vaccines against diphtheria are among the oldest and safest vaccines known. The basic principle of their production is the inactivation of purified diphtheria toxin by formaldehyde cross-linking, which converts the potentially fatal toxin in a completely harmless protein aggregate, which is still immunogenic. Since in addition to diphtheria toxin also other proteins may be secreted by *Corynebacterium diphtheriae* during cultivation, we assumed that diphtheria toxoid might not be the only component present in the vaccine. To address this question, we established a protocol to reverse formaldehyde cross-linking and carried out mass spectrometric analyses. Different secreted, membrane-associated and cytoplasmic proteins of *C. diphtheriae* were detected in several vaccine preparations from across the world. Based on these results, bioinformatics and Western blot analyses were applied to characterize if these proteins are immunogenic and may therefore support protection against *C. diphtheriae*. In frame of this study, we could show that the *C. diphtheriae* toxoid vaccines induce antibodies against different *C. diphtheriae* proteins and against diphtheria toxin secreted by *Corynebacterium ulcerans*, an emerging pathogen which is outnumbering *C. diphtheriae* as cause of diphtheria-like illness in Western Europe.



## 1. Introduction

Diphtheria is an infection of the upper respiratory tract of humans and was a major cause of morbidity and mortality especially of children until the beginning of the 20th century (for recent reviews, see [1–3]). In 1884 Löffler showed that *Corynebacterium diphtheriae* is the etiological agent of diphtheria and postulated that a toxin secreted by this bacterium is responsible for the often fatal damages observed to heart and kidneys [4]. This hypothesis was verified by Roux and Yersin (1888). When filter-sterilized supernatants of *C. diphtheriae* cultures were injected to guinea pigs, damages similar to those observed in cases of human diphtheria infections were found [5]. Classical diphtheria of the upper respiratory tract is spread from person to person by respiratory droplets produced by coughing. Additionally, other secretions and contaminated materials may be sources of infection especially in cases of cutaneous diphtheria. After infection and colonization of nasopharyngeal epithelia by the bacteria within two to five days, patients are infectious for two to three weeks. Today, penicillin and erythromycin are drugs of choice to stop the infection [6] and quickly render patients non-infectious [7]. Before introduction of mass

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vaccination, diphtheria was observed as an infection especially of children, indicating that *C. diphtheriae* was widely disseminated among the population leading to early contact with the pathogen.

The development of vaccines starting in the 1920s and the introduction of mass immunization using diphtheria toxoid vaccines led to a dramatic reduction of worldwide diphtheria cases. After the global introduction of the "Expanded Programme on Immunization" (EPI) in 1974, only relatively small and local outbreaks occurred until the 1990s [8]. However, with the breakdown of the former Union of Socialist Soviet Republics, a large scale outbreak was observed, leading to a diphtheria pandemic with more than 157,000 cases and over 5000 deaths reported between 1990 and 1998 [9–12]. The outbreak started in the Russian Federation [13–15], but it quickly spread to the Baltic States, former Asian Soviet Republics and other states such as Finland, Poland and Turkey [16]. In contrast to former epidemics, children were less affected, while diphtheria cases among adolescents and adults reached up to four fifths of total cases in some states [9,17]. The outbreak was finally stopped by mass immunization, especially of adults. Despite this success and the continuing global EPI, diphtheria is not eradicated today and its etiological agent *C. diphtheriae* is still present on the list of the most important global pathogens [18,19]. In fact, reported global cases increased from about seven thousand in 2016 to almost nine thousand in 2017 with a focus on countries with poor access to public health systems, for example India, Indonesia, Nepal, Pakistan, Venezuela and Yemen [20]. Furthermore, recent analyses of respiratory and cutaneous diphtheria cases among Spanish, Belgian, German and British citizens as well as in Asian and African refugees in Finland, Denmark, Germany and Sweden indicated the circulation of toxigenic *C. diphtheriae* not only among immigrants, but also among the indigenous population of the European Union [21–25]. Among toxigenic *C. diphtheriae* isolates, the highly virulent 'Sankt-Peters burg/Rossija' epidemic clone that caused the large diphtheria outbreak in Russia and neighboring countries in the 1990s is still in circulation in the European Union [26]. Moreover, in Europe the number of cases of human diphtheria-like disease associated with pet animals, i.e. cats and dogs, has increased, which are caused by *Corynebacterium ulcerans*, a close relative of *C. diphtheriae* [24,27].

Infections with diphtheria can be successfully treated with antitoxin and antibiotics [6]; however, an efficient vaccination regime is most effective to prevent this potentially fatal disease. The basis for vaccination was laid by scientists such as Ehrlich, Fraenkel, Park, Ramon, von Behring and others at the beginning of the 20th century, leading to the development of today's diphtheria toxoid vaccine (for review, see [28]). The vaccines are commonly injected intramuscularly as a 0.5 ml dose and typically combinations with tetanus and pertussis vaccines are administered (DT and DPT vaccines). The immunization schedule recommended by the World Health Organization (WHO) includes a primary immunization series of three doses for infants followed by optional booster immunizations for adults [19]. After the primary immunization series 94 to 100% of children develop at least minimal protective antibody levels (>0.01 IU/ml) [19]. For 2018 the WHO reported a global estimated diphtheria, tetanus and pertussis (DTP3) coverage of 86% [29], while almost 20 million infants worldwide did not receive a routine primary immunization series of DTP3 vaccine [30].

Although diphtheria vaccine is thought to be directed exclusively against the toxin and immunization is not expected to be prevent carriage of *C. diphtheriae* on epithelia and skin [31], an influence of vaccination on the increasing emergence of non-toxigenic strains was discussed [32]. In this case, diphtheria toxoid vaccines may also prevent bacterial infections due to the presence of trace amounts of other immunogenic proteins in the vaccine.

This hypothesis seems plausible, when the production process is analyzed in detail. After cultivation of the most common used toxigenic vaccine strain of *C. diphtheriae* PW8 [33] in beef-derived peptides or casein hydrolysate, the bacteria are removed by centrifugation [28,34,35]. For inactivation of the secreted diphtheria toxin, 0.75% of formaldehyde is added to the supernatant and the solution incubated for up to six weeks at 37 °C [35–37]. After the supernatant is fully detoxified, the toxoid-containing solution is filtered and as further purification and concentration step ammonium sulfate precipitation is often applied, before the toxoid is tested for potency [28,34,35,38]. To enhance the immune response, aluminum salts are added as adjuvant to the toxoid vaccine [28]. This well-established production process makes it very likely, that besides the toxin other secreted proteins, which were described earlier to be part of culture supernatants of *C. diphtheriae* [39], are also present in toxoid preparations. To investigate this hypothesis, we developed a protocol to purify proteins from commercially available vaccines and reverse the formaldehyde cross-linking, which was described to be irreversible previously [40]. A number of proteins was identified by mass spectrometry. Bioinformatics analyses and Western blotting experiments were carried out to elucidate, if these proteins may contribute to immune protection against *C. diphtheriae*.

## 2. Methods

### 2.1. Human ethics

Human blood was collected from three adult patients six months after hematopoietic stem cell transplantation before re-vaccinations (first serum) and one year after three vaccinations with Pentavac® (DTaP-Hib containing 30 IU of diphtheria toxoid and 40 IU of tetanus toxoid, SanofiPasteur) (second serum). First booster and second booster vaccinations were given 4 weeks after the primary or first booster vaccinations, respectively. All methods were performed in accordance with the relevant guidelines and were approved by the University Hospital Ethics Committee, Ethik-Kommission der Friedrich-Alexander-Universität Erlangen-Nürnberg" (Krankenhausstraße 12, 91054 Erlangen, Germany; https://www.ethikkommission.fau.de) under registration number 147_12B. All patients enrolled gave written informed consent before participation.

### 2.2. Preparation of vaccines for mass spectrometry analysis

Since the protein content of the commercial vaccines analyzed in this study (Table 1) was considered low, multiple vaccine doses (0.5 ml each) were pooled and precipitated by addition of 10% (w/v) trichloroacetic acid (TCA) and incubation at 4 °C for 16 h to get a concentrated sample for mass spectrometry analysis [41]. After incubating for 16 h at 4 °C the samples were centrifuged (8000*g*, 30 min, 4 °C). The precipitated proteins were dried on ice and solved in rehydration buffer (2% sodium deoxycholate, 10 mM dithiothreitol (DTT), 50 mM Tris, pH 8.0). To reverse the formaldehyde cross-linking of the inactivated toxins, the samples were incubated for 20 min at 95 °C [42]. Subsequently, the protein amount of rehydrated and heat-treated samples was determined using a spectrophotometer (NanoDrop LITE, Thermo Fisher Scientific, Bremen, Germany) at 280 nm.

### 2.3. Tryptic digest and C18 clean up

About 10 μg soluble proteins prepared from the vaccine samples (see above) were transferred to 10 kDa vivacon 500 membrane filters and the flow-through was discarded after centrifugation for

**Table 1**
Vaccines used for proteome analyses in this study. Sample size indicates the number of doses pooled and prepared for mass spectrometry analysis.

| Manufacturer/designation | Active components | Country | Sample size |
|---|---|---|---|
| Biological E (BE), Vacina contra a difteria e tétano | Tetanus toxoid ≥ 20 I.U., diphtheria toxoid ≥ 2 I.U. | India | 3 × 10 doses (0.5 ml per dose), two different lots |
| Butanan Institute, Vacina adsorvida difteria e tétano adulto (dT) | Tetanus toxoid ≥ 25 Lf/ml, diphtheria toxoid ≥ 2 Lf/ml | Brazil | 10 doses (0.5 ml per dose) |
| GlaxoSmithKline (GSK), Td-pur | Tetanus toxoid ≥ 20 I.U., diphtheria toxoid ≥ 2 I.U. | Germany | 10 doses (0.5 ml per dose) |
| InterVax for BB-NCIPD, Diftet Vacuna DT | Tetanus toxoid ≥ 20 Lf/ml, diphtheria toxoid ≥ 30 Lf/ml | Bulgaria | 10 doses (0.5 ml per dose) |
| Microgen, Diphtheria toxoid adsorbed (AD-M-toxoid) | Diphtheria toxoid ≥ 10 Lf/ml | Russia | 2 × 10 doses (0.5 ml per dose) |
| Microgen, Diphtheria-tetanus toxoid adsorbed (ADT-M-toxoid) | Tetanus toxoid ≥ 10 EC/ml, diphtheria toxoid ≥ 10 Lf/ml | Russia | 2 × 10 doses (0.5 ml per dose) |

30 min at 12,000*g* to remove all salts from the vaccine (e.g. aluminum adjuvant), TCA precipitation and rehydration step. The tryptic digest of the prepared vaccines samples occur within modified Filter Aided Sample Preparation (FASP) protocol [43]. The proteins were reduced by addition of 200 µl of reduction buffer (25 mM DTT, 8 M urea, 50 mM triethylammonium bicarbonate buffer (TEAB)) for 30 min at 37 °C. Alkylation of sulfhydryl groups was carried out with 200 µl alkylation buffer (25 mM chloroacetamide (CAA), 8 M urea, 50 mM TEAB) for additional 30 min on a shaker at 600 rpm in the dark. The proteins were subsequently washed with 300 µl of 8 M urea in 50 mM TEAB followed by another washing step with 200 µl 6 M urea in 50 mM TEAB. Afterwards 0.5 µg mass spectrometry grade LysC endopeptidase was added onto the filter unit and incubated on a shaker at 37 °C and 600 rpm for 3 h, followed by a second digest with 1 µg trypsin and 250 µl dilution buffer (50 mM TEAB) to reach a final concentration of 1 M urea. The sample was incubated over-night at 37 °C at 600 rpm on a shaker. Peptides were then collected by centrifugation at 12,000*g* for 20 min. For acidification of the peptide solution 20 µl of 10% trifluoroacetic acid (TFA) was added to reach a final concentration of 0.5% TFA. To remove all remaining salts a clean-up of the peptides with C18 stage tips were performed. Prior to LC-MS/MS analysis, peptides were vacuum dried and solved in 0.1% trifluoroacetic acid (TFA) [44].

### 2.4. Mass spectrometry

Mass spectrometric analyses were carried out as described [44,45] and resulting raw data files were analysed using the *C. diphtheriae* ATCC 700971/NCTC 13129/Biotype gravis database (Proteome Id: UP000002198) and the Proteome Discoverer 1.4 program package (Thermo Fisher Scientific, Bremen, Germany). As described by Schäfer and co-workers [46] theoretical masses for peptides were generated by trypsin digestion with a maximum of 2 missed cleavages. Product ions were compared to the measured spectra using the following parameters: carbamidomethyl modification on cysteine was set as fixed and oxidation of methionine as dynamic modification. Mass tolerance was set to 10 ppm for survey scans and 0.6 Da for fragment mass measurements. For protein identification the thresholds were set on 1% false discovery rate (FDR). For each vaccine, between two and six mass spectrometry runs were carried out (24 in total).

### 2.5. Label-free quantitative protein analysis

For protein quantification of vaccines, three single vaccine doses from the Russian vaccine were prepared and analyzed by mass spectrometry using approximately 250 ng of each sample. The peak area which correlates with the concentration of peptides was used to determine the quantity of proteins present in the vaccines [47–49]. Only peaks ranged from $2 \times 10^7$ up to $10^{11}$ where used for quantification as described previously [50].

### 2.6. Proteome prediction and prevalence of the proteins among other *C. diphtheriae* strains

Cellular localization and the characteristics of *C. diphtheriae* proteins identified in different samples of vaccines were extracted from the previously available data [51]. The prevalence of these proteins among diverse *C. diphtheriae* strains was inferred by searching the pan-genome of 117 strains [52].

### 2.7. Prediction of putative immunogenic proteins

To analyze the role of proteins present in vaccines as putative antigens the VaxiJen database (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used with a threshold of 0.4 [53].

### 2.8. Data availability statement

The mass spectrometry proteomics data of 24 runs carried out have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [54]. Data are available via ProteomeXchange with identifier PXD009289 and PXD012806.

### 2.9. Growth conditions

*C. diphtheriae* and *C. ulcerans* strains used in this study (Table 2) were grown in Heart Infusion (HI, Oxoid, Wesel, Germany) at 37 °C under shaking at 125 rpm in baffled flasks. Growth was monitored by measuring the optical density at 600 nm ($OD_{600}$). For preparation of protein extracts, bacteria were inoculated to an $OD_{600}$ of 0.1 and grown to an $OD_{600}$ between 0.4 and 0.6, which was reached after about 4 h. For toxin production 2–2́-bipyridyl was added at a final concentration of 0.5 mM during exponential phase and bacteria were incubated for further 2 h under iron starvation conditions [55].

### 2.10. SDS-PAGE and Western blotting

For SDS-PAGE of proteins in the Td-pur vaccine, proteins were precipitated, rehydrated (see Section 2.2 for buffers) and incubated at 95 °C for different time intervals. 125 µg of proteins were loaded per well and separated according to their apparent molecular mass using Tricine-buffered 12.5% (w/v) polyacrylamide gels [56]. After gel electrophoresis proteins were visualized by silver-staining [57].

For SDS-PAGE of cell extracts of the different *C. diphtheriae* and *C. ulcerans* strains studied, bacteria were harvested by centrifugation, resuspended in PBS (0.137 M NaCl, 2.7 mM KCl, 10 mM

**Table 2**
Bacterial strains used in this study.

| *C. diphtheriae* strain | GenBank accession no. | *tox* gene | Description/source | Reference |
|---|---|---|---|---|
| ATCC 27010 (NCTC 11397) | GCA_001457455.1 | –[1)] | | [70,71] |
| ATCC 27012 | CP003210 | +[2)] | Laboratory strain | [72] |
| DSM 43988 | GCA_000455785.1 | – | Throat culture | [73] |
| DSM 43989 | LJXS00000000.1 | + | | [70] |
| INCA 402 | CP003208 | – | Pneumonia (cancer patient) | [72] |
| ISS 3319 | JAQO00000000 | – | Severe pharyngitis/tonsillitis | [51,74] |
| ISS 4060 | JAQN00000000 | – | Severe pharyngitis/tonsillitis | [51] |
| ISS 4749 | JAQQ00000000 | – | Severe pharyngitis/tonsillitis | [51] |
| HC04 | CP003215 | – | Fatal case of endocarditis | [72] |
| NCTC 13129 | BX248353 | + | Diphtheria | [75] |
| *C. ulcerans* strain | GenBank accession no. | *tox* gene | Description/source | Reference |
| 809 | CP002790.1 | – | Human | [76] |
| BR-AD22 | CP002791.1 | – | Dog | [77] |
| KL 756 | – | + | Dog | This study |
| KL 758 | – | + | Human | This study |
| KL 785 | – | + | Human | This study |

[1)] –: absent.
[2)] +: present.

$Na_2HPO_4$, 2 mM $KH_2PO_2$, pH 7.4) to identical $OD_{600}$ equivalents ($OD_{600}$ of 0.8) and incubated at 95 °C for 20 min. Protein concentrations of these crude cell extracts were determined using a standard Bradford assay [58]. Equal amounts (approximately 2 μg protein per slot) of protein extracts from the bacteria were loaded onto Tricine-buffered 12.5% (w/v) polyacrylamide gels [56]. After separation by SDS-PAGE, proteins were transferred onto PVDF membranes using a Bio-Rad semi-dry apparatus for 1 h at 0.8 mA/cm$^2$. The membranes were washed for 15 min in TBS-T (19.8 mM Tris, 150 mM NaCl, pH 7.6, 0.1% Tween 20) and subsequently blocked in blocking solution (5% non-fat dry milk in TBS-T) for 1 h at 4 °C. Human serum from three different donors (K002, K003 and K009) and diphtheria antitoxin (DAT) (Microgen, Russia) was used as primary antibody (1:1,000 dilution) and incubated overnight at 4 °C in blocking solution. After three washing steps in TBS-T, the secondary antibody (anti human IgG (Fc-specific) alkaline phosphatase antibody, Sigma-Aldrich, Germany or anti-horse IgG (Fc-specific) alkaline phosphatase antibody, Sigma-Aldrich, Germany) was incubated in a 1:15,000 dilution in blocking solution for 1 h at 4 °C followed by three washing steps. Detection of immunereactive bands was performed with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) color development substrate according to the manufacturer's protocol.

## 3. Results

### 3.1. Reversibility of formaldehyde cross-linking

Diphtheria toxoid vaccines are among the safest vaccines known. During production, the potentially fatal diphtheria toxin is inactivated by extensive cross-linking with formaldehyde, carried out at 37 °C for several days. While short-term formaldehyde cross-linking is reversible, the long treatment in the vaccine production processes was described to be irreversible. Consequently, it was reported that mass spectrometric analyses of such vaccines are highly problematic or even impossible [40,59]. In this study, we developed a protocol to resolve cross-linking of toxoid preparations from different sources. When subjected to SDS-PAGE, untreated samples precipitated almost completely in the wells of the gel and did not enter the polyacrylamide matrix due to extensive cross-linking. Heat treatment of these samples for increasing time intervals resulted in the appearance of protein bands with apparent molecular masses between 10 and 70 kDa besides more faint bands in the higher molecular mass range. The most prominent bands with an apparent molecular mass of approximately 65, 55 and 45 kDa may represent subunits of diphtheria and tetanus toxin (i.e. the 62 kDa diphtheria toxin with a 22 kDa A and 40 kDa B subunit and the 150 kDa tetanus toxin with a 50 kDa A and 100 kDa B subunit), while the identity of the other bands was unclear (Fig. 1). Therefore, we started mass spectrometry analyses to identify the proteins present in vaccine preparations with emphasis on *C. diphtheriae*.

### 3.2. Mass spectrometric analysis of toxoid vaccines

For mass spectrometry analyses, heat-treated vaccine preparations were precipitated by TCA, purified and subsequently subjected to mass spectrometry. Samples were analyzed in respect to the total number of proteins present in the all vaccines and producer-specific variations. In the six vaccines analyzed, 665 different proteins from *C. diphtheriae* were identified. While the vaccine from Brazil contained 130 distinct proteins, in the vaccine from Germany 205 different proteins were found, 324 proteins were detected in the Bulgarian vaccine as well as 344 and 363 proteins, respectively, in the two different Russian vaccines analyzed.

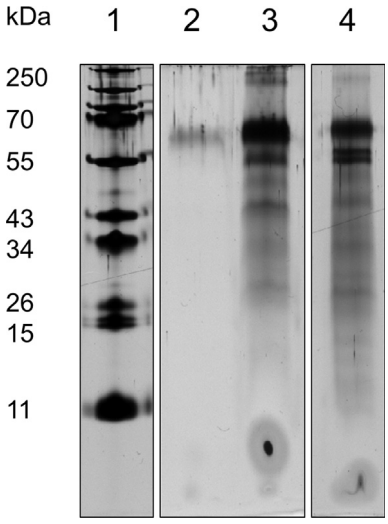


**Fig. 1.** Influence of heat-treatment on patterns of vaccine proteins form Td-pur (GSK). Proteins were separated by a 12.5% (w/v) Tricine-buffered SDS-PAGE. The proteins were precipitated by TCA extraction and resuspended in 50 μl rehydration buffer. Approximately 125 μg of protein was loaded per lane. The separated proteins were silver-stained. Lane 1: protein marker, lane 2: without boiling, lane 3: 95 °C for 5 min with loading dye, lane 4: 95 °C for 20 min in addition to 95 °C for 5 min with loading dye.

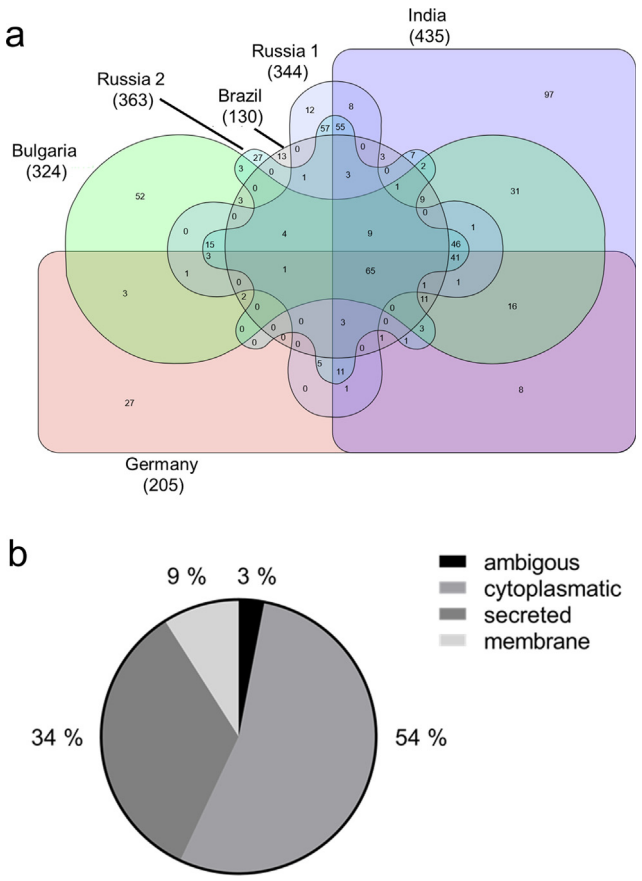


**Fig. 2.** Bioinformatics analysis of proteins identified in diphtheria toxoid vaccines. (a) Venn diagram [78] of identified proteins in the different samples. Country of origin is given (Russia 1: Diphtheria Toxoid, Russia 2: Diphtheria-Tetanus Toxoid). (b) Predicted localization of 65 proteins present in all vaccines studied. Proteins located in the cytoplasm are shown in medium grey, secreted proteins in dark grey, membrane proteins in light grey and proteins with ambiguous localization in black.

With 436 different proteins the highest number was detected in the vaccine from India (Fig. 2).

Predictions of protein localization showed a cytoplasmic localization for 456 (69%) proteins, 93 (14%) proteins were predicted to be secreted, 90 (13%) membrane-localized and 26 (4%) proteins showed an ambiguous localization. Forty-one percent of the secreted proteins are lipoproteins including 4% with a twin-arginine domain, 28% non-classical secreted proteins, 28% proteins with Spl signal peptide, 1% Tat-secreted proteins without signal peptide, 1% Tat-dependent proteins with transmembrane domain and 1% Esx substrate proteins. Twelve percent of the membrane-associated proteins were also detected with a signal peptide, 7% with a LPXTG domain and 1% were YidC-like proteins.

Further bioinformatics analyses revealed that out of the 665 distinct proteins detected in the different vaccines, 456 proteins were described before as a set of proteins conserved among 117 *C. diphtheriae* isolates, which was defined as core proteome of these strains [60]. These included 336 cytoplasmic proteins, 57 secreted proteins, 54 membrane proteins and 9 proteins with ambiguous localization. 65 proteins were present in all six vaccines (Fig. 2a, Table 3). Among these an enrichment of secreted and membrane proteins was observed compared to the overall identified proteins (43% versus 27.5%) (Fig. 2b). From the set of 65 proteins detected in all six vaccines 24 proteins were identified in a previously carried out secretome study [39]. Recently, in an *in silico* analysis by Hassan and co-workers [61] ten of the 65 proteins were found as conserved target proteins and four (DIP1902, DIP1303, DIP0470, DIP0281) of them as global drug targets of the conserved proteome form *C. diphtheriae* (Table 3) [61]. In addition, the DIP1680 protein was found in an *in silico* approach for the identification of therapeutic targets and putative virulence factor by Jamal and co-workers [62].

Besides their presence, also the relative amounts of *C. diphtheriae* proteins were analyzed for the Russian vaccine. As estimated by label-free protein determination, the peak-area of diphtheria toxin (DIP0222) represents up to 78.7 ± 7.6% of the total proteins from *C. diphtheriae*. The next prominent single proteins were glutamate dehydrogenase (DIP1547) with 1.1% ± 0.5% and catalase (DIP0281) with 0.8% ± 0.1%, while all other proteins accumulated to 19.4% ± 7.2%, (Fig. 3).

### 3.3. Prediction of putative immunogenic proteins

Due to the fact, that formaldehyde cross-linking for toxin detoxification is not selective for diphtheria toxin, each of the proteins present in the culture supernatant is able to react with each other to build protein complexes. These proteins identified besides the diphtheria toxin in all vaccine samples (see above) may function as additional antigens during vaccination. Of special interest in this respect were proteins of *C. diphtheriae*, which may have direct contact with the host immune system. Therefore, all secreted or cell surface-exposed proteins, which were identified in this study in all vaccines and were found to be conserved among more than hundred *C. diphtheriae* strains recently [60], were analyzed in respect to their putative immunogenicity. In fact, 15 out of 16 proteins were indicated as possible antigens when the VaxiJen database was used to predict immunogenicity of these proteins.

### 3.4. Immuno-reaction of diphtheria antitoxin

Although the diphtheria toxin is obviously the main component of diphtheria toxoid preparations, also the additionally observed proteins may be immunogenic as predicted by the bioinformatics analyses presented above. As a first approach to address this idea, commercially available diphtheria antitoxin (DAT) was tested in Western blotting experiments. The diphtheria antitoxin used was produced by immunization of horses with toxoid preparations for application in diphtheria cases to scavenge the diphtheria toxin from the patients' blood stream.

The immune reaction of DAT against a selection of three toxigenic and seven non-toxigenic *C. diphtheriae* isolates with different strain background and from different countries was tested (Table 2). Cells were grown in absence and presence of bipyridyl, a chelator of metal ions, which induces iron starvation and leads consequently to the induction of toxin synthesis, since transcription of the *tox* gene is regulated by the iron-dependent transcriptional regulator DtxR.

In response to bipyridyl addition, a band with an apparent molecular mass of 62 kDa was observed for toxigenic strains, which was absent without starvation and also not found in cell extracts of non-toxigenic strains. The corresponding protein for the toxigenic strain NCTC 13129 was only poorly visible, indicating a low toxin production (Fig. 4). In addition to the bands corresponding to diphtheria toxin, immune reactions of DAT with different proteins in toxigenic and non-toxigenic strains were observed. One protein with an apparent molecular mass marginally higher than the toxin was detected in all strains and under iron surplus and iron starvation. Furthermore, a number of bands corresponding to polypeptides with an apparent molecular mass between 25 and 65 kDa were observed, supporting the idea that toxoid preparations induce antibodies directed other proteins besides the inactivated diphtheria toxin.

**Table 3**
Proteins identified in all analyzed vaccine samples. Annotation and predicted localization as well as localization signals are given for the identified proteins. The number of strains in which the proteins are conserved among 117 *C. diphtheriae* sequences analyzed are indicated as "prevalence" and a previous identification of these proteins in different proteome analyses is shown.

| Identifier | Localization [51] | Prevalence | Antigen | Annotation | [39] | [62] | [61] |
|---|---|---|---|---|---|---|---|
| DIP0025 | Cyt | 117 | + | Peptidyl-prolyl cis-trans isomerase | Yes | No | Yes |
| DIP0108 | Sec-Lipo | 116 | + | Ferrisiderophore receptor Irp6A | Yes | No | Yes |
| DIP0154 | Cyt | 117 | – | Putative endopeptidase | No | No | No |
| DIP0169 | Tat-Lipo | 117 | – | Putative secreted protein | No | No | No |
| DIP0178 | Cyt | 117 | + | Putative phenylalanine aminotransferase | No | No | No |
| DIP0222 | Sec-SpI | 49 | + | Diphtheria toxin | No | No | No |
| DIP0225 | Sec-Lipo | 111 | + | Putative secreted polysaccharide deacetylase | Yes | No | No |
| DIP0257 | Cyt | 108 | + | Uncharacterized protein | No | No | No |
| DIP0281 | Sec-NC | 117 | + | Catalase | Yes | No | Yes |
| DIP0350 | Sec-SpI | 101 | + | Putative secreted protease | Yes | No | No |
| DIP0365 | Sec-SpI | 117 | + | Surface layer protein A | Yes | No | No |
| DIP0368 | Cyt | 117 | + | Dihydrolipoyl dehydrogenase | No | No | No |
| DIP0383 | Cyt | 117 | + | Uncharacterized protein | Yes | No | No |
| DIP0442 | TM-Sec | 117 | + | Putative membrane protein | No | No | No |
| DIP0469 | Cyt | 117 | + | Elongation factor G | Yes | No | Yes |
| DIP0470 | Cyt | 117 | + | Elongation factor Tu | No | No | Yes |
| DIP0483 | Amb | 116 | + | Putative secreted protein | No | No | No |
| DIP0491 | Sec-SpI | 116 | + | Putative secreted amino acid hydrolase | No | No | No |
| DIP0515 | Tat-Lipo | 117 | + | Putative transport system secreted protein | No | No | No |
| DIP0534 | Sec-Lipo | 117 | + | Putative sugar-binding secreted protein | Yes | No | No |
| DIP0543 | TM-Sec | 114 | + | Putative sialidase | Yes | No | No |
| DIP0575 | Cyt | 117 | + | 10 kDa chaperonine | Yes | No | No |
| DIP0582 | Sec-Lipo | 117 | + | Putative iron transport system binding (secreted) protein | Yes | No | No |
| DIP0611 | Sec-Lipo | 117 | + | Putative ABC transport system secreted protein | No | No | No |
| DIP0615 | Sec-Lipo | 117 | + | ABC transport system exported protein | No | No | No |
| DIP0631 | Cyt | 117 | + | Isocitrate dehydrogenase [NADP] | No | No | No |
| DIP0680 | Amb | 117 | + | Uncharacterized protein | Yes | No | No |
| DIP0775 | Sec-SpI | 114 | + | Uncharacterized protein | Yes | No | No |
| DIP0856 | TM | 117 | + | Putative serine protease | No | No | No |
| DIP0917 | Cyt | 117 | + | Enolase | Yes | No | No |
| DIP0956 | Sec-Lipo | 117 | + | Putative peptide transport system secreted protein | Yes | No | No |
| DIP1062 | Sec-Lipo | 117 | + | Putative iron siderophore uptake system exported solute-binding component | Yes | No | No |
| DIP1086 | Sec-Lipo | 117 | + | Putative iron transport system exported solute-binding component | No | No | No |
| DIP1100 | Cyt | 117 | + | Ketol-acid reductoisomerase (NADP(+)) | No | No | No |
| DIP1204 | Cyt | 117 | + | Oxoglutarate dehydrogenase inhibitor | No | No | No |
| DIP1229 | Cyt | 116 | + | Putative cobalamin biosynthesis related protein | No | No | No |
| DIP1303 | Cyt | 117 | + | Transaldolase | No | No | Yes |
| DIP1308 | Cyt | 117 | + | Triosephosphate isomerase | No | No | No |
| DIP1309 | Cyt | 117 | – | Phosphoglycerate kinase | Yes | No | Yes |
| DIP1310 | Cyt | 117 | + | Glyceraldehyde-3-phosphate dehydrogenase | Yes | No | No |
| DIP1390 | Sec-Lipo | 115 | + | Putative secreted protein | No | No | No |
| DIP1419 | Cyt | 117 | + | Alkyl hydroperoxide reductase AhpD | Yes | No | No |
| DIP1420 | Cyt | 117 | + | Iron repressible polypeptide (putative reductase) | Yes | No | No |
| DIP1482 | Cyt | 117 | + | Proline-tRNA ligase | No | No | No |
| DIP1505 | Cyt | 117 | + | Ribosome-recycling factor | Yes | No | No |
| DIP1586 | Sec-Lipo | 117 | + | Putative secreted protein | No | No | No |
| DIP1636 | Cyt | 117 | + | Branched-chain-amino-acid aminotransferase | No | No | No |
| DIP1637 | Cyt | 117 | + | Probable cytosol aminopeptidase | No | No | No |
| DIP1644 | Cyt | 117 | + | Glutamine synthetase | No | No | No |
| DIP1667 | TM | 116 | + | Putative membrane protein | No | No | No |
| DIP1680 | Cyt | 117 | + | GTP cyclohydrolase 1 type 2 homolog | No | Yes | No |
| DIP1783 | Cyt | 117 | + | Nucleoside diphosphate kinase | No | No | Yes |
| DIP1786 | Cyt | 117 | + | Valine-tRNA ligase | No | No | No |
| DIP1787 | Cyt | 117 | + | Malate dehydrogenase | No | No | Yes |
| DIP1902 | Cyt | 116 | + | Succinyl-CoA:coenzyme A transferase | No | No | Yes |
| DIP2010 | TM-Sec-LPXTG | 88 | + | Putative surface-anchored membrane protein | No | No | No |
| DIP2120 | Cyt | 117 | + | Chaperone protein DnaK | Yes | No | No |
| DIP2128 | Sec-Lipo | 116 | + | Putative substrate-binding transport protein | No | No | No |
| DIP2169 | TM | 111 | + | Putative membrane protein | No | No | No |
| DIP2180 | Cyt | 116 | – | Phosphoenolpyruvate carboxykinase | No | No | No |
| DIP2193 | Sec- SpI | 117 | + | Putative secreted protein | Yes | No | No |
| DIP2261 | Cyt | 117 | + | Superoxide dismutase [Mn] | No | No | No |
| DIP2290 | Sec-NC | 117 | + | Single-stranded DNA-binding protein | No | No | No |
| DIP2331 | Cyt | 117 | – | Putative aldehyde dehydrogenase | No | No | No |

### *3.5. Antibody response to corynebacterial proteins of humans vaccinated with diphtheria toxoid*

To determine the immune reaction of humans after vaccination with diphtheria toxoid vaccines, Western Blot analyses with human sera were carried out. The adult human donors of these blood samples had undergone a hematopoietic stem cell transplantation. As part of therapy, the patients' immune system was depleted before transplantation and had later to be rebuilt by the transplanted hematopoietic stem cells. Consequently, freshly vaccinated donors six month after stem cell transplantation were expected to show a negligible immune response, while one year after the first vaccination and carried out consecutive booster vaccinations, antibodies should be generated. The use of sera from

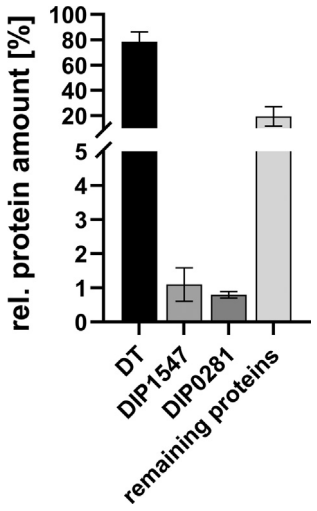


**Fig. 3.** Quantitative protein analysis. 250 ng of prepared vaccine samples were analyzed by mass spectrometry. The relative protein amount of diphtheria toxin (DT), the two most prominent proteins beside DT and of the remaining proteins was calculated. Only proteins in a range from $2 \times 10^7$ to $10^{11}$ where considered for calculation.

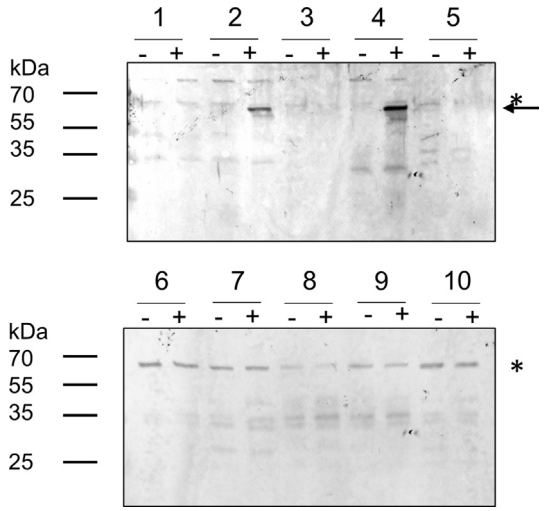


**Fig. 4.** Western blots of cell extracts from toxigenic and non-toxigenic *C. diphtheriae* strains. Bacteria were grown without (−) and with (+) bipyridyl, which induces iron starvation. 2 μg of protein extract were added per lane. Diphtheria antitoxin produced in horses for therapy was used as primary antibody. 1: ATCC 27010 (tox⁻), 2: ATCC 27012 (tox⁺), 3: DSM 43988 (tox⁻), 4: DSM 43989 (tox⁺), 5: INCA 402 (tox⁻), 6: ISS 3319 (tox⁻), 7: ISS 4060 (tox⁻), 8: ISS 4749 (tox⁻), 9: HC04 (tox⁻), 10: NCTC 13129 (tox⁺). The arrow indicates the diphtheria toxin with an apparent molecular mass of 62 kDa, the asterisk a protein with slightly higher apparent molecular mass present in all cell lysates.

these patients avoids the problem that healthy adult individuals vaccinated during childhood may have responded against environmental corynebacteria during their lifetime and show cross-reactions due to this contact. Any observed immunoreactivity therefore would not necessarily be directed against the proteins in the vaccine in this case.

When sera from three individuals taken six months after transplantation but before booster vaccination were tested, only minor immune reactions were observed (Fig. 5a). In contrast, when sera one year after primary vaccination and consecutive booster vaccinations were applied, a strong immune reaction against a protein with apparent molecular mass of 62 kDa was observed for cell extracts of all toxigenic strains, especially when iron starvation was induced (Fig. 5b, panels 2, 4 and 10). Besides this main reaction against the diphtheria toxin, additionally bands corresponding to proteins of an apparent molecular mass between 25 and 65 kDa were observed. In principle, these might be truncated forms of the toxin; however, additional bands were also found for cell extracts of four of the seven non-toxigenic isolates tested (Fig. 5b, panels 1, 3, 5 and 6). Obviously, the sera of the vaccinated adults tested contained antibodies directed against other proteins than the toxin. The number of these immune-reactive proteins correlated with the evolutionary distance of strains. Non-toxigenic *C. diphtheriae* strains, which are more closely related to PW8 production strains, showed more bands in Western blots compared to more distantly related isolates (Fig. S1).

To further support the idea that the PW8-derived toxoid vaccines induce antibodies directed against additional proteins only in related *C. diphtheriae* strains, we also tested three toxigenic and two non-toxigenic *C. ulcerans* isolates in Western blot experiments. In fact, when grown without bipyridyl, none of the strains showed an antibody reaction with the three human sera tested. In contrast, in case of the three toxigenic isolates KL756, KL758 and KL785, a clear band attributed to the toxin was observed, in extracts from iron-starved cells. Obviously, the diphtheria toxoid vaccine is also active against *C. ulcerans* diphtheria toxin while immunization does not induce significant levels of antibodies directed against other *C. ulcerans* proteins (Fig. 6).

## 4. Discussion

The development of diphtheria toxoid vaccine is without any doubt a milestone in history of medicine and mankind. Its introduction saved the life of millions of children; however, especially the large scale outbreak starting 1990 in the Russian Federation and Ukraine [14–16] demonstrated that diphtheria is not defeated and eradicated and that surveillance and research are still necessary [2,26].

In this study, we identified 665 distinct proteins in addition to the diphtheria toxin in six vaccines from different sources around the world. This astonishingly high number corresponding to about one fifths of the proteins encoded in the *C. diphtheriae* genome is the result of physiological protein secretion, shearing of cell surface proteins and cell lysis as indicated by the predicted localization of proteins. Since between 130 and 436 proteins were observed in the vaccine samples from the different manufacturers, cultivation and production process seem to have a major influence on the protein composition. Bioinformatics analyses of the 65 proteins common in all six vaccines studied here revealed that at least 60 proteins are predicted antigens which may induce antibody formation upon vaccination. In addition, Western blot experiments showed that antibodies of vaccinated persons are directed against different *C. diphtheriae* proteins besides the toxin. Putative target proteins identified by mass spectrometry, which may influence the outcome of infection since antibodies against these proteins may result in a decreased fitness of the pathogen, are components of iron uptake systems, proteases, superoxide dismutase and catalase. The latter enzyme, found in all vaccine sample and one of the most abundant proteins in the Russian vaccine, is crucial for detoxification of reactive oxygen species secreted by host cells upon infection. Effects of vaccination on general pathogen fitness were already suggested for *Clostridium botulinum* since in this case administration of toxoid vaccines to cows reduced the number of *C. botulinum* spores in feces significantly [63].

Although it was previously suggested that DT vaccines only induce immune responses against the toxin but are not effective against non-toxigenic strains that are increasing isolated from cases of systemic infections [24,32,52,64], our data indicate that also the distribution of *C. diphtheriae* strains may be affected by

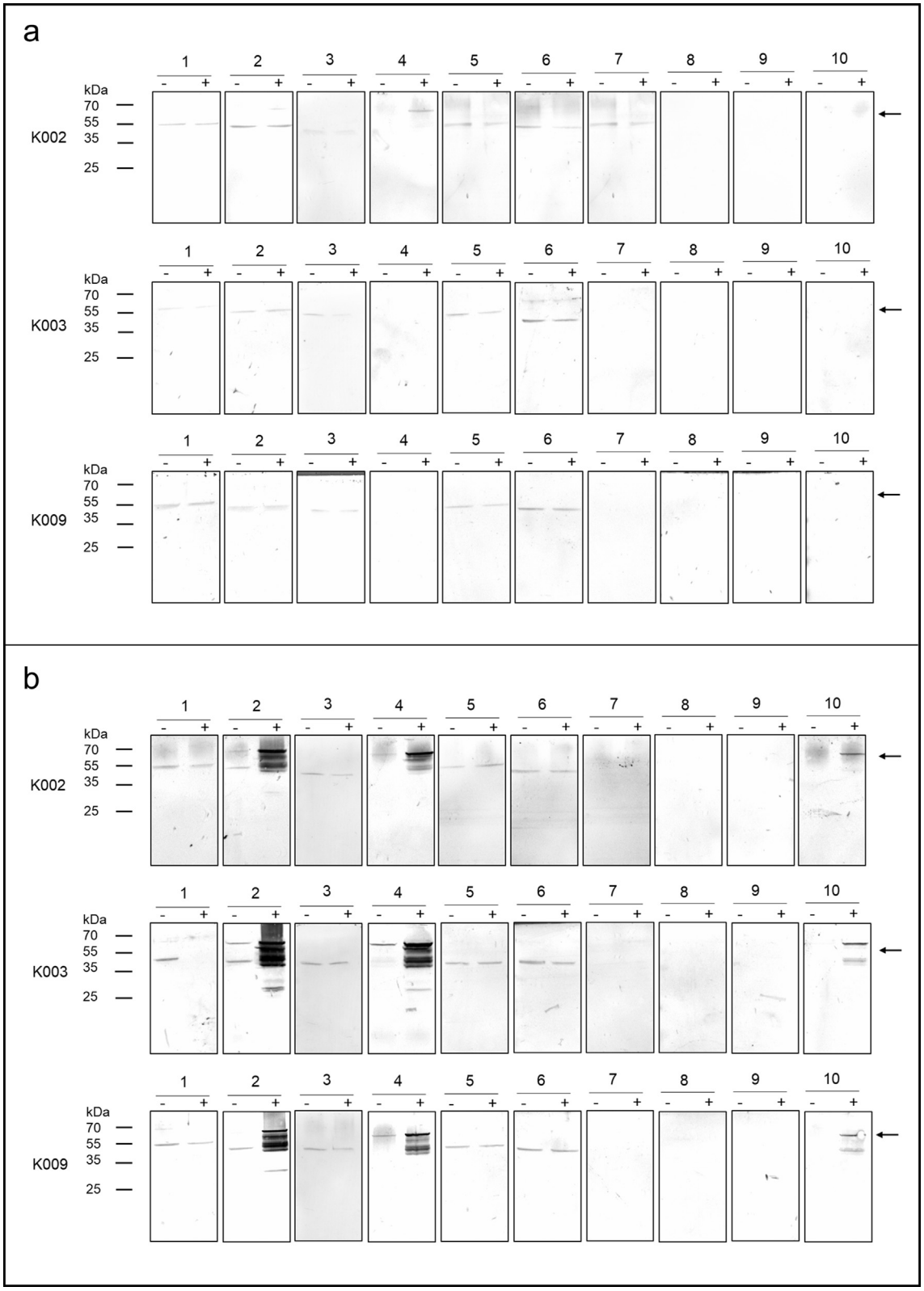


**Fig. 5.** Western blots of *C. diphtheriae* cell extracts. Toxigenic and non-toxigenic strains were grown without (−) and with (+) bipyridyl. 2 μg of protein extract were added per lane. Serum from three different donors (K002, K003 and K009), six months after hematopoietic stem cell transplantation (first serum) (Fig. 5a) and 1 year after primary and two booster vaccinations (second serum) (Fig. 5b) was used as primary antibody. 1: ATCC 27010 (tox$^-$), 2: ATCC 27012 (tox$^+$), 3: DSM 43988 (tox$^-$), 4: DSM 43989 (tox$^+$), 5: INCA 402 (tox$^-$), 6: ISS 3319 (tox$^-$), 7: ISS 4060 (tox$^-$), 8: ISS 4749 (tox$^-$), 9: HC04 (tox$^-$), 10: NCTC 13129 (tox$^+$). Diphtheria toxin is indicated by an arrow.

vaccination. The human antisera tested showed a strain-dependent antibody binding and diphtheria toxoid vaccines may consequently not only put toxigenic strains at disadvantage, but also impair non-toxigenic strains, especially in case that these are taxonomically closely related to the PW8 production strains. The phenomenon that vaccination influence population and genetic drift of strains is discussed for *Bordetella pertussis* [65,66]. In this species, vaccination is assumed to affect the evolution of strains based on

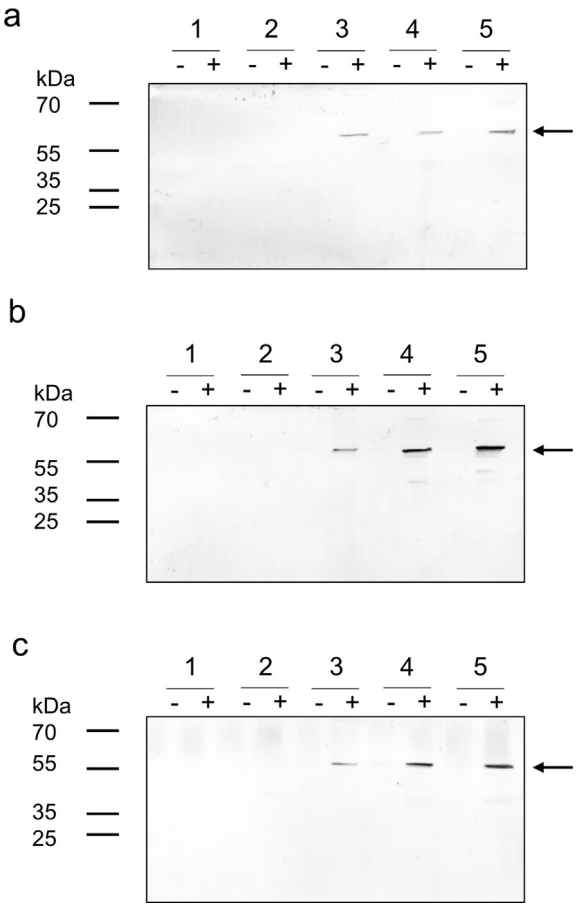


**Fig. 6.** Western blots of *C. ulcerans* cell extracts. Proteins from strains grown without (−) and with bipyridyl (+) were separated by SDS-PAGE. Human serum from three individual donors (a: K002, b: K003 and c: K009), collected 1 year after primary and two booster vaccinations (second serum) was used as primary antibody. 1: 809 (tox$^-$), 2: BR-AD22 (tox$^-$), 3: KL 756 (tox$^+$), 4: KL 758 (tox$^+$) and 5: KL 785 (tox$^+$). Diphtheria toxin is indicated by an arrow.

the observation that the molecular clock rate of mutations correlated with the vaccination coverage [67]. An effect of vaccination on *B. pertussis* strains was reported for the pertactin gene. The *prn1* allele, which is present in the vaccine strain, was predominant in the pre-vaccine era. After introduction of the whole-cellular vaccine, strains carrying the *prn2* allele increased from 39% (1993–1996) to 90% (1998–2004) [67, for review see 68]. Moreover, a study from Sweden showed a shift in the serotype of *B. pertussis* populations from serotype Fim2 in unvaccinated populations to serotype Fim3 and serotype Fim2, 3 in vaccinated populations [69].

In case of *C. diphtheriae*, studies on allelic replacements over time are not practical due to use of the toxoid vaccine for decades; however, the results obtained here may provide a basis for studies on genomic variations and the clonal distribution of toxigenic and non-toxigenic *C. diphtheriae* strains. From the amount of cross-reactions observed, the prediction can be made that strains closely related to PW8 should be at disadvantage compared to more distantly-related strains, a hypothesis, which can be tested in future taxonomical investigations.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Authors' contributions

Möller: protein preparation, Western blot experiments, data collection/analysis and manuscript preparation.

Kraner: mass spectrometric analyses.

Sangal: bioinformatic analyses.

Tittlbach, Winkler, Winkler: provided and characterized the human sera.

Melnikov: involved in study design.

Melnikov, Lang, Matttos-Guaraldi: provided the vaccines.

Sing: isolation and characterization of *C. ulcerans* strains used in this study.

Burkovski: data analysis/ interpretation and manuscript preparation.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/ 10.1016/j.vaccine.2019.04.059.

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