Veterinary and Animal Science 21 (2023) 100305

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

Evaluation of humoral immune responses against *C. perfringens* epsilon toxin in Iranian sheep and goats after vaccination

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ARTICLE INFO

Keywords:
Antibody titer
Sheep
Goat
Indirect ELISA

ABSTRACT

Clostridium perfringens is a common cause of death in domestic animals worldwide. However, vaccination on a regular basis is an economically beneficial means for controlling clostridial contamination.The objective of the current investigation was to evaluate the humoral immune responses using iELISA in Iranian sheep and goats following the vaccination programs administered by the bacterin-toxoid polyvalent entrotoxemia vaccine. A total of one-hundred-and-twenty animals, consisting of sixty sheep and sixty goats, were randomly divided into three groups. These animals were vaccinated with clostridial vaccine on days 0 and 14 using two different dosages. Blood samples were collected on day zero, 15, 30, 45, 60, 75, and 90 following vaccination. The sera samples were then separated and antibody titers were measured using an in-house enzyme-linked immunosorbent assay (ELISA) against *C. perfringens* epsilon toxin. The titers of antibodies in sheep were notably higher than those in goats, particularly after receiving the booster dose. No statistically significant variations were identified in the immune responses of Iranian sheep and goat breeds. ($p>0.05$). Overall, the duration of the humoral immune response in goats upon administration of the clostridial vaccine was relatively brief, requiring multiple booster injections.

Introduction

Exototoxemia caused by *C. perfringens* type D is a significant reason for sudden mortality or extended diarrhea in sheep and goats (Uzal & Songer, 2008). The epsilon toxin released by *C.perfringens* can be secreted as an inactive prototoxin, which can be activated through the action of intestinal proteases (Garcia et al., 2013). The absorption of epsilon toxin into the systemic circulation increases the permeability of capillaries in the intestinal mucosa, resulting in kidney and brain damage (Miyashiro et al., 2007). Epsilon Toxin, produced by *C. perfringens* type D in animal intestines, does not typically cause any harm, though it may provoke the development of antibodies in subclinical animal infections (Blackwell et al., 1983). Vaccinations have proven to be an extremely successful tool in the prevention and control of clostridial disease in small ruminants (Abdolmohammadi Khiav & Zahmatkesh, 2021). There is not specific clostridial vaccine for goats (Uzal et al., 1997). In a study conducted by Uzal et al. (2016), it was found that the vaccination of goats led to a low frequency and severity of the disease. However, the antibody titer of goats was comparatively low and short-lasting when compared to those of sheep and cattle (Uzal et al., 2016). Consequently, the goat breeds need to receive booster doses every 3 or 4 months in order to stay completely safeguarded throughout their life (Uzal & Kelly, 1999). No existing data on the immunological responses of sheep and goats to Clostridial vaccine exists in Iran. Therefore, this study was designed to evaluate the humoral immune response to *C. perfringens* epsilon toxin in sheep and goats following vaccination with the bacterin-toxoid polyvalan entrotoxemia vaccine using an Indirect ELISA assay.

Materials and methods

Experiment design

In this cross-sectional study, conducted in Shahrbabak, Kerman, Iran,

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https://doi.org/10.1016/j.vas.2023.100305

Available online 21 July 2023

120 clinically healthy animals - sixty sheep (Qashqai and Kermani sheep breeds) and sixty goats (Pakistani and Shahrbabak goat breeds) - aged 7–9 months were divided into three equal experimental groups. The first group (sheep I and goat I) served as a negative control, as they were not administered any vaccine. The second group (sheep II and goat II), however, was administered 3.0 mL of bacterin-toxoid Enterotoxemia vaccine subcutaneously on one occasion. The third group (sheep III and goat III) was administered the same vaccine twice, with an interval of fourteen days. Animal injections and blood sampling in this study were conducted in accordance with the Animal Welfare Act and Regulations, adhering to the principles outlined in the Guide for the Care and Use of Animals. The animals didn't receive the clostridial vaccine in the previous year and were healthy. Throughout the entirety of the trial, the animals were maintained in a same condition. On day 0, 15, 30, 45, 60, 75 and 90 after the vaccination, 10 ml of blood was collected from the jugular vein.Three replicates of hyperimmune serum were used for jn house ELISA. Blood samples were centrifuged at 4000 rpm (rpm) for 1 min to separate the sera samples. These samples were then heated and inactivated to 56 °C for 30 min before being stored at −20 °C for further analysis. During the study, the antibody titers against *C. perfringens* epsilon toxin were measured by an iELISA assay in three groups.

iELISA

In this study, an indirect Enzyme-Linked Immunosorbent Assay (iELISA) was designed to measure the antibody titer of *C. perfringens* type D epsilon toxin, reported in International Units per milliliter (IU/mL). The toxin was purified by ammonium sulfate precipitation, dialysis, and neutralization with alpha antitoxin. It was then highly purified using a chromatography system (Abdolmohammadi Khiav et al., 2022). For his purpose, purified epsilon toxin (0.25 ng/mL) was coated to a plate and left to incubate overnight at 4 °C. The plate was washed with PBS 1X containing 0.05% v/v Tween 20 (PBST) three times, then blocked with 3% BSA for two hours. Next, a blocking solution of 1% w/v Bovine Serum Albumin (BSA) in PBS 1X (Sigma) was added, with hyperimmune epsilon antitoxin (8 IU/mL) serving as a positive control. Serum from unvaccinated animals was also used as a negative control. The plate was then incubated for 60 min at 37°C. Following this, the wells were washed and 100 μL of a horseradish peroxidase-conjugated anti-goat antibody (1:5000) was added and incubated again for 60 min at 37 °C. After washing the wells one more time, 100 μL of substrate solution containing 40 mg/mL o-Phenylenediamine dihydrochloride (Sigma-Aldrich) in 10 mL o-Phenylenediamine dihydrochloride buffer and hydrogen peroxide 30% v/v (Merck, Germany) was added at room temperature until coloring occurred. The reaction was stopped with 50 μL of 3 M H2SO4 solution, and read at 490 nm (Fathi Najafi et al., 2020). The standard deviations were determined using SPSS ver 18. The cross-reactivity of the iELISA was evaluated using *C. novyi* alpha toxin (CN 804), *C. septicum* alpha toxin (CN 913), and *C. chauvoi* (CN 701) as a control. Subsequently, antibody titers were plotted after administration of a single dose and a booster dose of vaccination for sheep and goats.

Data analysis

A *t*-test was conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA) to compare the results of iELISA between different groups. The results were reported statistically significant, with a p-value of less than 0.01

Results

Repeatability and specificity of ELISA assay

The quantification of humoral immune responses demonstrated reliable repeatability with low standard deviations (SD=0.033). The cutoff point of the iELISA was 0.435 and the cross-reactivity findings indicated that epsilon toxin has no cross-reactivity with other clostridial species (Table 1).

Sera epsilon antibody titres

Analysis of our results revealed that serum concentrations of 2 IU/mL or more of epsilon antitoxin were protective against the effects of epsilon toxin of C. perfringens type D. Pre-vaccination antibody titers, observed on day zero, were not enough to induce an immune response (measured at 0.4 IU/mL).In the second group, on day 15 after the clostridial vaccine, antibody titers in sheep II and goat II were 1.7 and 0.4 IU/mL respectively. On days 30 and 45, the titers increased, and a maximum titer of 3.5 and 1.1 IU/mL were observed in sheep and goats respectively on day 45. However, on day 60 the titers declined rapidly in goats and reached zero. The titers in sheep also declined on day 75 (as shown in Fig. 1).In the third group of sheep, the antibody titer gradually increased on days 15 and 30 (2.2 and 3.1 IU/mL) before significantly increasing to 4.4 IU/mL on day 45 and 6.6 IU/mL on day 60 after the booster dose. The maximum titer was observed on day 60 . On day 75 to 90 (1.8 IU/mL) post-vaccination, the titer of antibody was declined in sheep breeds (Fig. 2). Ultimately, the antibody titer was higher than it would have been for single-dose vaccination.The antibody titer in the third group of goats was higher and long-lasting compared to a single-dose of vaccination. After 75 days of post-vaccination, the antibody titer dropped to 1.7 IU/mL. On day 90, the antibody titer was lower than 60 days after vaccination (Fig. 2). The comparison of immune responses between single and booster-doses of vaccination in the sheep and goats is shown in Figs. 3 and 4. Tottaly, the antibody titer against clostridial infection was lower and less durable in goats than in sheep. However, our results indicated that there was no significant difference between sheep and goat breeds ($p > 0.05$).

Discussion

Clostridium is a natural inhabitant of the gastrointestinal tract of animals, generally harmless unless given the opportunity to proliferate due to stress or dietary changes, which can lead to the release of toxins (Carroll, 2023). Epsilon toxin, produced by *C. perfringens* types D and B, is one of the most potent toxins known. Vaccination against clostridium species is strongly recommended for small ruminants, as this will provide protection through the stimulation of the humoral immune system and the prompting of significant antibody production (Abdolmohammadi Khiav, Emadi & Zahmatkesh, 2022). The primary aim of a vaccine program is to guarantee that the vaccine is effective in the intended target animals. In Iran, a vaccination program for cattle, sheep, and goats against enterotoxemia involves two vaccine doses, administered at two to three-week intervals. Recent studies on cattle have indicated that the humoral protection offered by commercial vaccines diminishes within a year (de Oliveira Júnior et al., 2019).

This issue emphasizes the requirement for further inquiry into vaccine formulation and immunization schedules to heighten the immune response to this toxin and minimize the need for additional dosages. Studies have demonstrated that utilizing vaccine formulations containing purified and recombinant antigens alongside novel adjuvants can enhance the immune system's response (Ferreira et al., 2016; Zaragoza et al., 2019). A recent study that used recombinant botulinum vaccines found that none of the formulations tested could provide protection for one year in buffaloes, which indicates a need for additional research into clostridial vaccine formulations and improved immunization regimens

Table 1
Mean ± standard deviations of clostridial species was assessed by iELISA.

Control group	Mean ± standard deviations
C. novyi alpha toxin	0.062 ± 0.042
C. septicum alpha toxin	0.060 ± 0.038
C. chauvoi	0.169 ± 0.060

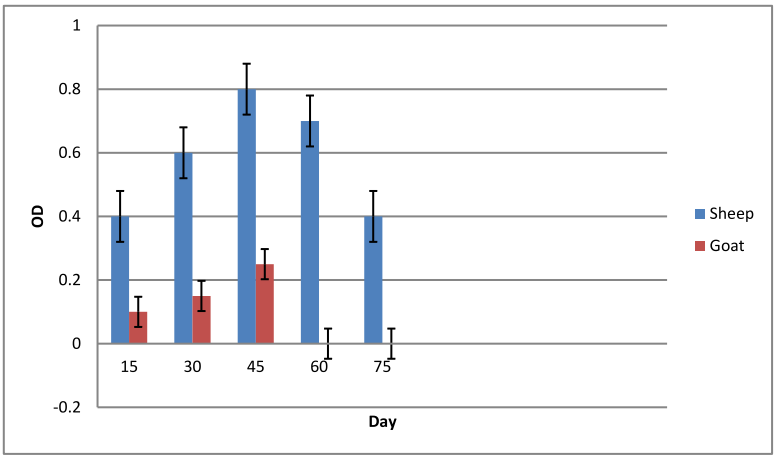

Fig. 1. Comparison of immune responses between sheep and goat after single-dose of vaccination
On days 15 to 60, the antibody titers were increased. On day 45 post-vaccination, the maximum titer of antibody was observed in sheep and goat. On day 60 antibody titers were declined rapidly in goat compared with sheep and reached in zero. On the 75th day, the antibody titer decreased to the amount on the 15th day.

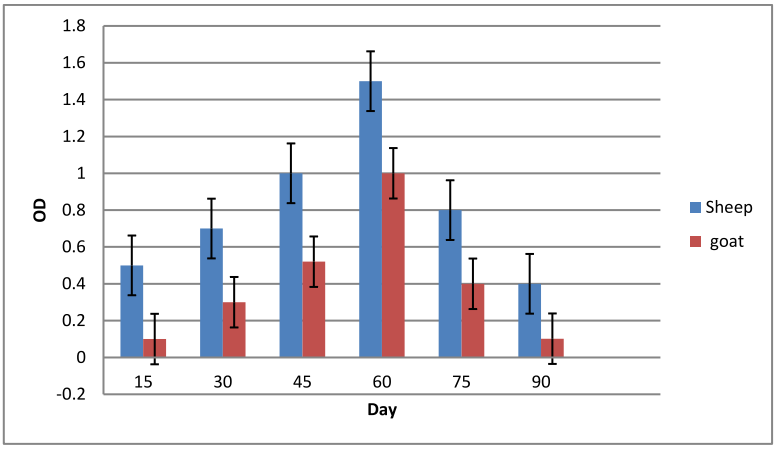

Fig. 2. Comparison of immune responses between sheep and goat after booster dose of vaccination
On days 15 to 60, the antibody titers were increased in both of them. The maximum titer was observed on day 60. On day 75 to 90 post-vaccination, the antibody titer was declined in sheep. Totally, booster vaccination dose produces more durable immunity compared to single-dose of vaccination.

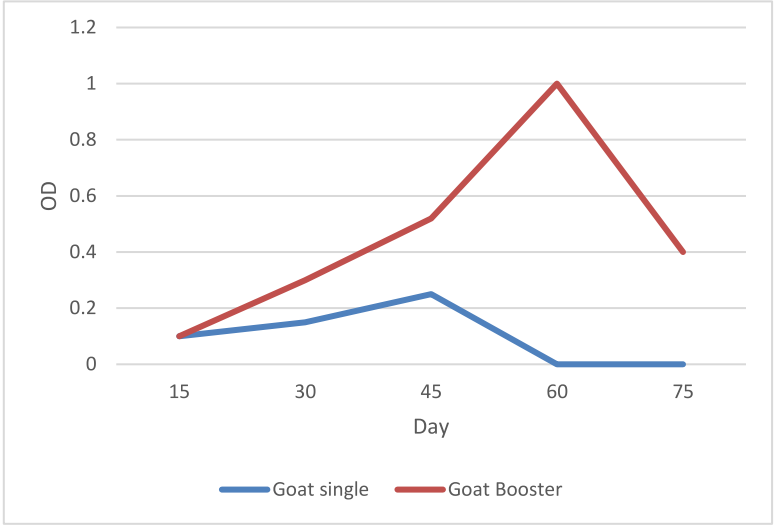

Fig. 3. Comparison of immune responses between single and booster-dose of vaccination in the goats.

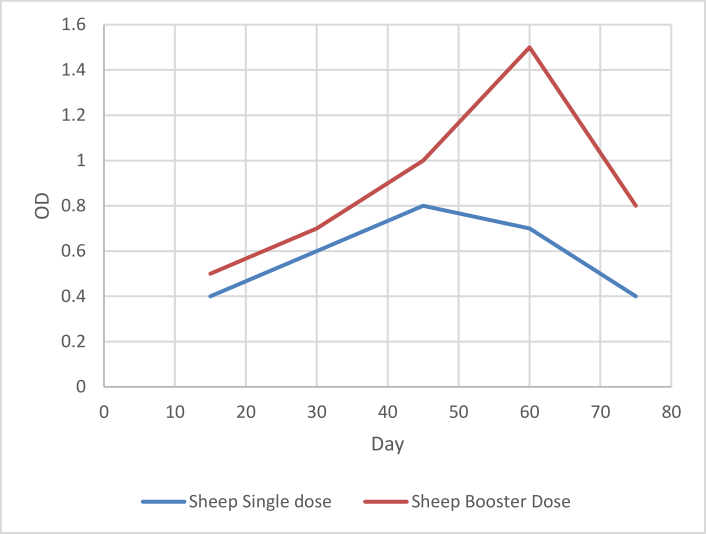

Fig. 4. Comparison of immune responses between single and booster-dose of vaccination in the sheep.

(Otaka et al., 2020). In Iran, no research has been done on this issue and this is the first study to evaluate the immune system against one of the important toxins of *C. perfringens* following vaccination. Therefore, this study was conducted with the aim of measuring the durability of immunity in the two dominant hosts of sheep and goats in Iran. There are various methods to measure humoral immunity, including iELISA. Thus, biological assays such as ELISA assays, which are capable of measuring serum antibodies, can be extremely useful.This study demonstrated the ability to detect serum antibodies with good repeatability and low standard deviations using iELISA based on epsilon toxin. Our results showed that the antibody titer against epsilon toxin was present in animals, suggesting Clostridia presence on the Iranian farm even though the animals had not received any vaccine, as seen in the Blackwell and Veschi studies of goat herds (Blackwell, Butler & Bell, 1983; Veschi et al., 2006).

The antibody titer increased for 45 days following a single dose vaccination. However, on day 60, the antibody titers dropped significantly, which was previously observed in goats (Finnie, 2003; McClane et al., 2006). Thus, goats needed to be given booster doses faster than sheep in order to boost their humoral immunity (Uzal & Kelly, 1999, Veschi, Dutra, Miyakawa, Perri and Uzal 2006). In group sheep III, the antibody titer was increased until 60 days after booster dose. The maximum titer was observed on day 60. The antibody titer decreased until 90 days and it was a little lower than the protection titer.

In Oliveira's 2021 study, the maximum antibody titer for both cattle and sheep hosts when given a semi-purified toxoid vaccine, purified toxoid plus mineral adjuvant, or chitosan was reported to be on the 60th day with a decrease afterward. Despite the initial decrease in antibody titer, cattle and goat hosts were still protected for 240 and 90 days after their respective vaccinations; after this point, a significant decrease was observed in some vaccine groups (de Castro Oliveira et al., 2021). Blackwell and her colleagues reported that, six months after the vaccination of the goats in their study, the antitoxin titer was reduced to less than 0.10 IU/mL in 72% of the cases (Blackwell, Butler & Bell, 1983). In Freitas's 2021 study of horses that were vaccinated with recombinant alpha and beta *C.perfringens* toxoid and a commercial vaccine, it was seen that similar to other studies, the highest antibody titer was recorded 60 days after vaccination then decreased within all groups until reaching zero at 120 days. The concentration of 200 and 400 micrograms of the recombinant vaccine was found to greatly stimulate the immune system. Unfortunately, none of the recombinant vaccines studied were able to maintain detectable titers against *C. perfringens* alpha and beta toxin throughout the year, demonstrating the need for further research into formulating and utilizing adjuvants. (Ferreira, Moreira, Cunha, Mendonça, Salvarani, Moreira and Conceição 2016). A further study by Rousselet on animal species revealed that primary vaccination and booster injections of multiple clostridial vaccines at an interval of four weeks can create a higher level of immunity against epsilon toxin, which then gradually decreases over time - a result which is also mirrored in our own study. It was also suggested that providing booster injections every six months may be necessary in order to sustain the antibody response to epsilon toxin (Rousselet et al., 2021). In Oliveira's study, the maximum antibody titer for the semi-purified toxoid vaccine with either aluminum hydroxide or chitosan as adjuvant was observed 60 days after vaccination similar to our study. However, immunity sharply decreased on the 90th and 120th day and reached its lowest level. An interesting finding was that the same level of immunity was observed up to 210 days after vaccination with purified toxoid vaccine and the same adjuvant (de Castro Oliveira, de Oliveira Júnior, Alves, Assis, Silva, de Sousa Xavier and Lobato 2021). A further study was conducted to assess the immune response of buffaloes 56 days after receiving vaccinations with recombinant proteins of two different serotypes of the *C. botulinum* bacteria (Type C and Type D) at differing concentrations (100, 200, and 400 micrograms), which was then compared with a bivalent commercial toxoid vaccine. The findings of the study indicated that the recombinant vaccine containing 400 micrograms of the recombinant protein was the most advantageous out of all the vaccines assessed (Otaka et al., 2017). Our study showed that goats which received two injections at four-week intervals had higher levels of antibodies than those which had a single-dose vaccination, which is in line with other reports (Uzal & Kelly, 1999, Veschi, Dutra, Miyakawa, Perri and Uzal 2006). The antibody titer in goat was lower and shorter-lasting than expected, which means animals need booster shots every 3 or 4 months for the rest of their lives. This is the first study in Iran to evaluate the immune response of small ruminants following clostridial vaccination schedules.

Contributions

Akbar Asadi and Anahita Emadi performed the project, conducted the trials. Lida Abdolmohammadi Khiav performed the literature search and prepared the original draft. Maryam Dadar edited and revised the manuscript.All of the author read and approved the final manuscript.

Funding

This work was supported by Shahrbabak Islamic Azad University

[grant number 52105930511001]

Ethics approval

Minimum number required to maintain the quality of the experiment were included. Throughout the experiment good animal welfare was provided.

Research regarding animal injections and blood sampling was performed in compliance with the Animal Welfare Act and Regulations following the principles in the Guide for the Care and Use of Animals.

Ethics statement

Research regarding animal injections and blood sampling was performed in compliance with the Animal Welfare Act and Regulations following the principles in the Guide for the Care and Use of Animals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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