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Pathogenesis and toxins

Production of neutralizing antibodies against the secreted *Clostridium chauvoei* toxin A (CctA) upon blackleg vaccination

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ABSTRACT

Clostridium chauvoei is the etiologic agent of blackleg in cattle, inducing fever, severe myonecrosis, oedemic lesions and ultimately death of infected animals. The pathogen often results in such rapid death that antibiotic therapy is futile and thus vaccination is the only efficient strategy in order to control the disease. The β-barrel pore forming leucocidin *Clostridium chauvoei* toxin A (CctA) is one of the best characterised toxins of *C. chauvoei* and has been shown to be an important virulence factor. It has been reported to induce protective immunity and is conserved across *C. chauvoei* strains collected from diverse geographical locations for more than 50 years. The aim of this study was to identify the location of the CctA toxin during liquid culture fermentation and to use CctA to develop an *in vitro* assay to replace the current guinea pig challenge assay for vaccine potency in standard batch release procedures. We report that CctA is fully secreted in *C. chauvoei* culture and show that it is found abundantly in the supernatant of liquid cultures. Sera from cattle vaccinated with a commercial blackleg vaccine revealed strong haemolysin-neutralizing activity against recombinant CctA which reached titres of 1000 times 28 days post-vaccination. Similarly, guinea pig sera from an official potency control test reached titres of 600 times 14 days post-vaccination. In contrast, ELISA was not able to specifically measure anti-CctA antibodies in cattle serum due to strong cross-reactions with antibodies against other proteins present pre-vaccination. We conclude that haemolysin-neutralizing antibodies are a valuable measurement for protective immunity against blackleg and have the potential to be a suitable replacement of the guinea pig challenge potency test, which would forego the unnecessary challenge of laboratory animals.

1. Introduction

Clostridium (C.) chauvoei is the causative agent of blackleg also referred to black quarter and quarter evil, a severe infectious disease predominately affecting cattle [1]. It causes a fulminant myonecrosis, which rapidly leads to the death of the animal [2–4]. Blackleg causes significant losses in livestock production globally due to its high lethality rate [1,3]. *C. chauvoei* is a strict anaerobic, spore forming, monomorphic and motile Gram-positive bacterium that is spread worldwide [5]. Grazing ruminants become infected due to *C. chauvoei* spores persistently contaminating the soil of pastures either from perished animals or via manure [4,6]. Despite the fact that blackleg is one of the oldest clinically known diseases affecting cattle, the exact mechanisms of its pathogenesis remain unclear [1,3,7–10]. Similarly, knowledge gaps also exist concerning the role of the toxins and virulence factors produced by *C. chauvoei* but various virulence factors have been recently characterised [1,3] and genomic studies have contributed to a better understanding of virulence in *C. chauvoei* [5,11]. The best characterised virulence

Abbreviations: CctA, *Clostridium chauvoei* leucocidin toxin A; rCctA-NusA, recombinant *Clostridium chauvoei* leucocidin toxin A fused to *E. coli* NusA protein; rCctA-cent, recombinant antigenic central fragment of CctA (amino acids 177–271).

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factor is *Clostridium chauvoei* toxin A (CctA) belonging to the β-barrel pore forming leucocidin family [12]. This factor has been shown to be the main cytotoxic and haemolytic agent of *C. chauvoei* [13]. Along with a highly active DNAse, hyaluronidase and sialidase, they seem to constitute the central virulence arsenal of *C. chauvoei* [1,3,7,13–15]. Furthermore, flagella have also been reported to be involved in the pathogenesis of *C. chauvoei* infections [11,16–23].

C. chauvoei is one of the most toxic of the clostridial species, often inducing such a rapid death in animals that antibiotic therapy tends to be a fruitless treatment for blackleg. However, vaccines containing *C. chauvoei* valences are highly successful at preventing the disease and are used worldwide to control blackleg [3,24]. Only sporadic outbreaks of blackleg occur globally each year [1]. Vaccines against blackleg have been available since the 1930s and consist of chemically inactivated bacteria and bacterial culture supernatants from relatively old strains [24]. Even though the current blackleg vaccines, consisting of bacterin and toxoided culture supernatants are effective, it is not clear which antigens in the current formulations actually provide the protection. Indeed, identity of the protective antigens as well as their location has never been conclusively resolved. For a long time it was proposed that flagella and various outer membrane proteins were the protective immunogens against *C. chauvoei* infections [18–20,25] while more recently it was reported that the extracellular proteins of *C. chauvoei* are necessary for protection [26].

As standard procedure, the potency of vaccine batches must be monitored by a challenge model in guinea pigs [27]. In this test, the vaccine under assessment is administered to at least 10 guinea pigs, which are later challenged with a virulent strain of *C. chauvoei* alongside a non-vaccinated control group of guinea pigs. For the test to be valid, all of the control animals must die within three days and for the vaccine to pass the test, at least 90% of the vaccinated guinea pigs must survive for at least five days post-challenge. This procedure induces great distress in the animals.

CctA is fully conserved between *C. chauvoei* strains isolated worldwide [5] and it was recently shown that a recombinant version of the toxin (rCctA) provided full protection in the guinea pig infection model used for official potency tests as part of blackleg vaccine batch release procedures [13]. Furthermore, serum from guinea pigs vaccinated with inactivated rCctA neutralized both the cytotoxic activity of *C. chauvoei* to embryonic calf nasal epithelial cells (ECaNEP) and the haemolytic activity of *C. chauvoei* culture supernatants [13]. Hence, CctA must be considered as one of the main protective antigens in vaccines against blackleg [3]. Our aim here was to further characterize the leucocidin CctA and make it central to the development of an *in vitro* assay to potentially replace the current guinea pig challenge potency test as standard for blackleg vaccine batch releases. This would remove the need for experimental infections of animals.

2. Materials and methods

2.1. Cattle information, vaccination and serum preparation

Ten cattle were vaccinated with a single dose of the vaccine Bovilis® Blackleg (ad us. vet. MSD Animal Health GmbH, Switzerland) according to the instructions of the manufacturer. The vaccination protocol consists of a single subcutaneous application *supra scapulae* of Bovilis® Blackleg. One vaccine dose is 2 mL and consists of 2.5×10^8 inactivated bacteria and toxoid of the five *C. chauvoei* strains (655, 656, 657, 658 and 1048), 400 mg aluminium hydroxide (adjuvants), 0.013% thiomersal (conservatives) and traces of formalin (inactivation), according to Swissmedic, the registration office of Switzerland. Blood samples were taken from the *vena caudalis mediana* with standard vacutainers prior to the vaccination and once per week for 4 weeks post-vaccination. Blood samples were immediately cooled to 4 °C and serum was separated by coagulation and centrifugation using standard procedures. The decision to vaccinate 10 animals was calculated using the Stata statistics software (StataCorp, 2011, Stata statistical software: release 12.0. Statcorp LP, College Station, TX, USA) using the following parameters: alpha, 0.05; power, 0.80; number of follow-up measurements 4; correlation between follow up measurements, 0.70. The 10 cows used in this study are part of a herd of 25 cows owned by the department of Farm Animals of the University of Zürich. This herd is used for educational purposes at the Veterinary faculty. All cows were pluripar and in the age range of 3–8 years. The breeds were Brown Swiss and Holstein Friesian. The cows were kept in a tie barn and fed hay and grain but no silage. The herd was established in 1970, it has no history of blackleg disease and this was the first time that the dams were vaccinated against blackleg. This study was conducted under the research animal approval of the Veterinary Office of the canton of Zurich (permission number eTV 27763).

2.2. Serum of vaccinated guinea pigs

Serum from 10 guinea pigs taken pre- and 14 days post-blackleg vaccination was kindly donated to JF by the Swiss Federal vaccine control authority after performing an official blackleg vaccine control test according to international standards [27].

2.3. C. chauvoei culturing conditions and preparation of bacterial supernatants and cell pellets

C. chauvoei reference strain JF4335 [5,28] which was fully genome sequenced (GenBank/EMBL accession number LT799839), was grown anaerobically in Brain Heart Infusion (BHI) broth (Oxoid Microbiology products, Thermo Scientific, Waltham, MA, USA) supplemented with 0.05% L-cysteine (Sigma-Aldrich chemical, St. Louis, MO, USA) or on standard blood agar medium (Thermo Scientific™ R01198, Blood Agar TSA with 5% Sheep Blood Medium) in an anaerobic chamber at 37 °C for up to 72 h.

2.4. Production of recombinant antigenic domain of CctA

Haemolytically active recombinant CctA protein fused to the *E. coli* NusA protein which acts as a stabilizer (rCctA:NusA) was produced as previously described [13]. To express an antigenically active but not haemolytically active CctA derivative for analytical purposes, we designed a peptide based on the central domain of CctA, named CctA-cent. The antigenic domain of CctA was determined based on hydrophilicity scores and the charged amino acid content in the peptide structure [29]. To express the CctA-cent peptide, the gene fragment for the antigenic central fragment of CctA (AA 177–271) was amplified by PCR using genomic DNA of strain JF4335 and primers: CctAcentF *CATCCATGGGC*ATAACTAAAAGTATCCCAACTAATC and CctAcentR *CATGGATCCA*TATTGATCATTAAAACGATTATATTC (letters in italics are additional nucleotides for *Nco*I and *Bam*HI recognition and to ensure fusion with the poly-His site in the pET-HIS1 plasmid). The generated PCR fragment was cloned into pET-HIS1 [30] using the restriction sites *Nco*I and *Bam*HI. Expression and purification of rCctA-cent was performed as described before [30] using BL21 (DE 3) *E. coli* cells yielding a peptide of 18.7 kDa.

2.5. Immunological detection of CctA

C. chauvoei cells were grown for 24 h to mid-log phase as specified in section 2.3 above, followed by centrifugation at

10′000×*g* for 30 min at 4 °C. The bacterial pellet was re-suspended in a volume of Phosphate-Buffer Saline (PBS) (10 mM Na-phosphate pH 7.4, NaCl 144 mM) equal to the volume of the collected supernatant from any given culture and both fractions were mixed 1:1 (v/v) with SDS-PAGE loading buffer (65.8 mM Tris-HCl, pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue) containing 5% β-mercapto-ethanol. The samples were separated by SDS-PAGE. Recombinant CctA [13] and a purchased recombinant (r)CctA (Antibodies-online.com, PX-P3067-20) were used as positive controls. The proteins were transferred onto polyvinylidene diflouride (PVDF) membranes (Millipore, IPVH10100) using the Trans-Blot SD semidry transfer system (Bio-Rad) at 18 V in Towbin transfer buffer (25 mM Tris, 192 mM Glycine and 20% (v/v) methanol). The membranes were blocked for 30 min using TBST (20 mM Tris, 137 mM NaCl and 0.1% Tween-20 detergent, pH 7.6) with 5% skimmed-milk powder and then probed with rabbit anti-CctA serum [13] diluted 1:1000 in TBST and for 2 h at room temperature with phosphatase labelled goat anti-rabbit (KPL 4751-1516) diluted 1:5000 in TBST. The signals were detected by incubating the blot in fresh alkaline phosphatase substrate buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) containing BCIP (Roche 11585029001) and NBT (Roche 10760994001) staining solutions (Fig. 1). The blots shown in Fig. 2 were performed as stated above except, rCctA-cent was loaded in to one lane spanning a 15% SDS polyacrylaminde gel, the PVDF membrane was cut into equally sized strips and probed with the serum (diluted 1:50 using TBST) collected at day 0 and day 28 post-vaccination for each cow, followed by incubation with a monoclonal anti-bovine IgG–alkaline phosphatase secondary antibody produced in mouse (Sigma-Aldrich, #A7554), diluted 1:3000 using TBST.

2.6. Triton X-114 membrane protein extraction for C. chauvoei

C. chauvoei JF4335 cells were grown as above for 24 h and 1 mL was centrifuged at 10′000×*g* for 30 min at 4 °C, the supernatant and bacterial pellet were treated as described above. After washing with PBS, the bacterial pellet was re-suspended in 900 μL cold sterile TS + buffer (154 mM NaCl, 10 mM Tris, pH 7.4 + cOmplete EDTA Protease inhibitor cocktail (Roche, 11873580001). Triton X-114 fractionation was performed according to Bordier [31].

2.7. Tryptic shaving experiments

JF4335 *C. chauvoei* cells were grown as above for 24 h and centrifuged as above. Four 2 mL samples were prepared from the same culture. The bacterial pellet of sample 1 was re-suspended in cold buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) equal to the volume of the collected supernatant from any given culture. Sample 2 was treated as samples 1, except the cells were washed before their re-suspension. The bacterial pellet of sample 3 was re-suspended in 2 mL of incubation buffer (20 mMTris-HCl, 150 mM NaCl, 10 mM $CaCl_2$ and 0.5 M sucrose) supplemented with 10 μg trypsin re-constituted in 100 μL acetic acid (Promega V5111) and incubated at 37 °C for 2 h. Sample 4 was treated as sample 3, supplemented with 100 μL acetic acid but lacking trypsin. Samples 3 and 4 were centrifuged at 10′000×*g* for 15 min at 4 °C. The supernatants were retained, and the cells were re-suspended in cold buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6). The cells and supernatant samples were mixed 1:1 with 2 × sample buffer and SDS-PAGE followed by immunoblotting was performed as detailed above.

2.8. LC-MS/MS analysis and data interpretation

SDS-PAGE and protein transfer to PVDF membrane of *C. chauvoei* samples was performed as described above. The membrane was thoroughly washed in PBS and the middle section of the membrane was retained at 4 °C. The membrane on either side was used for immunoblotting as described before. The probed membrane and middle clean membrane were aligned, and areas of the non-blotted membrane were excised using a sterile scalpel according to the bands of interest in the immunoblotted membranes. The excised membrane pieces were further cut into six 1 mm^3 pieces and transferred to a micro-centrifuge tube. The membranes were blocked with 100 μL 0.5% PVP-40, reduced with 50 mM DTT/50 mM Tris pH 8.0 and alkylated with 50 mM IAA/50 mM Tris pH 8.0 prior to trypsin (Promega, V511A) digestion for 6 h at 37 °C. The peptides were extracted by adding 10 μL of 20% formic acid and transferred in a new tube.

Each sample was analysed by nano-liquid chromatography tandem mass spectrometry at the Proteomics & Mass Spectrometry Core Facility of the University of Bern using a Fusion LUMOS mass spectrometer (Thermo Fischer, Bremen; Germany) via a Nano spay ESI source, or for a second set of samples on a QExactive Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific) [32] (see Table 1).

2.9. Analysis of CctA haemolytic activity

Recombinant (r)CctA:NusA was produced as described previously [13] or purchased (Antibodies-online.com, PX-P3067-20). The haemolytic activity of rCctA:NusA was determined by 2-fold serial dilution in 0.9% NaCl - 10 mM Tris hydrochloride (pH 7.5) buffer and mixing 100 μL of each dilution with 100 μL of 1% sheep erythrocytes (approximately 7×10^7 erythrocytes/mL) (Oxoid Limited, Thermo Scientific, Basingstoke, Hampshire, England UK; SR0051C). The mixtures were incubated for 2 h at 37 °C and then sedimented by centrifugation at 5′000×*g* for 15 min. The optical adsorption at 540 nm of the supernatants was measured using an ELISA reader. Negative control samples contained equal volumes of buffer and erythrocyte suspensions. One haemolytic unit (HU) is defined as the amount of material that fully lyses a 0.5% sheep erythrocyte suspension under the assay conditions described above.

2.10. Haemolysin neutralization assay

The haemolysin neutralization capacity of bovine and guinea pig serum was measured by pre-incubation of 100 μL of rCctA:NusA titrated to 1 HU/100 μL with 10 μL of two-fold serially diluted bovine serum samples from vaccinated and non-vaccinated animals prior to addition to 100 μL of 1% sheep erythrocytes for 10 min at 37 °C for 10 min. The neutralizing capacity is given by the maximum serum dilution that results in full neutralization of the haemolysis under the given experimental conditions.

3. Results

3.1. Leucocidin CctA is fully secreted by C. chauvoei and not retained in or on the bacterial surface

Blackleg vaccines are based on a mixture of detoxified culture supernatant of *C. chauvoei* and inactivated bacteria but there is no conclusive information concerning the protective antigens and where they reside in this vaccine. In a previous study, we characterised CctA as a major virulence factor of *C. chauvoei* and demonstrated that it is one of the main protective antigens in vaccines against blackleg [13]. However, the exact location of the CctA toxin in liquid culture has never been determined. To this end, we employed a variety of biochemical methods to address the exact location of CctA in liquid culture; cell membrane bound, totally

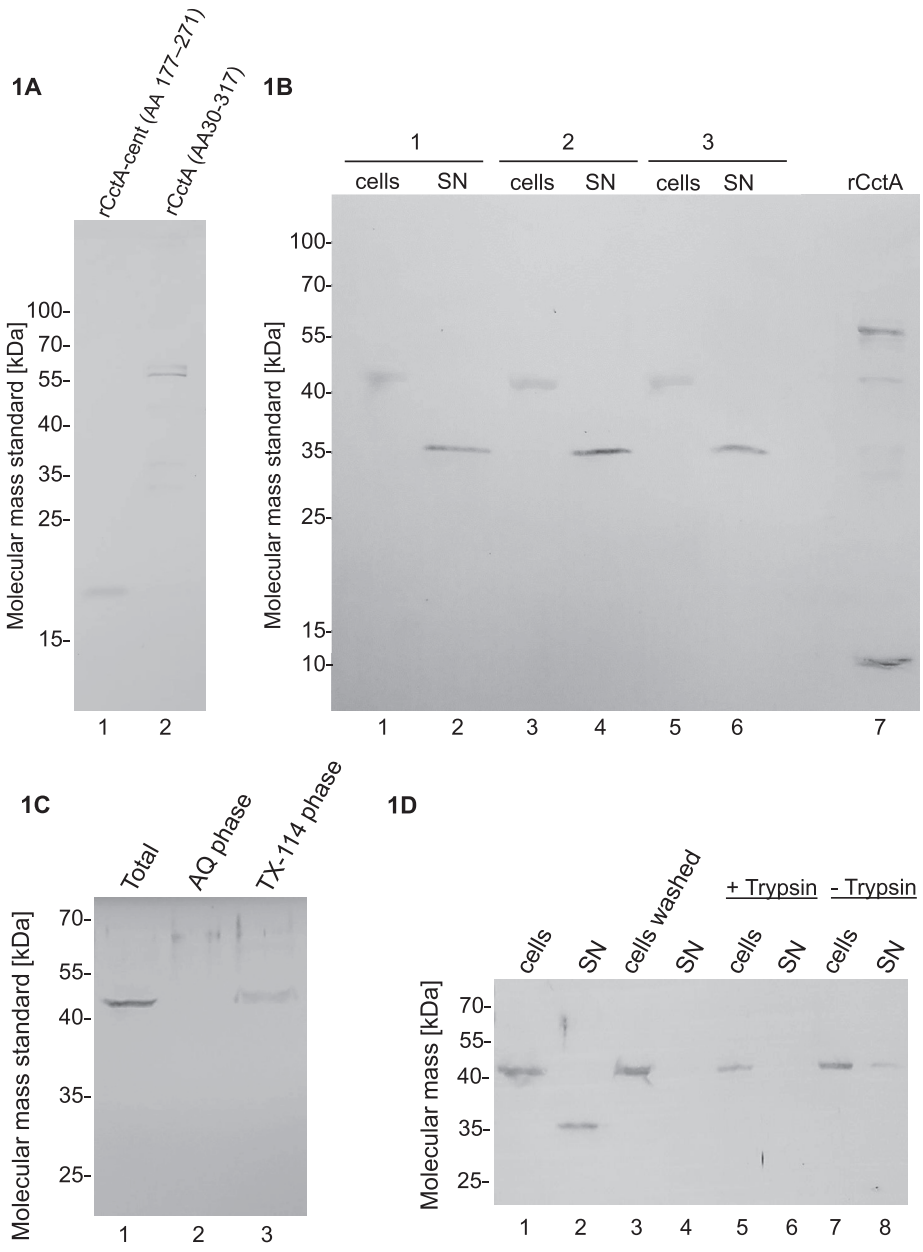

Fig. 1. Immunoblot analysis of recombinant CctA and of antigens from *C. chauvoei* liquid cultures. A. recombinant rCctA-cent representing the central part (AA 117–271) and recombinant mature size NusA-6xHis tagged CctA fusion protein were subjected to SDS-PAGE, followed by immunoblotting with monospecific rabbit anti CctA hyper-immune serum. The expected molecular masses of 18.7 kDa for rCctA (AA177-271) and 59.52 kDa rCctA (AA30-317–NusA-6xHis) were detected in lanes 1 and 2, respectively. B. Three independent *C. chauvoei* cultures (1–3) were separated in to cells (lanes 1, 3 and 5) and supernatant (SN; lanes 2, 4 and 6) by centrifugation. The cells were re-suspended in PBS to the equal volume of the collected supernatant. Each fraction was separated by SDS-PAGE followed by immunoblotting using anti-CctA serum. Recombinant CctA (Antibodies online.com) served as a positive control for the anti-CctA specificity (lane 7). C. Triton X-114 phase partitioning. *C. chauvoei* cells were partitioned into an aqueous fraction (AQ phase; lane 2) containing hydrophilic proteins and a TX-114 detergent fraction (TX phase; lane 3) containing amphiphilic integral membrane proteins. The total cells (lane 1) along with the AQ and TX phases were separated by SDS-PAGE followed by immunoblotting using anti-CctA serum. D. Tryptic shaving of *C. chauvoei* cells. Four 2 mL samples from a culture of *C. chauvoei* culture were used in this experiment. Sample 1 was separated in to cells (lane 1) and supernatant (SN) (lanes 2). Sample 2 was treated as sample 1 except the cells from 2 mL of *C. chauvoei* culture were washed (lanes 3 & 4). Sample 3 was treated as sample 2 except the washed *C. chauvoei* cells were treated with trypsin for 120 min (lane 4) Samples 4 was treated as sample 3 except trypsin was omitted from the incubation buffer (lane 7). Samples 3 and 4 were centrifuged after the incubation and the supernatants were collected (lanes 6 and 8, respectively) All designated samples were subjected to SDS-PAGE followed by immunoblotting using anti-CctA serum.

secreted or a combination of both. First, to demonstrate the specificity of our anti-CctA antibody [13] which provides the basis of our experiments, we loaded the recombinantly generated CctA produced in this study, encompassing the central antigenic domain of CctA-cent (rCctA-cent comprising amino acids (AA) 177–271), with a predicted molecular mass of 18.7 kDa and a commercially purchased recombinant mature CctA (AA29-317) with a stated molecular mass of 59.53 kDa on to a 15% SDS-polyacrylamide gel followed by Western blotting. Immunoblotting analysis shows that the anti-CctA antibody was able to specifically detect both the partial recombinant protein rCctA-cent and the tagged mature rCctA at the correct molecular masses (Fig. 1A). Therefore, independent mid-log *C. chauvoei* bacterial cultures were centrifuged and the bacterial pellet were resuspended in an equal volume of buffer as the volume of collected supernatant. Both the cell lysate and supernatant were subjected to SDS-PAGE followed by

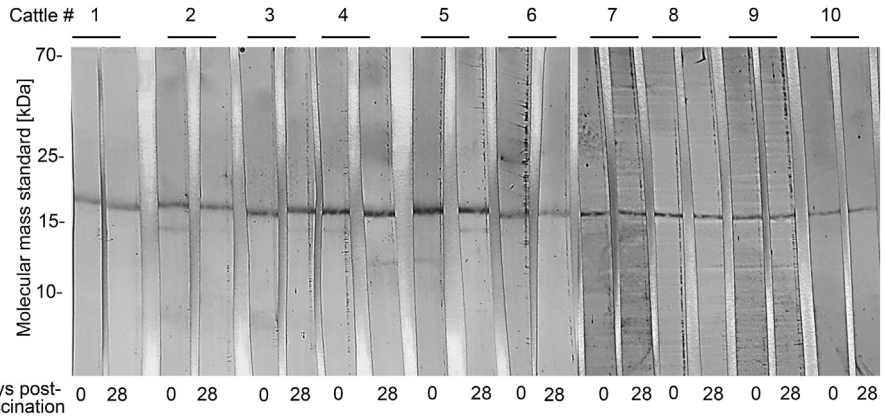

Fig. 2. Immunoblot analysis of cattle serum before and after vaccination with Bovilis® Blackleg. Immunoblots contain purified recombinant CctA-cent antigen (18.7 kDa) and were immunoblotted with serum from cattle taken pre-(0) or 28 days post-vaccination (serum dilution 1:50). Sera from 10 animals are denoted at the top of the blots.

Table 1
Results of mass spectrometry analysis using C. chauvoei cell and supernatant components to identify potential proteins immunodetected using anti-CctA.
Cell fractionation, SDS-PAGE, immunoblotting, mass spectrometry and data acquisition were performed as stated in the materials and methods section 2.8. PMSS is Protein Match Score Summation wherein all scores from peptide spectral matches (PSM) to one particular protein are added up irrespective of the fact if a peptide was identified several times, with or without modifications. PSM defines a potentially correct interpretation of a single fragment spectrum. Coverage is stated in percentage (%) and protein mass is given in Daltons (Da).

	PMSSi	Cover-age (%)	PSM	Pep tides	Protein Mass (Da)	Description
A. Supernatant						
1.	298.39	46.06	51	11	35572.50	Putative CctA
B. Cells						
2.	2617.952	71.03	235	27	43646.7	Elongation factor Tu
3.	2374.507	70.94	194	23	43822.2	Flagellin
4.	518.7743	73.27	50	25	46866.1	Putative Pyrimidine-nucleoside phosphorylase
5.	708.3453	58.31	71	23	49436.9	Putative ABC transporter substrate-binding protein
6.	496.5089	73.4	51	23	42050.2	Phosphoglycerate kinase
7.	308.5164	62.89	31	16	42742.6	Phosphopentomutase
8.	258.9276	48.37	26	15	44860.4	Glucose-1-phosphate adenylyltransferase
9.	148.2079	36.98	17	12	43932.1	3-oxoacyl-[acyl-carrier-protein] synthase 2
10.	171.114	38.74	19	10	45590.9	Putative FAD-dependent pyridine nucleotide-disulfide oxidoreductase
11.	101.7717	39.9	12	11	43733.8	Putative Electron transfer flavoprotein, alpha subunit-
12.	96.14735	22.71	12	9	45088.9	Gamma-glutamyl phosphate reductase
13.	65.68321	27.94	8	7	46089.2	Putative Aminopeptidase II
14.	93.78725	21.65	12	7	45569.4	Putative Dihydroorotate dehydrogenase family protein
15.	75.39014	20.49	9	6	41174.5	Putative Ribosomal protein S1
16.	51.20397	17.75	6	6	43278.5	Probable tRNA sulfurtransferase
17.	56.38388	14.62	7	6	47419.6	M18 family aminopeptidase
18.	40.69621	14.53	4	4	45546.5	Tyrosine–tRNA ligase
19.	44.87383	15.46	5	4	42997.4	Putative NADH-dependent butanol dehydrogenase
20.	27.22858	12.17	3	3	36671.6	Uncharacterized protein
21.	25.59351	8.71	2	2	40994.4	Putative Acyl-CoA dehydrogenase
22.	24.89473	7.95	3	3	47687.0	Histidine–tRNA ligase
23.	22.24853	8.36	3	3	43457.9	Putative UDP-N-acetylglucosamine 2-epimerase
24.	29.65345	6.41	4	3	44337.3	Putative Flavo-diiron protein FprA1
25.	19.29608	9.42	2	2	40714.1	GDP-mannose 4,6-dehydratase
26.	19.12764	6.16	2	2	45913.2	Putative Hypothetical oxidoreductase YeiT
27.	16.68868	9.94	2	2	35732.8	Glyceraldehyde-3-phosphate dehydrogenase
28.	14.13798	11.75	2	2	43535.1	Aspartokinase
29.	16.12659	6.28	2	2	47004.6	Enolase
30.	15.671	5.79	2	2	42530.6	Putative perosamine synthetase

immunoblotting with the anti-CctA. Fig. 1B revealed, at first glance, that CctA appears to be secreted in to the culture supernatant as well as being located on the cells since we detected a signal around 35 kDa in the supernatant corresponding to endogenous mature CctA protein [13] and another signal at 45 kDa in the cell lysate (Fig. 1B).

Triton X-114 partitioning was performed to investigate if this 45 kDa protein detected using anti-CctA is located on the cell membrane. Phase separation of integral membrane proteins in Triton X-114 solution using *C. chauvoei* cells from a mid-log culture followed by resolving the samples on SDS-PAGE and probing with anti-CctA demonstrated that the 45 kDa signal is most likely membrane associated since the signal was observed in the detergent fraction (TX) and not in the aqueous fraction (AQ) (Fig. 1C). This detergent based method of fractionation is limited in that it may be cross-contaminations of the separated phases. Therefore, to overcome this limitation and also to provide insight into the topological organisation of this protein detected by the CctA antibody on the cell surface, we employed tryptic shaving experiments, a technique established by others to examine the bacterial cell

surface proteome [33–36]. Washed *C. chauvoei* cells were incubated with trypsin and we found that the 45 kDa signal was reduced upon trypsin treatment compared to cells incubated using the same experimental conditions, except lacking trypsin (Fig. 1D). Collectively, these results indicate that the protein detected by anti-CctA in *C. chauvoei* cell lysates is cell membrane located and can be shaved from the surface using trypsin.

To conclusively identify this *C. chauvoei* membrane protein, we performed on-membrane tryptic digestion of the proteins corresponding to the anti-CctA detected bands in the cell lysate and supernatant shown in Fig. 1B, followed by mass spectrometry (MS) analysis. This method is preferable to in-gel enzymatic digestion followed by MS where trypsin may have limited accessibility to proteins implanted into the gel and there is a low recovery of hydrophobic digested proteins from SDS-polyacrylamide gels [37]. The MS results showed that the band detected using *C. chauvoei* supernatant samples is unequivocally CctA at a molecular mass of 35.57 kDa and was the only protein identified (Table 1). However, to our surprise, the protein that we observed in the cell lysate fraction likely belongs to *C. chauvoei* flagellum or elongation factor Tu. In fact, over 20 protein were identified using the membrane pieces corresponding to the proteins identified in the cell lysate using anti-CctA. However, elongation factor Tu (EF-Tu) and Flagellin (43.65 and 43.82 kDa, respectively) were by far top of the list in terms of Protein Match Score Summation (PMSSi), coverage and total number of peptides (Table 1). Therefore, taking into account the TX114 experiments in Fig. 1C, the anti-CctA generated signal using *C. chauvoei* cell lysates is most likely is due to a non-specific cross-reaction of the rabbit serum with flagellin. EF-Tu is an abundant protein involved in multiple cellular functions and is found both in the cells and on the cell surface [38] and thus would be in both the TX and AQ phase of such an experiment. Indeed, commercially purchased rabbit serum resulted in the same signal as seen in Fig. 1B using the cell lysate samples (Supplementary Fig. 1). We conclude that CctA is completely secreted into the supernatant of *C. chauvoei* mid-log cultures and does not appear to be retained on or in the cell membrane.

3.2. Serological cross-reactions of CctA

Based upon our knowledge from previous studies and the data above increasing our understanding of CctA, we made it central to establishing a lab-based test for assessing vaccine potency with the ultimate aim of replacing the current guinea pig challenge test. Thus, we sought to develop a suitable *in vitro* immunoassay to measure the antibody response induced by CctA after vaccination against blackleg. In our initial attempt, we generated purified recombinant CctA corresponding to the central amino acids (AA) of CctA (rCctA-cent: see section 2.4 and Fig. 1A) and used this as an antigen to develop an immunoassay to measure specific antibody responses post blackleg vaccination. However, our approach, even after optimisations, to quantitate the induction of specific antibodies towards CctA in cattle vaccinated for the first time with a commercial blackleg vaccine (full cattle, vaccination procedure and exact vaccine details are stated in section 2.1) was unsuccessful. In brief, an indirect ELISA prepared with purified rCctA-cent did not produce specific signals for vaccinated animals since non-vaccinated animals showed high background signal (data not shown). To remedy this, we attempted a variety of peptides based around the central domain of CctA but the specificity was not improved. Immunoblotting with rCctA-cent as an antigen confirmed these findings since sera from vaccinated cattle compared to sera taken pre-vaccination showed virtually identical reactions on immunoblots (Fig. 2). Dilution of the cattle sera from the 10 cows did not improve the specificity, as signals from both the pre and post-vaccination sera reduced uniformly upon dilution. The same results were obtained with rabbit and guinea pig sera that were positive in the CctA ELISA or immunoblots prior to vaccination (data not shown). BLASTp analysis of the amino acid sequence of rCctA revealed similarity of the peptide with proteins of several Gram-positive bacteria, in particular: the β-channel forming cytolysin of *Clostridium septicum* (77% AA identity), the α-haemolysin of *Clostridium botulinum* (65% identical aa) and the β-barrel channel forming cytolysin NetE of *Clostridium perfringens* (56% AA identity). Hence, such widespread antigens belonging to the β-barrel channel forming protein family [12] may be responsible for the observed cross-reactions of sera from non-vaccinated and non-infected cattle.

3.3. Vaccinated cattle and guinea pigs induce neutralizing antibodies against the haemolytic activity of r-CctA

C. chauvoei cultivated on blood agar medium shows a very strong haemolytic halo (Fig. 3) and this has been shown to be due to CctA [13]. Using this simple observation, we tested if our anti-CctA antibody would be able to neutralize the strong haemolysis due to secreted CctA. Thus, *C. chauvoei* was spread on to blood agar and anti-CctA, anti-rabbit serum, as a control since anti-CctA was produced in rabbit and a PBS control to ensure aseptic technique, were dropped in equal volumes onto defined areas of the blood agar before incubation (Fig. 3). Compared to PBS and anti-rabbit serum, anti-CctA was able to completely neutralize the activity of CctA and thus no haemolysis was observed in the defined anti-CctA droplet areas (Fig. 3). In fact, we observed that the addition of anti-CctA seemed to inhibit growth of the bacteria indicating that substances of lysed erythrocytes are required by *C. chauvoei* for efficient growth on this medium. Therefore, given this clear result and the observed problematic serological cross reactions associated with the ELISA assay, we decided to establish a haemolysis-neutralizing assay. The assay that we developed is based on haemolytically active rCctA:NusA (see section 2.4 for details) [13]. In this assay (explained in sections 2.9-2-10), sera of all cattle and guinea pigs taken prior to vaccination revealed virtually no haemolysin-neutralization activity. Two weeks post-vaccination, the neutralizing titres started to rise in all cattle and at 4 weeks post-vaccination they had reached titres between 1000 and 2000 in all 10 animals vaccinated (Fig. 4). On average, vaccination of cattle with a commercial vaccine resulted in neutralization titres of 1000 four weeks post-vaccination (Fig. 5A). Similarly, sera from guinea pigs taken at 14 days post-vaccination scored neutralization titres of 600 (Fig. 5B). These results demonstrate the high specificity of this *in vitro* assay for the determination of neutralizing seroconversion to CctA in both target animals and experimental animals used for standard blackleg vaccine potency tests.

4. Discussion

Vaccines against *C. chauvoei* have been used for many decades and continue to be a successful preventive measure against blackleg. However, there is relatively little knowledge regarding the identity of the protective antigens, where they reside, and crucially, how to measure these antigens conferring protection to the vaccination as an assessment of vaccine potency. CctA was shown to be the main virulence factor of *C. chauvoei* and recombinant CctA used as a vaccine showed protective immunity in the standard guinea pig challenge potency assay which is used internationally for blackleg vaccine batch release [27]. To extend our characterisation of CctA, we analysed anaerobic liquid culture of *C. chauvoei*, equally split into culture supernatants and sedimented cells after exponential growth of *C. chauvoei* in axenic medium. We found that

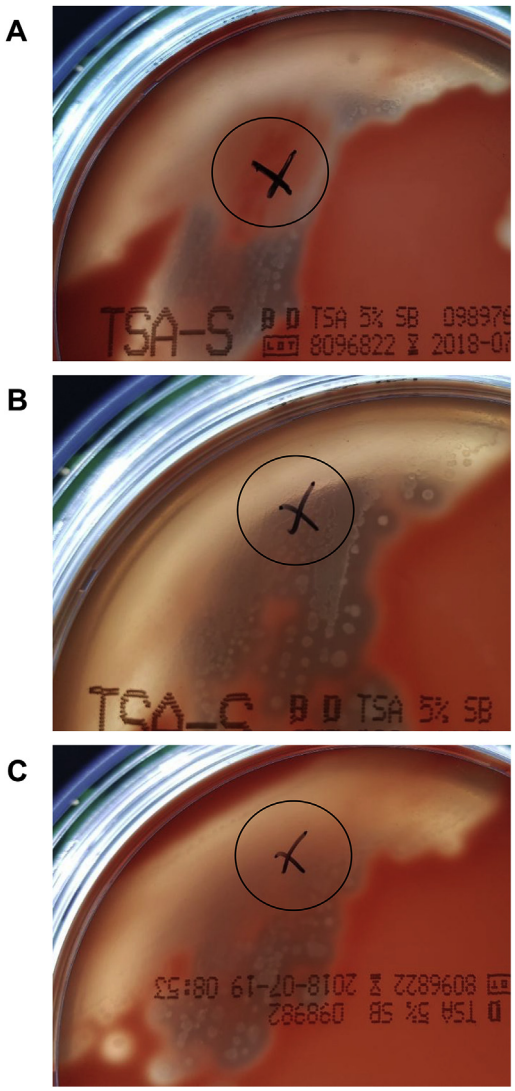

Fig. 3. Characteristic haemolytic halo around *C. chauvoei* colonies due to secreted CctA is neutralized by anti-CctA. A 10 μL inoculation loop of frozen JF4335 *C. chauvoei* stock was spread on to Tryptic soy agar supplemented with 5% sheep blood. Concurrently, 10 μL of either anti-CctA serum, anti-rabbit serum or PBS was carefully dispensed at the designated circled points (X) prior to incubation.

CctA was fully secreted and that no CctA appeared to be retained by the bacterium (Fig. 1B). Indeed, the only band observed in the supernatant by immunoblotting with anti-CctA was confirmed to be CctA by mass spectrometry (Table 1). This is in full correlation with the strong and large haemolytic halo caused by the haemolytic-toxic activity of CctA that is typically found around the tiny colonies of *C. chauvoei* on standard blood agar solid medium, which is used in common bacterial diagnostics (Fig. 3). It can be speculated that a rapid and thorough secretion of the β-barrel channel forming CctA toxin which has a very strong pore forming activity [3] is necessary, otherwise it would interact with the membrane of the growing bacterium and potentially interfere with growth of the organism.

An antigen that was initially thought to represent cell-bound CctA, based on our immuno-blotting experiments using the cell fraction (Fig. 1B–D) was clearly identified as a membrane component of *C. chauvoei* and according to our mass spectrometry results, it is most likely flagellin or EF-Tu (Table 1). Both of these proteins are inherent antigens of many commensal as well as pathogenic bacteria which due to their conserved structures reveal strong antigenic cross-reactions across species level. This was further supported by the fact that immunoblotting using standard rabbit serum also resulted in the same 43 kDa antigen of *C. chauvoei* cells (Supplementary Fig. 1). The data presented here is in line with a recent study from Jayaramaiah and colleagues who investigated *C. chauvoei* cell surface associated proteins using various proteomics techniques [39]. They did not find CctA to be cell surface bound and although we did not have the same widespread proteomics approach, we found many of the same proteins in the range of 35–50 kDa such as flagellin, Electron transfer flavoprotein components, ribosomal proteins and Enolase (Table 1). A deviation from their study is that we clearly identified cell associated EF-Tu (Table 1) but this interesting finding correlates very well with the multitude of reports showing EF-Tu to be at the cell surface of many Gram-negative and -positive pathogenic bacteria where it appears to have various moonlighting activities such as involvement in host cell binding [34,40–48]. Some years ago, EF-Tu was identified as being an antigenic *C. perfringes* protein which can be used as a target antigen to detect serum antibodies in chicken with or without clostridial infections [48]. In fact, most of the cell surface proteins that we identified correlate very well to the major functional groups of proteins that have been reported in recent years as moonlighting proteins in pathogenic bacteria [38].

In our first attempt to assess the induction of CctA specific antibodies in vaccinated cattle, we produced and purified the antigenic 18.7 kDa central domain of CctA as a recombinant peptide by standard recombinant gene technology in *E. coli*. This rCctA-cent peptide showed strong serological cross reactions with serum from non-vaccinated cattle both in an ELISA assay and on immunoblots (Fig. 2). Hence, this approach cannot be used for detection of specific anti-CctA antibodies. Therefore, based on the simple observations in Fig. 3, we developed a serum haemolysin-neutralization assay using ovine erythrocytes. In this assay, serum from all cattle showed specific post-vaccination neutralizing activity reaching high levels of approximately 1000 times above background in samples taken at four weeks post-vaccination, while no neutralization was measured in the pre-vaccination or immediate post-vaccination samples (Figs. 4 and 5). Results from a previous study using serum of guinea pigs vaccinated with recombinant CctA:LTB (LTB was fused to CctA as a biological adjuvant) neutralized the cytotoxic activity at a dilution of 1:128 [13]. The haemolysin-neutralizing titres obtained with serum of cattle vaccinated with a commercial vaccine in this study were approximately 8 times higher than observed in the previous experiment (Fig. 5A). Hence, vaccination of cattle with a commercial blackleg vaccine leads to a considerable haemolysin-neutralizing activity in cattle sera four weeks post-vaccination. Furthermore, guinea pigs that were vaccinated with blackleg vaccine and survived the subsequent *C. chauvoei* challenge, successfully passing the official potency test, also show seroconvert to rCctA neutralizing serum 14 days post-vaccination. Collectively, our results show that the determination of the of haemolysin-neutralizing levels in vaccinated animals can be used to assess the potency of blackleg vaccines which would eliminate the need for experimental infections of laboratory animals. The assay developed here is based on recombinant CctA and will be universally applicable since CctA is fully conserved in *C. chauvoei* [5].

5. Conclusions

CctA, a major protective antigen of *C. chauvoei*, is exclusively found in culture supernatants. This is an important finding for optimizing production procedures of future blackleg vaccines. In our

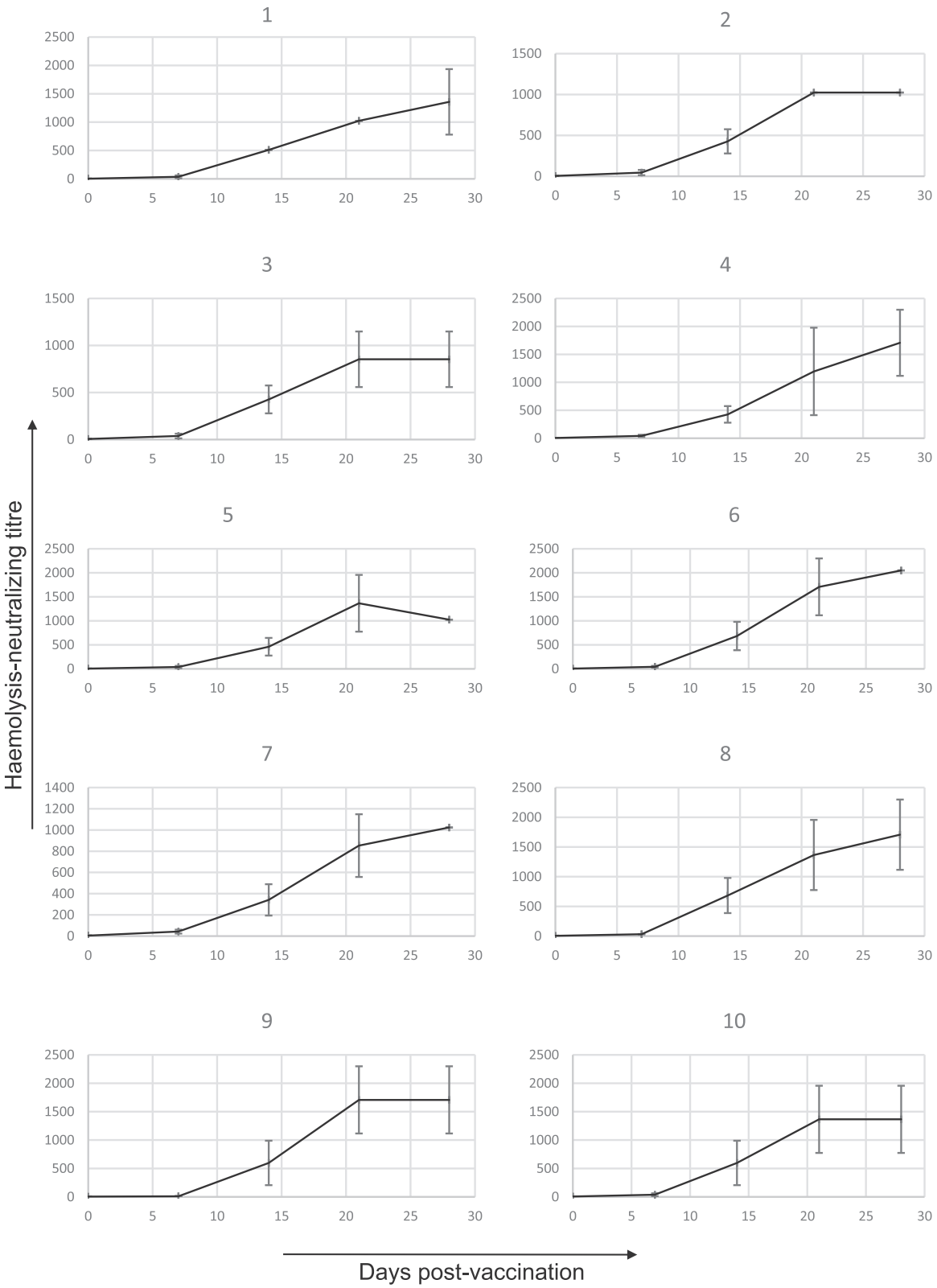

Fig. 4. CctA haemolysis-neutralizing activity of sera from cattle vaccinated with Bovilis® Blackleg. Data shown are the mean values of 3 independent assays and the standard deviations are represented by the error bars. The numbers on the x-axis denote the days post-vaccination, while the numbers on y-axis correspond to the haemolysis-neutralizing titres. The numbers at the top of each graph refers to the cow.

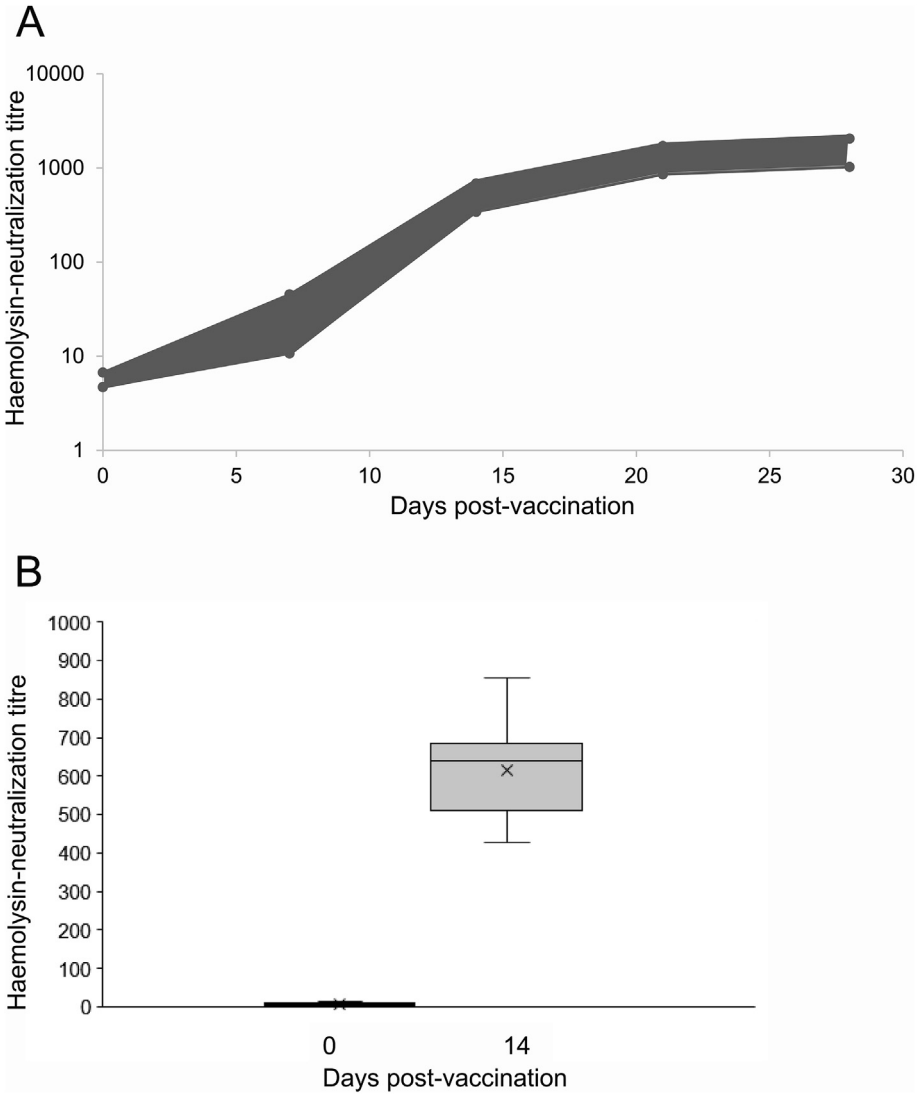

Fig. 5. Average haemolysis-neutralizing activity of sera from 10 vaccinated cows and guinea pigs. A) Range plot of the CctA haemolysis-neutralizing activity titres of all sera from all 10 vaccinated cows represented on a semi-logarithmic diagram. B) Box plot of haemolysis-neutralizing titres of sera from 10 guinea pigs vaccinated during an official governmental blackleg vaccine control potency test. All individual neutralizing titres were determined by 3 independent measurements and the standard deviations are represented by the error bars.

quest to elucidate the location of CctA we also identified EF-Tu, a protein that has been shown to be cell surface located with multiple virulence associated activities in a plethora of other bacteria, including the closely related *C. perfringes*. Thus, EF-Tu expression on *C. chauvoei* cell surface warrants further study since it may be an additional potential target for neutralizing antibodies, cytotoxic leukocytes or as a subunit vaccination for blackleg, in combination with CctA. In addition, we used recombinant CctA to develop a potential replacement test for the standard guinea pig challenge potency assay used for blackleg vaccine batch releases. This novel assay would remove the current requirement of animal testing.

Author contributions

JFr designed the study and performed haemolysin neutralization tests, PN did the immunological and functional analyses. JFr and PN wrote the manuscript. JFu and CS performed cloning and expression of CctA proteins and peptides, MHä performed the cattle vaccination and MHe and SBL performed mass the spectrometry analyses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2019.02.011.

Conflict of interest disclosure

The authors have no conflicts of interest to declare.

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