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# Sheep immune response against a novel recombinant enterotoxemia and infectious necrotic hepatitis vaccine in Türkiye

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## Abstract

**Background** Clostridial diseases are common in ruminants all over the world. This study investigated the efficacy of a novel recombinant vaccine developed against enterotoxemia and infectious necrotic hepatitis in sheep.

**Results** Plasmids carrying the *Clostridium novyi* type B alpha toxin gene (*CnBtcn-alpha*), *Clostridium perfringens* type C beta toxin gene (*CpCcpb*) and *C. perfringens* type D epsilon toxin gene (*CpDetx*) were constructed and the plasmids were transferred to *Escherichia coli*. Unpurified protein obtained from *E. coli* cell lysate supernatant was used to prepare the recombinant vaccine. The vaccine was prepared in three different formulas (RV100, RV200 and RV400), with 100, 200 and 400 µg of each protein in one vaccine dose. RV400 was preferred to immunise sheep as the antitoxin titres in the pooled blood serum of rabbits administered with this vaccine were determined to be above the minimum values specified in European Pharmacopoeia (10 IU/mL for beta, 5 IU/mL for epsilon, and 3.5 IU/mL for alpha). A total of 24 Akkaraman breed sheep with no antibodies against the relevant toxins were used for the experiment. All the animals in three groups (recombinant vaccine group, commercial polyvalent vaccine group and negative control group), each consisting of eight sheep, were vaccinated twice with an interval of 21 days and, the antitoxin titres were measured 14 days after the second vaccination by the mouse toxin neutralization test. The average antitoxin titres in sheep immunised with RV400 were calculated as 9.75 ± 1.28 IU/mL for *C. perfringens* beta, 13.75 ± 1.98 IU/mL for *C. perfringens* epsilon and 5.50 ± 0.93 IU/mL for *C. novyi* alpha toxins. On the other hand, the average values in sheep immunised with commercial vaccine were detected as 8.00 ± 2.14 IU/mL, 4.25 ± 1.67 IU/m L and 6.50 ± 0.93 IU/mL for *C. perfringens* beta, *C. perfringens* epsilon and *C. novyi* alpha, respectively. No antitoxin titre was detected in sheep in the negative control group (PBS). A statistically significant difference was observed between the recombinant and commercial vaccine groups in terms of *C. perfringens* epsilon antitoxin titres ($P = 0.0002$).

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**Conclusions** The present study was the first to investigate the efficacy of a combined recombinant vaccine prepared from unpurified proteins against enterotoxemia and infectious necrotic hepatitis and, the results suggested that it shows promise for protecting sheep against these diseases.

**Keywords** Enterotoxemia, Infectious necrotic hepatitis, Immune response, Recombinant vaccine, Sheep

## Background

Clostridial diseases are common in ruminants worldwide and cause high mortality rates [1]. Enterotoxemia is a disease characterised by toxaemia in sheep and lambs, but also in goats, cattle, pigs and horses [2]. Various types of *Clostridium perfringens* are involved in the aetiology of the disease. *C. perfringens* is divided into seven toxin types (A-G) according to the production of alpha, beta, epsilon, iota, enterotoxin and NetB toxins [3]. Type B causes lamb dysentery; type C causes enterotoxemia (struck) in lambs, sheep, goats, cattle, pigs and horses; type D causes enterotoxemia in sheep, goats and rarely in cattle. Enterotoxaemia caused by *C. perfringens* type D is also known overeating disease or pulpy kidney disease [2, 4]. Beta and epsilon toxins, which are the main virulence factors of *C. perfringens* types B, C and D, have lethal and necrotic effects [5]. Infectious necrotic hepatitis caused by *C. novyi* type B is an acute, toxaemic disease characterised by liver necrosis. Although the disease is usually seen in sheep, it can also be seen in cattle, pigs and horses [6, 7]. *C. novyi* type B produces alpha, beta and zeta toxins [8, 9]. Alpha toxin plays an active role in infectious necrotic hepatitis [10].

Conventional vaccines against clostridial infections in animals have been used for many years. However, recombinant vaccines using purified or unpurified recombinant proteins have recently been developed as an alternative to conventional vaccines [11–18]. It is reported that recombinant vaccines have some advantages such as little or no toxic effects, good immunogenicity, use of non-pathogenic strains and easy production on a large scale [1, 16, 19]. In order for recombinant vaccines to be widely used in veterinary medicine, their cost and effectiveness should be advantageous compared to conventional vaccines. The use of purified recombinant proteins in large-scale vaccine preparation increases the cost of vaccines. It has been reported that vaccines developed using unpurified recombinant proteins against botulism disease in animals produce higher antibody titres than conventional vaccines in previous studies [12, 13, 15]. To our knowledge, no studies are available investigating the efficacy of a combined recombinant vaccine using unpurified recombinant proteins against enterotoxemia or infectious necrotic hepatitis. This study was therefore carried out to investigate the effectiveness of a novel recombinant vaccine developed against enterotoxemia and infectious necrotic hepatitis in sheep.

## Results

### Expression and purification of recombinant protein

*C. perfringens* type C beta toxin gene (*CpCcpb*), *C. perfringens* type D epsilon toxin gene (*CpDetx*) and *C. novyi* type B alpha toxin gene (*CnBtcn-alpha*) cloned into the bacterial expression vector pET-28a (+) were cloned in *Escherichia coli (E. coli)* C43 (DE3) strain and confirmed with restriction endonucleases and primers (Fig. 1). Additionally, plasmids containing toxin genes were confirmed by sequence analysis. Expression of recombinant proteins was visualized by SDS-PAGE method using soluble and insoluble fractions of *E. coli* culture (Fig. 2). Recombinant strains were purified by nickel-nitrilotriacetic acid (Ni-NTA) spin kit (Qiagen - Germany, Cat. Number: 31314) (Fig. 3).

### Sterility and safety tests of recombinant vaccines

No microorganism growth was detected in the sterility test of the prepared recombinant vaccines during the 21-day period. In the safety test, no local or systemic reactions or deaths were observed.

### Detection of antitoxin levels in vaccinated rabbits and sheep

Antitoxin titres detected for *C. perfringens* beta, *C. perfringens* epsilon and *C. novyi* alpha in the pooled blood sera of rabbits immunised with the recombinant vaccines (RV100, RV200 and RV400) prepared using unpurified protein obtained from *E. coli* cell lysate supernatant and a commercial vaccine were presented in Table 1. According to the European pharmacopoeia [20], antitoxin titres should not be less than 10 IU/mL, 5 IU/mL and 3.5 IU/mL for *C. perfringens* beta, *C. perfringens* epsilon and *C. novyi* alpha, respectively. 100 µg of all toxin proteins and 200 µg of *C. perfringens* beta and *C. novyi* alpha toxin proteins produced antitoxin titres lower than the minimum values specified in the European Pharmacopoeia. On the other hand, 400 µg of all toxin proteins and 200 µg of *C. perfringens* epsilon toxin protein produced adequate immune responses. No antitoxin titre was detected in rabbits in the negative control group (data not shown).

The mean antitoxin titres detected against *C. perfringens* beta, *C. perfringens* epsilon and *C. novyi* alpha toxins in sheep vaccinated with RV400 were 9.75 ± 1.28 IU/mL, 13.75 ± 1.98 IU/mL and 5.50 ± 0.93 IU/mL, respectively. On the other hand, the mean antitoxin titres detected against *C. perfringens* beta, *C. perfringens* epsilon and *C. novyi* alpha toxins in sheep vaccinated with

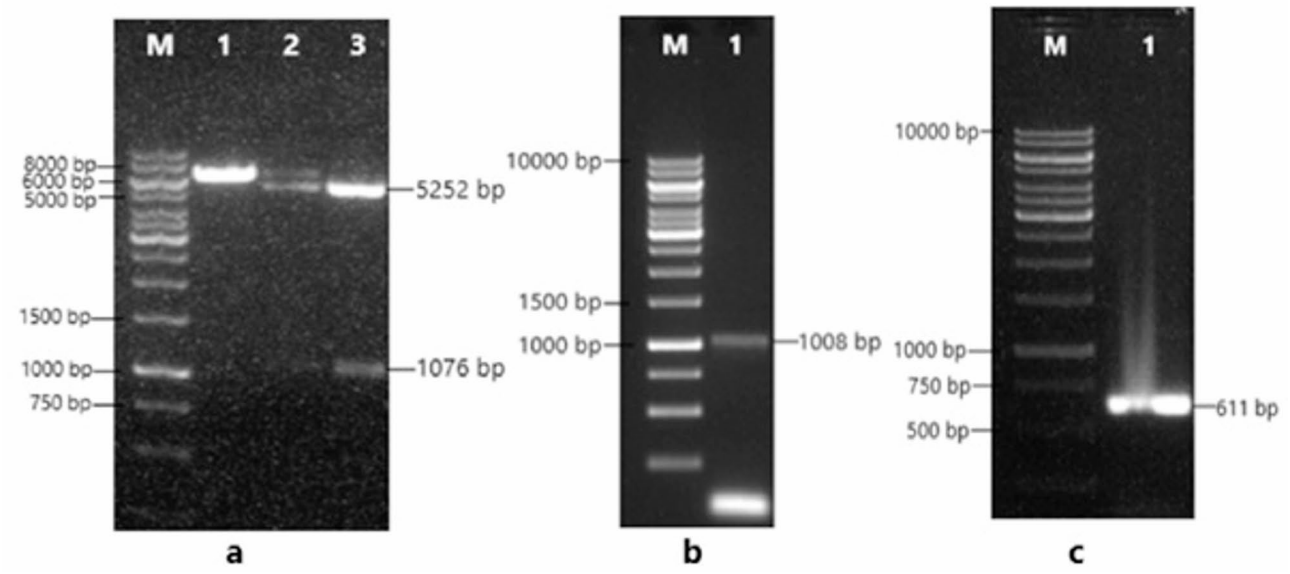


**Fig. 1** (**a**-**c**) Confirmation of plasmids containing toxin genes, analyzed on agarose gel 1%. (**a**) Lane M: DNA size marker; lane 1: negative colony; lane 2: negative colony; lane 3: pET-28a (+)-CpDetx plasmid digested by MluI and HindIII restriction enzymes. (**b**) Lane M: DNA size marker; lane 1: pET-28a (+)-CpCcpb plasmid confirmation by PCR primers. (**c**) Lane M: DNA size marker; lane 1: pET-28a (+)-CnBtcn-alpha plasmid confirmation by PCR primers

commercial vaccine were 8.00 ± 2.14 IU/mL, 4.25 ± 1.67 IU/mL and 6.50 ± 0.93 IU/mL, respectively (Table 2). No antitoxin titre was detected in sheep in the negative control group (data not shown). The difference between alpha ($P = 0.1436$) and beta ($P = 0.1251$) antitoxin titres in the recombinant and commercial vaccine groups was not statistically significant, but the difference between epsilon antitoxin titres was significant ($P = 0.0002$) (Fig. 4).

## Discussion

Clostridial infections cause significant economic losses in animal husbandry all over the world [2]. The most effective way to protect animals from clostridial infections is vaccination. Commercial vaccines used to protect animals against diseases caused by clostridial agents such as *C. botulinum*, *C. tetani*, *C. perfringens*, *C. sordellii*, *C. chauvoei*, *C. septicum* and *C. novyi* are produced by conventional methods [7, 21]. Although existing commercial vaccines create an adequate immune response, recombinant vaccines have been reported to be more advantageous in terms of cost and easy production [1, 16, 19]. In the present study, combined recombinant vaccines were prepared for the first time by using different concentrations (100, 200 and 400 µg) of unpurified protein obtained from *E. coli* cell lysate supernatant against enterotoxemia and infectious necrotic hepatitis diseases and, the level of humoral immune response in vaccinated sheep was measured.

According to the European Pharmacopoeia, potency testing of *C. perfringens* and *C. novyi* vaccines to be used for immunisation of target animals should be performed using pooled sera of rabbits. In the potency testing of rabbits, 100 µg of all three recombinant proteins and 200 µg of *C. novyi* alpha and *C. perfringens* beta toxin proteins did not produce an adequate immune response. However, the titres obtained in rabbits immunised with RV400 for *C. novyi* alpha (8 IU/mL) and *C. perfringens* beta (12 IU/mL) antitoxins were above the minimum values specified in the European Pharmacopoeia. On the other hand, antitoxin titres of 8 IU/mL and 20 IU/mL were detected for *C. perfringens* epsilon protein in pooled blood sera of rabbits immunised with RV200 and RV400, respectively. These values were above the minimum value (5 IU/mL) specified for epsilon antitoxin in the European Pharmacopoeia. Antitoxin titres against *C. perfringens* beta, *C. perfringens* epsilon and *C. novyi* alpha toxins in pooled blood sera of rabbits vaccinated with commercial vaccine were determined as 10 IU/mL, 6 IU/mL and 10 IU/mL, respectively. Since antitoxin was produced at a protective level against all of the related toxins in rabbits immunised with RV400, this vaccine formula was used to immunise sheep.

In this study, RV400 vaccine caused lower levels of neutralising antibodies in sheep when compared with rabbits. Similar to our findings, previous studies reported that conventional and recombinant clostridial vaccines produced higher antitoxin titres in rabbits than in ruminant species [22, 23]. In a study by Salvarini et al. [24], it was reported that the bivalent vaccine prepared using recombinant alpha and beta toxin proteins of *C. perfringens* produced 9.6 IU/mL and 20.4 IU/mL neutralising antibodies against alpha and beta toxins in rabbits and 6 IU/mL and 14.5 IU/mL in pigs, respectively. Recombinant botulism vaccines have been reported to produce 10

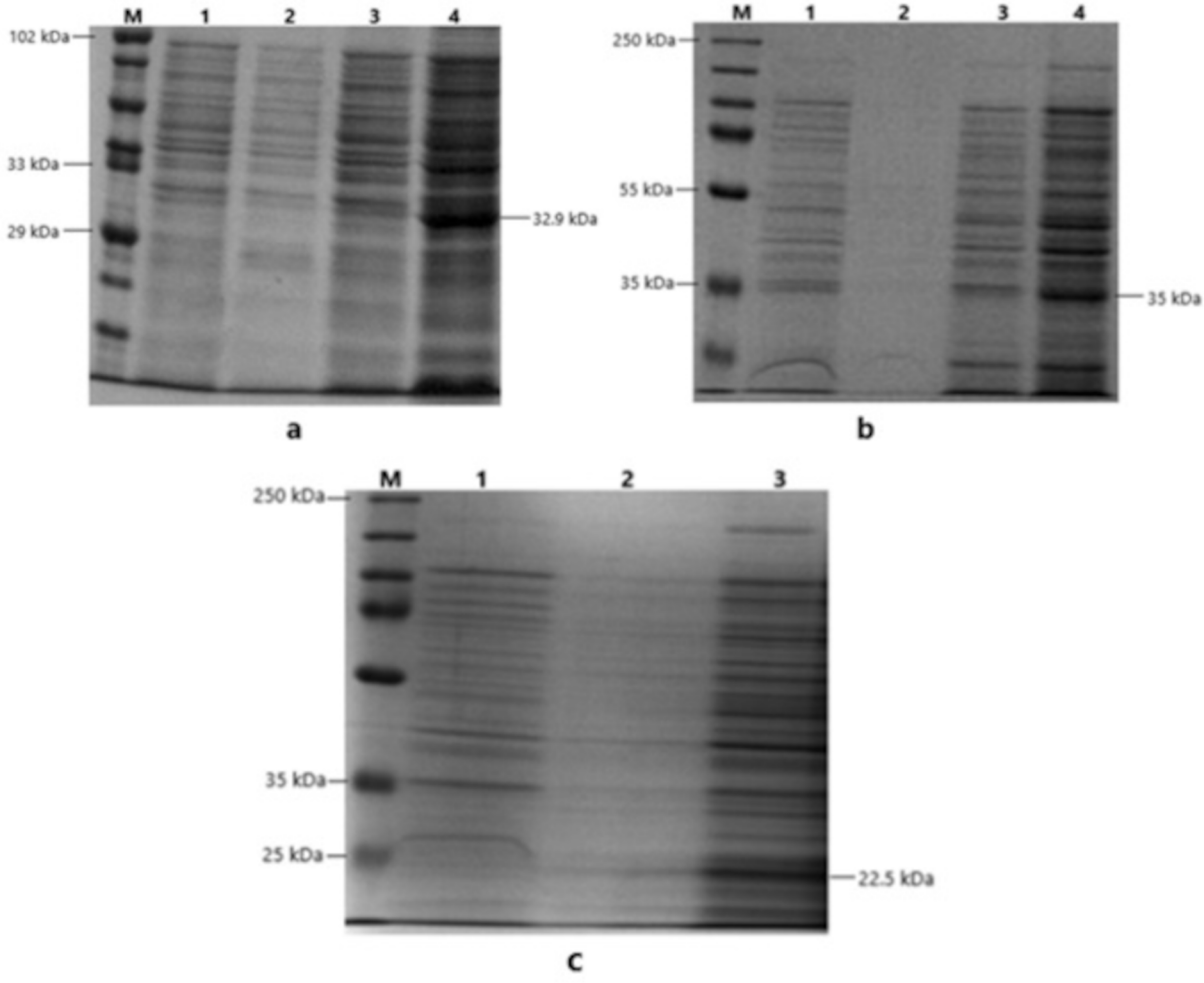


**Fig. 2** (**a**-**c**) Expression of plasmids containing toxins stained with coomassie blue on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10%. (**a**) Lane M: protein size marker; lane 1: non- transformed *E. coli* C43(DE3) strain; lane 2: water soluble components of the pET-28a (+)-CpDetx plasmid; lane 3: 8 M urea-soluble protein components of the pCpDetx plasmid constructed into the pVC-UGT plasmid containing the *CpDetx* toxin gene; lane 4: 8 M urea-soluble protein components of the pET-28a (+)-CpDetx. (**b**) Lane M: protein size marker; lane 1: non-transformed *E. coli* C43(DE3) strain; lane 2: water soluble components of the pET-28a (+)-CpCcpb plasmid; lane 3: 8 M urea-soluble protein components of the pCpCcpb plasmid constructed into the pVC-UGT plasmid containing the *CpCcpb* toxin gene; lane 4: 8 M urea-soluble protein components of the pET-28a (+)-CpCcpb plasmid. **c**) Lane M: protein size marker; lane C43(DE3): non-transformed *E. coli* C43(DE3) strain; lane 1: water soluble components of the pET-28a (+)-CnBtcn-alpha plasmid; lane 2: 8 M urea-soluble protein components of the pET-28a (+)-CnBtcn-alpha plasmid. The original gels are presented in additional file 1. The cropped areas of gel images were marked with a red box line

IU/mL neutralising antibodies against *C. botulinum* type D in guinea pigs and 6.14 IU/mL in cattle [25, 26]. On the other hand, Chadran et al. [27] reported that the combined vaccine prepared using recombinant epsilon toxin protein of *C. perfringens* and attenuated sheep smallpox virus produced approximately the same level (8 IU/mL) of epsilon antitoxin titre in rabbits and sheep. It should be underlined that immunological and body weight differences between animal species might be responsible for the differences in antitoxin titres [15]. Interestingly, recombinant epsilon protein induced a stronger immune response than other proteins, both in rabbits and sheep. Similarly, previous studies [18, 22] have also reported that the recombinant vaccine induced higher levels of neutralising antibodies against epsilon toxin than beta toxin in cattle. A detailed investigation is needed to understand why the epsilon toxin protein reveals a stronger immune response.

In previous studies, clostridial vaccines were prepared using unpurified [12–16, 28] and purified recombinant proteins [11, 18, 24, 29, 30]. Purification of recombinant proteins is carried out by chromatography, which is a cost-increasing factor in large-scale vaccine production. Therefore, the use of purified recombinant proteins for vaccine production has not been found plausible in veterinary medicine [31, 32]. The vaccine prepared from

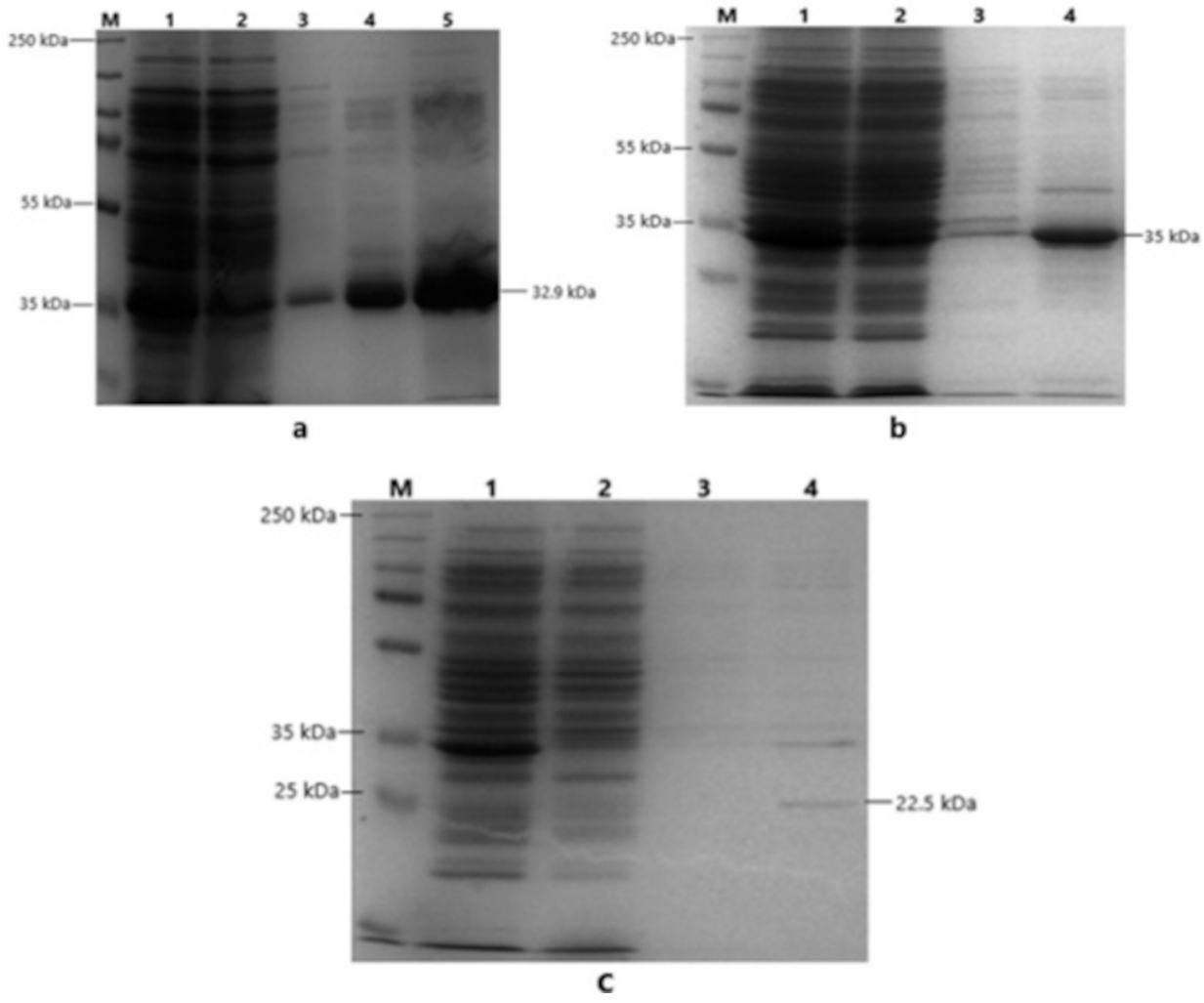


**Fig. 3** (**a**-**c**) Ni-NTA purification of toxins with specific anti-His tag. (**a**) Lane M: protein size marker; lane 1: Filtrate product of the pET-28a (+)-CpDetx in *E. coli* C43(DE3) strain after digestion with Ni-NTA purification kit lysis buffer; lane 2: Filtrate product of the pET-28a (+)-CpDetx in *E. coli* C43(DE3) strain after the first precipitation through the column with the Ni-NTA purification kit; lane 3: Filtrate product of the pET-28a (+)-CpDetx in *E. coli* C43(DE3) strain after column washing with Ni-NTA purification kit; lane 4: Purified 1st eluate of the pET-28a (+)-CpDetx in *E. coli* C43(DE3) strain with Ni-NTA purification kit; lane 5: Purified second eluate of the pET-28a (+)-CpDetx in *E. coli* C43(DE3) strain with Ni-NTA purification kit. (**b**) Lane M: protein size marker; lane 1: Filtrate product of the pET-28a (+)-CpCcpb in *E. coli* C43(DE3) strain after digestion with Ni-NTA purification kit lysis buffer; lane 2: Filtrate product of the pET-28a (+)-CpCcpb in *E. coli* C43(DE3) strain after the first precipitation through the column with the Ni-NTA purification kit; lane 3: Filtrate product of the pET-28a (+)-CpCcpb in *E. coli* C43(DE3) strain after column washing with Ni-NTA purification kit; lane 4: Purified first eluate of the pET-28a (+)-CpCcpb in *E. coli* C43(DE3) strain with Ni-NTA purification kit. (**c**) Lane M: protein size marker; lane 1: Filtrate product of the pET-28a (+)-CnBtcn-alpha in *E. coli* C43(DE3) strain after digestion with Ni-NTA purification kit lysis buffer; lane 2: Filtrate product of the pET-28a (+)-CnBtcn-alpha in *E. coli* C43(DE3) strain after the first precipitation through the column with the Ni-NTA purification kit; lane 3: Filtrate product of the pET-28a (+)-CnBtcn-alpha in *E. coli* C43(DE3) strain after column washing with Ni-NTA purification kit; lane 4: Purified first eluate of the pET-28a (+)-CnBtcn-alpha in *E. coli* C43(DE3) strain with Ni-NTA purification kit

unpurified recombinant proteins was observed to produce higher beta and epsilon antitoxin titres in sheep than the commercial vaccine employed in the present study. This may be explained by the fact that recombinant vaccine contains more specific proteins that stimulate the immune system with less antigenic competition. On the other hand, conventional polyvalent vaccines contain a large number of structurally related antigens from different microorganism species which may lead to more antigenic competition [33]. Consistent with our findings, previous studies have also reported that recombinant toxin vaccines developed against clostridial infections in animals produce higher antibody titres than conventional vaccines [12–16, 18, 22, 29].

One of the striking results of the study was that RV400 vaccine induced a lower immune response against *C.*

**Table 1** Results of potency testing in rabbits

| Vaccine | Antitoxin titres (IU/mL) | | |
|---|---|---|---|
| | C. *perfringens* beta | C. *perfringens* epsilon | C. *novyi* alpha |
| RV100[a] | 2 | 4 | 2 |
| RV200[b] | 5 | 8 | 3 |
| RV400[c] | 12 | 20 | 8 |
| Commercial vaccine | 10 | 6 | 10 |

[a]RV100: Recombinant vaccine 100 µg; [b]RV200: Recombinant vaccine 200 µg; [c]RV400: Recombinant vaccine 400 µg.

*novyi* alpha toxin when compared with the commercial vaccine. This is thought to be due to the transfer of a partial of the alpha toxin gene to the plasmid vector instead of the whole gene. Although the beta (1011 bp) and epsilon (987 bp) toxin genes of *C. perfringens* were successfully transferred to plasmids constructed in our laboratory, the *C. novyi* alpha toxin gene (6576 bp) could not be transferred to the plasmid vector as a whole probably due to the size of the gene. It has been reported that the genetic material of *Clostridium* species is difficult to easily transfer into cells using conventional plasmid vectors and electroporation techniques [34]. The entire *C. novyi* alpha toxin gene has not been cloned so far. However, a part of the gene encoding highly epitopic regions of alpha toxin has been cloned before and, alpha toxin-specific antibodies have been produced in mice and rabbits immunised with the recombinant proteins obtained [35, 36]. Alpha toxin of *C. novyi* consists of 2178 amino acids. The N-terminal part of the toxin contains approximately 550 aminosides and this part shows enzymatic activity. The C-terminal part has antigenic activity and is the part where the toxin binds to receptors [10]. In the present study, a 570 bp long nucleotide sequence encoding a large number of epitope regions in the C-terminal part of alpha toxin was transferred to plasmid and the recombinant proteins obtained produced immune response against alpha toxin, though not at a desired level.

It is reasonable that the level of immune response increases as the amount of proteins used in the production of recombinant vaccines increases. In the present study, although desirable results were obtained with 400 µg of all the recombinant proteins, adequate immune responses were not produced by the administration of 100 µg of the all recombinant proteins and 200 µg of *C. perfringens* beta and *C. novyi* alpha toxin proteins. Most of the previous studies testing various amounts of proteins in recombinant vaccines prepared for *C. perfringens* toxins in different animal species have generally reported that sufficient protection was observed with the amount of 200 µg and above [18, 22, 24, 37]. However, an amount of 100 µg recombinant protein has been showed to induce sufficient protection in rabbits and sheep in several studies [11, 38]. The use of purified or unpurified proteins and type of adjuvant might play role in these differences. Aluminum-based adjuvants have been reported as the safest, and have the best efficacy due to the triggering of a strong humoral response [39]. $Al(OH)_3$ is the most commonly used adjuvant in recombinant vaccines against clostridial diseases [19, 29, 40, 41]. In the present study, recombinant vaccines were prepared from unpurified proteins using $Al(OH)_3$ as adjuvant. It is plausible to suggest that in addition to specific proteins, other proteins can be present in the total amount of unpurified protein. Therefore, the low amount of specific proteins in the vaccine may cause low levels of neutralising antibodies to be produced. It is also reported that the type of adjuvant used in recombinant vaccines may affect the amount of antibody formed [28]. As a matter of fact, Moreira Jr. et al. [42] reported that the recombinant botulism vaccine containing Montanide ISA 50 adjuvant produced higher antitoxin titre than the vaccine containing $Al(OH)_3$.

**Table 2** Antitoxin titres determined in blood sera of sheep vaccinated with RV400 and commercial vaccine

| Sheep | Antitoxin titres (IU/mL) | | | | | |
|---|---|---|---|---|---|---|
| | RV400[a] | | | Commercial vaccine | | |
| | C. *perfringens* beta | C. *perfringens* epsilon | C. *novyi* alpha | C. *perfringens* beta | C. *perfringens* epsilon | C. *novyi* alpha |
| 1 | 12 | 16 | 6 | 8 | 4 | 6 |
| 2 | 10 | 14 | 6 | 4 | 2 | 6 |
| 3 | 10 | 14 | 6 | 8 | 4 | 6 |
| 4 | 8 | 10 | 4 | 6 | 2 | 6 |
| 5 | 10 | 14 | 6 | 10 | 6 | 8 |
| 6 | 10 | 12 | 6 | 8 | 4 | 6 |
| 7 | 10 | 16 | 6 | 10 | 6 | 6 |
| 8 | 8 | 14 | 4 | 10 | 6 | 8 |
| **Mean titres** | **9.75 ± 1.28** | **13.75 ± 1.98** | **5.50 ± 0.93** | **8.00 ± 2.14** | **4.25 ± 1.67** | **6.50 ± 0.93** |

[a]RV400: Recombinant vaccine 400 µg

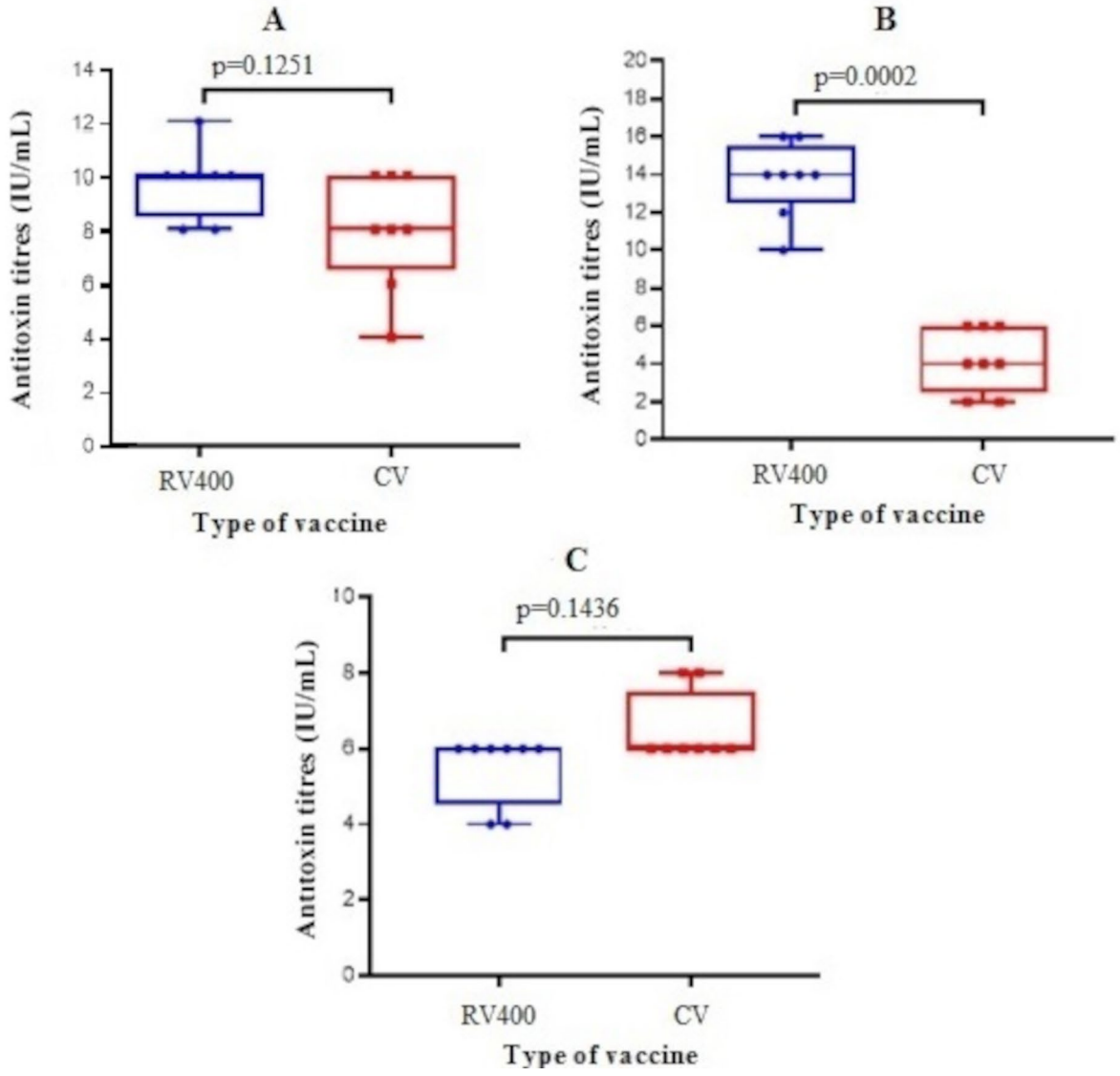


**Fig. 4** Antitoxin titres against *C. perfringens* beta (**A**) and *C. perfringens* epsilon (**B**) and *C. novyi* alpha (**C**) toxins in sheep immunised with commercial (CV) and recombinant (RV400) vaccines. Each data is shown as a dot in the whisker-plot graph. Comparison between groups was performed by Mann Whitney U test ($P < 0.05$)

## Conclusion

Conventional vaccines are commercially produced against clostridial infections such as enterotoxemia, blackleg, infectious necrotic hepatitis, bacillary haemoglobinuria, tetanus, braxy and gas gangrene in animals. In this study, a combined recombinant vaccine against enterotoxemia and infectious necrotic hepatitis diseases using unpurified recombinant proteins was developed for the first time as an alternative to the existing conventional vaccines and, tested in sheep. It was demonstrated that the vaccine prepared using 400 µg amounts of unpurified recombinant *C. novyi* alpha, *C. perfringens* beta and epsilon toxin proteins produced an adequate immune response in sheep. Because recombinant vaccines are prepared more quickly, easily and potentially at a lower cost, it was suggested that they can be used successfully against enterotoxemia and infectious necrotic heptatis diseases. However, comprehensive studies are needed to investigate the duration of immunisation of recombinant vaccines in target animals and to determine the effectiveness of recombinant vaccines prepared using different adjuvants. In addition, different epitope regions forming the toxin gene of *C. novyi* should be cloned into separate plasmids and the immunogenic properties of the toxins formed by each region should be examined separately.

## Materials and methods

### Kits, enzymes and reagents

The pVC-UGT (constructed for another study), pET-28a (+) plasmids and *E. coli* C43 (DE3) strains were prepared from the Synthetic Biology and Metabolic Engineering Laboratory at Firat University (Türkiye). The EcoRI, SalI, XhoI, HindIII, and MluI restriction enzymes (NEB, ABD) were used for cloning the epsilon, beta and alpha toxin genes into the pET-28a (+) plasmid. "QIAprep Spin Miniprep Kit (Catalog Number: 27106)" used for plasmid DNA isolation and "QIAquick Gel Extraction Kit (Catalog Number: 28706)" used for purification of polymerase chain reaction (PCR) products after agarose gel electrophoresis were supplied by QIAGEN (Germany). "E.Z.N.A Bacterial DNA Kit (Cat. Number: D3350-00)" used for genomic DNA (gDNA) isolation was supplied by OMEGA and "1 kb DNA Ladder (Catalog Number: MG-LDR-1000)" used for determination of molecular weights of DNA bands during agarose gel electrophoresis was supplied by Thermo. 50xTAE Buffer Solution (Tris-Acetate-EDTA) used for agarose gel electrophoresis was supplied by Fisher Scientific. dNTP, NAD+ (β-Nicotinamide adenine dinucleotide), T5 exonuclease, phusion polymerase, Taq ligase solutions used for the Gibson assembly method were supplied by NEB, and trisma base chemicals were supplied by Sigma, HCl solution and $MgCl_2$ were supplied by Merck, nuclease free water was supplied by Wisent Canada, Difco SOB medium (Super Optimal Broth) used for the transformation step was supplied by BD Difco, and glucose was supplied by Wisent Canada.

### Vaccine strains and standard toxins

*C. novyi* type B (Genbank: JENV01000129.1), *C. perfringens* type C (NCBI Reference Sequence: NZ_ABDU01000064.2) and *C. perfringens* type D (NCBI Reference Sequence: NZ_VFIZ01000005.1) vaccine strains and standard toxins used in the mouse toxin neutralisation test were purchased from Pendik Veterinary Control Institute (Türkiye).

### Constructions of recombinant CpDetx, CpCcpb, CnBtcn-alpha plasmids

#### *Construction of CpDetx plasmid*

The *CpDetx* toxin gene, obtained from the previously constructed [43] pCpDetx plasmid (*CpDetx* in pVC-UGT plasmid) with EcoRI and SalI restriction enzymes, was constructed by ligation to the pET-28a (+) plasmid to which the sticky end was created with EcoRI and XhoI restriction enzymes. The construction was transformed into the *E. coli* C43(DE3) through the electroporation method.

#### *Construction of CpCcpb plasmid*

The *CpCcpb* toxin gene was amplified from *C. perfringens* type C genomic DNA by PCR using a pair of primers (5'-GCGAATTCCTCGAGGGTACCATGAAGAAAAAATTTATTTCATTAG-3' and 5'– GCCTGCAGGTCGACTCTAGAAATAGCTGTTACTTTGTGAGTAAG-3'). The reaction was performed with 0.5 µM of each primer and 50 ng of genomic DNA (pre-denaturation at 98 °C for 30 s, followed by denaturation at 98 °C for 10 s, annealing at 63 °C for 30 s, and extension at 72 °C for 20 s for 35 cycles, with a final extension at 72 °C for 5 min.) in a total volume of 50 µl. The PCR product was subjected to electrophoresis with 1% agarose gel and staining with ethidium bromide. Following the purification from the gel, the *CpCcpb* toxin gene was obtained by digestion with EcoRI and SalI restriction enzymes. The obtained *CpCcpb* toxin gene was ligated into the pET-28a (+) plasmid constructed with the sticky end EcoRI and XhoI restriction enzymes. The construction was transformed into the *E. coli* C43(DE3) through the electroporation method.

#### *Construction of CnBtcn-alpha plasmid*

The *CnBtcn-alpha* toxin gene was amplified from *C. novyi* type B genomic DNA by PCR using a pair of primers (5'- TGGTGGTGCTCGACTCTAGATTCTTCCCAATTTCCGTTTATATTATC – 3' and 5'- TCCGAATTCCTCGAGGGTACCAATGGAGAGCTTCATTACAAAAATATAC – 3'). The reaction was performed with 0.5 µM of each primer and 50 ng of genomic DNA (pre-denaturation at 98 °C for 30 s, followed by denaturation at 98 °C for 10 s, initial temperature of 70 °C for 4 s decreasing by 0.1 °C per cycle (or ramp rate of 0.1 °C per cycle) until reaching 67 °C, then holding at 67 °C for 20 s for annealing, and extension at 72 °C for 35 s for 35 cycles, with a final extension at 72 °C for 5 min.) in a total volume of 50 µl. The PCR product was subjected to electrophoresis with 1% agarose gel and staining with ethidium bromide, followed by purification from the gel. For cloning into pET-28a (+) plasmid, pET-28a (+)-CpDetx plasmid was amplified by PCR using a pair of primer (5'-TTGTAATGAAGCTCTCCATTGGTACCCTCGAGGAATTCGGATCC-3' and 5'- TAAACGGAAATTGGGAAGAATCTAGAGTCGAGCACCACCACCAC-3'). The reaction was performed with 0.5 µM of each primer and 50 ng of genomic DNA (pre-denaturation at 98 °C for 30 s, followed by denaturation at 98 °C for 10 s, initial temperature of 70 °C for 4 s decreasing by 0.1 °C per cycle (or ramp rate of 0.1 °C per cycle) until reaching 67 °C, then holding at 67 °C for 20 s for annealing, and extension at 72 °C for 35 s for 35 cycles, with a final extension at 72 °C for 6 min and 20 s) in a total volume of 50 µl. The PCR product was subjected to electrophoresis with 1% agarose gel and staining with ethidium bromide, followed by purification from the gel. The obtained fragments were

combined using the Gibson method [44]. The construction was transformed into the *E. coli* C43(DE3) through the electroporation method.

#### Expression and purification of the recombinant proteins

*E. coli* C43(DE3) strains containing pET-28a (+) plasmids carrying *CpDetx*, *CpCcpb*, *CnBtcn-alpha* toxin genes were grown in LB containing 30 mg/mL kanamycin. When the $OD_{600}$ value of the culture reached the range of 0.6–0.8, it was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 1 mM. After 16 h, the pellet of the culture was collected by centrifugation (4000 x rpm; 20 min; 4 °C), suspended in 8 M urea and sonicated for 10 s at 60 kHz, 3 times, and collected by centrifugation (4000 x rpm; 20 min; 4 °C). The expressions of the proteins were confirmed by 12% SDS-PAGE and their amounts were measured using Lowry method as 39.298 µg/mL, 3.996 µg/mL, 8.420 µg/mL, respectively.

#### Experimental animals

New Zealand rabbits aged 3–6 months used in the potency test and mice (Swiss albino) weighing 18–20 g used in the toxin neutralisation test were obtained from the Experimental Animals Unit of Elazığ Veterinary Control Institute. Akkaraman breed sheep (1–2 years old) were obtained from the Agriculture and Livestock Research Centre of Fırat University. Ethical approval for experimental studies on animals was obtained from the Local Ethics Committee for Animal Experiments of Elazığ Veterinary Control Institute (Approval no. 2021/05).

#### Vaccine preparation and safety

Unpurified protein obtained from *E. coli* cell lysate supernatant was used for vaccine preparation. Three different recombinant vaccines (RV100, RV200, RV400) were prepared with 100, 200 and 400 µg each of *C. novyi* alpha, *C. perfringens* beta and *C. perfringens* epsilon proteins in each vaccine dose (2 mL). Aluminum hydroxide [2.5–3.5% Al $(OH)_3$] was used as adjuvant [18]. For sterility of the vaccine, 0.5 mL of the vaccine formula was transferred into thioglycolate and Sabouraud broths. Thioglycolate broths were incubated under aerobic and anaerobic conditions at 37 °C and Sabouraud broths were incubated under aerobic conditions at 25 °C. Growth was checked daily for 21 days by spectrophotometry [22]. The safety test was performed as described in the European Pharmacopoeia [20].

#### Potency test in rabbits

The potency test was performed using New Zealand rabbits that did not carry antibodies against the relevant toxins [22, 27]. The number of rabbits ($n = 35$) was determined with an effect size of 0.71, an alpha error of 0.05 and a power of 85% using the G*Power package program (Version 3.1. 9.2). Five groups were formed with 7 rabbits in each group: RV100 (G1), RV200 (G2), RV400 (G3), Commercial Vaccine-CV (G4) and negative control (G5). Rabbits from G1, G2 and G3 were vaccinated subcutaneously with 2 mL of recombinant vaccines RV100, RV200 and RV400 (concentrations of 100, 200 and 400 µg), respectively. Rabbits from G4 were vaccinated subcutaneously with 2 mL of commercial vaccine. Rabbits from G5 received 2 mL of PBS. After 21 days, the rabbits were revaccinated with the same dose. Blood was collected from the rabbits 14 days after the second vaccination and sera were removed and pooled serum samples were obtained for each group. The antitoxin level in the pooled rabbit blood sera was determined by the mouse toxin neutralisation test [20, 24]. Briefly, serum dilutions (from 1:1 to 1:32) and standard toxin mixes were incubated at 37 °C for 1 h for neutralisation, 0.2 mL of *C. novyi* alpha toxin and serum dilution mixtures were administered intramuscularly to 2 mice, 0.2 mL of *C. perfringens* beta and epsilon toxin and serum dilution mixtures were administered intravenously to 2 mice. The number of mice was determined based on the recommendation of the European Pharmacopoeia that suggests the use of at least two mice for serum dilutions and standard toxin mixes (for *C. perfringens* and *C. novyi* vaccines) [20]. The mice were observed for 72 h, and the number of dead and surviving animals was recorded. The 50% neutralization titre was calculated according to the method reported by Reed and Muench [45] and expressed in international units per milliliter (IU/ mL).

#### Immunization of sheep

The number of sheep was determined to be 24 sheep in total with an effect size of 0.78, an alpha error of 0.05 and a power of 85% using the G*Power package program (Version 3.1. 9.2). Twenty four sheep without detectable antibody levels against *C. novyi* alpha, *C. perfringens* beta and epsilon toxins were divided into 3 groups containing 8 sheep each (RV400, CV and negative control group). RV400 vaccine was used for immunisation of sheep because the antitoxin titers in pooled blood sera of rabbits given vaccine containing 400 µg of each recombinant protein were found to be above the limits specified in European Pharmacopoeia. The animals in the RV400 and CV groups were vaccinated twice by inoculating 2 mL of vaccine at 21-day intervals, while those in negative control group received 2 mL of PBS. Blood was collected from the sheep 14 days after the second inoculation and the antitoxin titres in individual blood sera were determined by the mouse toxin neutralisation test.

### Statistical analysis

SPSS statistical package programme [46] was used to evaluate the study data. Pairwise comparisons of the groups were evaluated by the two-tailed Mann Whitney U test. Statistical significance level was accepted as $P < 0.05$.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04841-6.

Supplementary Material 1

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Not applicable.

**Author contributions**
HK and VÇ contributed to the conception, design and writing of the study. DNE, VÇ and EG performed gene cloning and DNA analysis. HK and HÖ carried out recombinant vaccine production and immunization experiments. BÇ conributed interpretation of the data and writing. *: First authors (DNE, VÇ, HK and EG)&: Co-corresponding authors (HK and VÇ).

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**Data availability**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**
All experiments were performed in accordance with relevant guidelines and regulations. All methods were reported in accordance with ARRIVE guidelines for the reporting of animal experiments. The approval for the study was obtained from Animal Experiments Local Ethics Committee of Elazığ Veterinary Control Institute (Approval No: 2021/05). The informed consent was obtained from owners of Animals Unit of Elazığ Veterinary Control Institute.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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