



## QUALITY CONTROL TESTING OF THE CLOSTRIDIAL VACCINE, ADVANTAGES AND CHALLENGES – A COMPREHENSIVE REVIEW

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### Summary

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*Clostridium perfringens* causes severe damage in the gastrointestinal tract of ruminants and birds leading to death. So, *C. perfringens* infection can influence livestock and poultry industry, especially fat animals. Vaccination can highlight and improve animal health by preventing the clostridial disease. The quality control of veterinary vaccines mentioned by regulatory agencies are described in their guidelines. Each batch of these biological products is subjected to multiple testing and must meet a series of requirements, including sterility, safety, purity, and potency. Clostridial exotoxins, especially major exotoxins, are the main virulence factor of the clostridial vaccine; therefore, determination of the biological activity in each batch series of vaccine is necessary. There are two categories of efficacy tests (*in vitro* and *in vivo*) that are performed during the vaccine manufacturing, and on the final product. This comprehensive study reviews briefly the QC methods for determination of the biological activity, advantages, challenges, and possible problems associated with quality control testing of each veterinary *C. perfringens* vaccine. In spite of different sensitivity, specificity, and accuracy, the quality control of vaccine should be well done in accordance with pharmacopoeial monographs or other references in order to gain reliable results.

**Key words:** *Clostridium perfringens*, major toxin, potency, quality control, vaccine

### INTRODUCTION

*Clostridium perfringens* (*C. perfringens*) are anaerobic and spore-forming bacteria found in the soil and body of animals for a long time. Ruminants and birds can be-

come infected with *C. perfringens*, which is associated with high morbidity and mortality. *C. perfringens* is classified into seven isotypes A, B, C, D, E, F, and G, by

major toxins that produce disease in humans and animals. *C. perfringens* type D and *C. perfringens* type B epsilon toxin are responsible for enterotoxaemia (pulpy kidney or overeating disease), especially in sheep and goat, which is caused by consuming high-energy food and change of diet. *C. perfringens* type B beta toxin causes lamb dysentery. *C. perfringens* type C beta toxin causes necrotising enteritis in young domestic animals and struck in adult sheep. *C. perfringens* type A alpha toxin causes necrotic enteritis in birds, yellow lamb disease, and enterotoxaemia in domestic animals.

The protective immunity against *C. perfringens* disease is associated with efficient vaccine. Vaccines against clostridial disease used to immunize animals worldwide are prepared as a whole formalin-inactivated vaccine, bacterin-toxoid vaccine, toxoid vaccine (available as a commercial vaccine). Genetically engineered vaccine, recombinant chimeric vaccine, and mutant vaccine from  $\epsilon$ -toxin gene (Abdolmohammadi Khiav & Zahmatkesh, 2021) and nanovaccine (PLGA Nanoparticle from beta toxin) (Abbasi *et al.*, 2022) have been prepared as an experimental vaccine. The quantity of the

major *C. perfringens* toxins in vaccine and followed by improving of immunological response in animals is crucial. Quality control (QC) process of the vaccine (upstream and downstream process) against *C. perfringens* alpha, epsilon and beta toxins is given on Fig 1. The results of in-process QC for each major toxin are commonly different from final products (Metz *et al.*, 2007). Practical approaches based on literature review, design of validation studies, and accurate evaluation of the QC test results are crucial. The efficacy of a biological product such as a vaccine is mentioned in the Code of Federal Regulations (CFR) (Taffs, 2011). The efficacy test for animal clostridial vaccines is performed according to the pharmacopoeia by animal-based method. However, many researchers developed alternative techniques and compared them to the golden standard. The different techniques to control of the quality of clostridial vaccines are briefly listed in Table 1. This comprehensive study will review briefly the QC methods for determination of the biological activity, advantages, challenges, and possible problems associated with quality control testing of each veterinary *C. perfringens* vaccine.

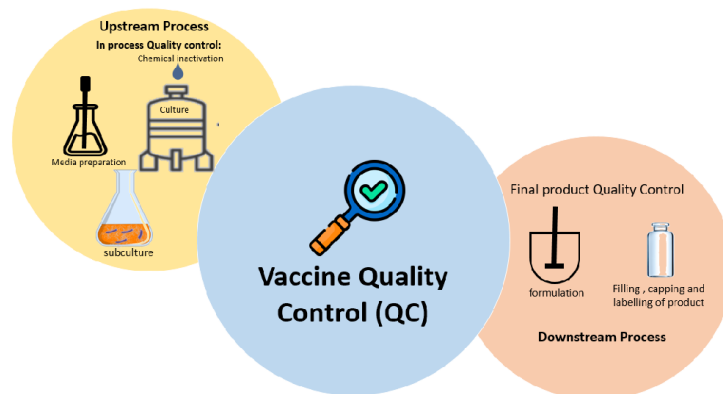


Fig. 1. Quality control steps of *C. perfringens* vaccine.

**Table 1.** *In vitro* and *in vivo* techniques for quality control of clostridial vaccines

Technique	<i>In vitro</i> / <i>in vivo</i>	QC method	Active/inactive toxin	Indicator
MLD	<i>In vivo</i>	In process	Active	Mice
LD <sub>50</sub>	<i>In vivo</i>	In process	Active	Mice
Potency	<i>In vivo</i>	Final product	Inactive	Mice
Challenge test	<i>In vivo</i>	Final product	Inactive	Mice, Chicken, Rabbit
TCP	<i>In vivo</i>	Final product	Inactive	Mice
LF	<i>In vitro</i>	Final product	Inactive	Floccules development
ELISA	<i>In vitro</i>	Final product/ in process	Active/ inactive	Colour development by enzyme
ToBI	<i>In vitro</i>	Final product/ in process	Active/ inactive	Colour development by enzyme
Cell culture	<i>In vitro</i>	In process Final product	Active/ inactive	Cellular metabolic activity
Haemolysin assay	<i>In vitro</i>	In process	Active	RBC
Lecithinase assay	<i>In vitro</i>	In process	Active	Lecithin

## METHODS

To obtain more information and understand the major toxins in veterinary vaccines and challenges to their quality control testing, a structured search of the literature was conducted across on the electronic international databases PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), Scopus (<https://www.scopus.com/home.uri>), CABDirect (<http://www.cabdirect.org/>) using English keywords “Clostridium perfringens” AND “toxin” AND “Quality control” AND “Vaccine” AND “efficacy”. In addition, all relevant manuscripts in Iranian databases, including Scientific Information Database (SID) ([www.sid.ir](http://www.sid.ir)), Magiran ([www.magiran.com](http://www.magiran.com)), conference proceedings, and conference papers were searched. Not only these references, but also the references from these manuscripts were searched to gain more information.

## RESULTS

### *Multivalent clostridial vaccine efficacy*

Multivalent clostridial vaccines efficacy is the ability of the multiple antigens to raise immunity response against virulent types of clostridia in a target model. Vaccines' efficacy is measured by *in vivo* and *in vitro* methods. Several multivalent clostridial vaccines are currently available, but their efficacy is variable. *C. perfringens* veterinary vaccines are a category of combined vaccines. Their complicated nature and combination of *C. perfringens*, formulation, adjuvant, excipients and delivery systems, pose significant challenges to evaluation of efficacy tests (Taffs, 2001). In a study, three groups of experimental vaccine were produced as follows. Group I # was a bacterin pentavalent vaccine with aluminum hydroxide adjuvant in glass bottle (small scale) with the follow-

ing formulation: *C. perfringens* type B, C, and *C. septicum*: 10%; *C. perfringens* type D: 40%; *C. novyi* type B 20%; and aluminum hydroxide gel 10%. Group II # bacterin – pentavalent vaccine with aluminum hydroxide adjuvant in fermenter (large scale) with the following formulation: *C. perfringens* type B, and C: 7.5%; *C. septicum*, *C. novyi*: 15%; *C. perfringens* type D: 45%; and aluminum hydroxide gel 10%. Group III # was a bacterin-toxoid pentavalent vaccine (purified by microfiltration and ultrafiltration system) with aluminum hydroxide adjuvant in fermenter (large scale) with the following formulation: *C. perfringens* type B (bacterin) 3%, *C. perfringens* type C (toxoid): 3%; *C. septicum* (toxoid): 4%; *C. novyi* (bacterin): 4%; *C. perfringens* type D (toxoid): 5%; aluminum hydroxide gel: 10%; physiological serum 70.6%, and formaldehyde 0.4%. Then, potency tests were done for all three groups of vaccines and compared with each other. The results showed that the bacterin-toxoid pentavalent vaccine (Group III) was selected as the formulation with high-quality attributes for large scale production as it met technical requirements including sterility, safety, freedom from abnormal toxicity, and potency (Pilehchian Langroudi *et al.*, 2015).

In another study, a pentavalent clostridial vaccine was produced according to L+ dose of *C. perfringens* type B, and D, *C. septicum*, *C. novyi*, the optical density (OD) of *C. chauvoei*, and limit of flocculation (LF) of *C. tetani*. After inactivation, bacterin-toxoid vaccine was prepared with the following formulation: *C. perfringens* type B (toxoid) (24%), *C. perfringens* type D (toxoid) 30%; *C. septicum* (toxoid) 14%; *C. novyi* (toxoid) 14%; *C. chauvoei* (anaculture) 10%; *C. tetani* (toxoid) 8%, and aluminum potassium sulfate was

added as an adjuvant. The result of potency of the pentavalent vaccine in rabbits and especially in sheep showed the effective amount of antibody titre. The potency test of the vaccine also showed that the *C. chauvoei* was completely protective in guinea pigs. The determination of L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. novyi*, and OD of *C. chauvoei* is also very useful for formulation and production of pentavalent vaccine with suitable induce of immune response, especially in target animals (Osman *et al.*, 2010).

In the study of Tariq *et al.* (2021), monovalent and multivalent clostridial vaccine was produced according to haemolysin assay (HU/mL) of alpha and epsilon toxins and cell culture assay (cytotoxic units per milliliter, CU/mL) of beta toxin. After inactivation, multiple toxoid vaccines plus adjuvant were prepared with the following formulation: Group 1 # *C. perfringens* type A + oil (Montanide ISA 70); Group 2 # *C. perfringens* type A + alum; Group 3 # *C. perfringens* type A + D + oil; Group 4 # *C. perfringens* type A + D + alum; Group 5 # *C. perfringens* type A + B + D + oil; Group 6 # *C. perfringens* type A + B + D + alum. Then ELISA was performed on serum samples from vaccinated rabbits. The results showed that the multivalent toxoid vaccine plus oil adjuvant was selected as the formulation with high-quality attributes for the prevention of clostridial diseases.

As mentioned, the efficacy of a vaccine depends not only on the contents of toxins but also on the adjuvants that are used in the clostridial vaccine. A study compared the immunogenicity of *C. perfringens* type B toxoid and foot-and-mouth combined vaccine along with adjuvant MF59 or Montanide ISA70. The results showed that the formulation with

MF59 adjuvant demonstrated significantly increased immunogenicity against the disease (Araghi *et al.*, 2023). In another study (Araghi *et al.*, 2022), *C. perfringens* type B, C, and D toxoids and foot-and-mouth combined vaccine along with adjuvant aluminum hydroxide and Montanide ISA206 were compared in terms of immunogenicity. The formulation with ISA206 adjuvant had significantly increased immunogenicity ( $P < 0.05$ ) against enterotoxaemia and FMD. This finding showed that ISA206 was more effective than aluminum hydroxide for immunisation against *C. perfringens* beta and epsilon toxins. Elhelw *et al.* (2022) prepared *C. perfringens* type A toxoid and bovine viral diarrhoea virus (BVDV) combined vaccine with Montanide as an adjuvant for immunisation of camels and reported that the combined vaccine produced high antibody titre against clostridial disease. Several studies carried out on chitosan adjuvants in vaccines showed significant increase in humoral and cellular immunity (Khalili *et al.*, 2015). Fathi Najafi *et al.* (2020) reported that a pentavalent toxoid vaccine with chitosan adjuvant stimulated antibody production more than the non-chitosan vaccine. On the contrary, de Castro Oliveira *et al.* (2021) affirmed that the purified toxoid vaccine with aluminum hydroxide adjuvant induced greater humoral immunity than adjuvant chitosan in cattle and goats. On the other hand, treatment of *C. perfringens* with chemical reagents such as formaldehyde makes the condition more complicated. The one-carbon structure of highly water-soluble formaldehyde can both destroy the ability of replication of *Clostridium* species, and neutralise the clostridial toxins (Mitkus *et al.*, 2013). Formaldehyde also prevents from reversion to toxicity form. Thimerosal, a mercury-based preservative, is

another chemical reagent used in *C. perfringens* type C vaccine (Saadh *et al.*, 2020). Furthermore, in the study of Pultov *et al.* (2021), thimerosal was used for tetravalent toxoid *C. perfringens* vaccine as a preservative. The results approved the harmlessness along with suitable stimulation of the immune system against clostridial disease in dairy animals. In another study, thimerosal was also used in *C. perfringens* type D vaccine as a combination with sheep pox (Chandran *et al.*, 2010). A literature summary with information is presented in Table 2.

#### *Efficacy testing of C. perfringens vaccines*

Most of the tests are designed and validated using laboratory animals for quantitative assay of toxins including minimum lethal dose (MLD), Lethal dose 50% (LD50), and L+ (Reed and Muench, 1938; Sobrinho *et al.*, 2014).

##### 1) Minimum lethal dose (MLD)

This *in vivo* assay measures the minimum concentration of active toxin in the fermenter culture supernatant, which is lethal for mice. MLD is in the group of in-process QC assays. After the growth, toxins production, and inactivation of bacteria, because of toxin conversion to inactivated toxoid form, it is no longer considered a QC method. For epsilon toxin of *C. perfringens*, the samples of fermenter suspension are centrifuged and trypsin added to supernatant and incubated for 45 min at 37 °C. After activation, serial dilutions are made and inoculated intravenously (IV) into mice. Activation is not required for other *C. perfringens* toxins. Monitoring of mice is performed over three days. The minimum lethal dose is a dilution containing the least amount of toxin (not toxoid), which causes the death of injected mice

**Table 2.** Summary information of *C. perfringens* vaccines

Delivery system	Adjuvant/ Preservative	Quality control method	Reference
Major toxin: Alpha; <i>C.perfringens</i> type: A, B, C, D, E, F LD50 <sup>a</sup> = 3 µg/kg; Potency <sup>b</sup> = 0.9 IU/mL			
Lipid particles	Oil/Montanide	SN	Elhelw <i>et al.</i> (2022)
Mineral salts	Alum	Haemolysin, Cell culture, ELISA, Challenge test	Tariq <i>et al.</i> (2021)
Lipid particles	Montanide ISA		
Mineral salts	Aluminum hydroxide/Thiomersal	SN	Pulotov <i>et al.</i> (2021)
Mineral salts	Aluminum hydroxide/Thiomersal	LD50, lecithovitellinase, SN	Saadh <i>et al.</i> (2022)
Lipid particles	ISA 15A VG	ELISA, L100, SN	Jiang <i>et al.</i> , 2014)
Mucosal adjuvant	Cholera Toxin B (CTB)	ELISA, Challenge test	Peng <i>et al.</i> (2020)
–	Without adjuvant	ELISA, Pathogenicity test, MLD, Challenge, SN	Song <i>et al.</i> (2018)
Mineral salts	Aluminum hydroxide	LD100- SN	Salvarani <i>et al.</i> (2013)
	Aluminum hydroxide	LD100- SN - ELISA, Challenge test	Zeng <i>et al.</i> (2011)
Mineral oil	Freund's adjuvant	Cell culture, Challenge test	Bokori-Brown <i>et al.</i> (2014)
Immune potentiators	Quil-A	Lecithinase	Verherstraeten <i>et al.</i> (2016)
Immune potentiators	Quil-A	ELISA, Challenge test	Fernandes da Costa <i>et al.</i> (2016)
Major toxin: Beta; <i>C.perfringens</i> type: B, C LD50 <sup>a</sup> <400 ng/kg; Potency <sup>b</sup> = 10 IU/mL			
Mineral salts	Aluminum hydroxide	MLD, SN	Pilehchian Langroudi <i>et al.</i> (2015)
Lipid particles	MF59/ISA70	ELISA	Araghi <i>et al.</i> (2023)
	ISA206	ELISA	Araghi <i>et al.</i> (2023)
Mineral salts	Aluminum hydroxide	ELISA	Araghi <i>et al.</i> (2022)
	Aluminum potassium sulfate	MLD, L+ dose, SN, Challenge	Osman <i>et al.</i> (2010)
Mucosal adjuvants	Chitosan	ELISA	Fathi Najafi <i>et al.</i> (2020b)
Mineral salts	Aluminum hydroxide/Thiomersal	LF, LD50, SN	Saadh <i>et al.</i> (2020)
Mineral salts	Aluminum hydroxide/Thiomersal	SN	Pulotov <i>et al.</i> (2021)

<sup>a</sup> Li *et al.* (2013); <sup>b</sup> European Pharmacopoeia (2019).

**Table 2 (cont'd).** Summary information of *C. perfringens* vaccines

Delivery system	Adjuvant/ Preservative	Quality control method	Reference
Polymeric nanoparticles	PLGA	MLD	Abbasi <i>et al.</i> (2022)
Lipid particles	ISA 15A VG	ELISA, LD100, SN	Jiang <i>et al.</i> (2014)
–	Without adjuvant	SN	Pilehchian Langroudi <i>et al.</i> (2013)
Lipid particles	Oil	SN	Milach <i>et al.</i> (2012)
Mineral oil	Freund's adjuvant	ELISA, LD100, SN, Cell culture	Das <i>et al.</i> (2016)
Mineral salts	Aluminum hydroxide	LD100- SN	Salvarani <i>et al.</i> (2013)
Major toxin: Epsilon; <i>C.perfringens</i> type: B, D LD50 <sup>a</sup> = 100 ng/kg; Potency <sup>b</sup> = 5 IU/mL			
Mineral salts	Aluminum hydroxide	MLD, SN	Pilehchian Langroudi <i>et al.</i> (2015)
Lipid particles	ISA206	ELISA	Araghi <i>et al.</i> (2022)
Mineral salts	Aluminum hydroxide	ELISA	Araghi <i>et al.</i> (2022)
	Aluminum potassium sulfate	MLD, L+ dose, SN, challenge	Osman <i>et al.</i> (2010)
Mucosal adjuvants	Chitosan	ELISA	Fathi Najafi <i>et al.</i> (2020b)
Mineral salts	Aluminum hydroxide/ Thiomersal	SN	Pulotov <i>et al.</i> (2021)
Lipid particles	ISA 61VG	Cell culture, ELISA, Challenge test	Morcrette <i>et al.</i> (2019)
Mineral oil	Freund's adjuvant	Cell culture, ELISA, Challenge test	Yao <i>et al.</i> (2016)
Mineral salts	Aluminum hydroxide	Cell culture, ELISA, Challenge test	Li <i>et al.</i> (2013b)
Polymeric nanoparticles	PLGA	ELISA	Wan <i>et al.</i> (2023)
–	Without adjuvant	SN	Pilehchian Langroudi <i>et al.</i> (2013)
Mineral oil	Freund's adjuvant	ELISA, LD50, SN	Souza <i>et al.</i> (2010)
Mineral salts	Aluminum hydroxide/Thiomersal	SN	Chandran <i>et al.</i> (2010)
Lipid particles	ISA 15A VG	ELISA, L100, SN	Jiang <i>et al.</i> (2014)
Polymeric nanoparticles	PLGA	Cell culture	Pudineh Moarref <i>et al.</i> (2023)

<sup>a</sup> Li *et al.* (2013); <sup>b</sup> European Pharmacopoeia (2019).

(Redhead *et al.*, 2011). Previously, researchers have reported the minimum lethal dose for epsilon toxin of *C. perfringens* type D isolates from 1:10 to 1:1200 (El-Sehamy, 2011). Fahmy *et al.* (2010) performed MLD for *C. perfringens* toxins and determined the range of MLD in mice. The reported MLD for this toxin was from 1:10 to 1:400 (El-Sehamy, 2011). MLD is a quantitative method for calculating toxin concentration in the vaccine strain and isolates. In another study, MLD value was also determined for semi-purified epsilon toxin of *C. perfringens* type D reference strain as 1:6000 (Abdolmohammadi Khiav *et al.*, 2022) although, in another study, MLD for this purified toxin was determined as 1:3000 after the chromatography step (Zayerzadeh & Fardipour, 2015). In the study of Pilehchian Langroudi (2016), MLD was determined as 1:19000, 1:12500, and 1:40000 mg/mL for filtrate, freeze-dried toxin, and freeze-dried prototoxin, respectively that was higher than previous mentioned study. In another study, MLD for purified epsilon toxin of *C. perfringens* type D vaccine strain for filtrate, precipitation, gel chromatography, and ultrafiltration was determined to be 1:8000, 1:14000, 1:19000, and 1:24000 mg/mL, respectively (Pudineh Moarref *et al.*, 2022). Purification steps (precipitation method, chromatography, ultrafiltration, and gel filtration) are based on multiple factors such as the nature of toxin, time, cost, equipment, and final objective of purification which influence on MLD value.

2) Lethal dose 50% (LD50) or the median lethal

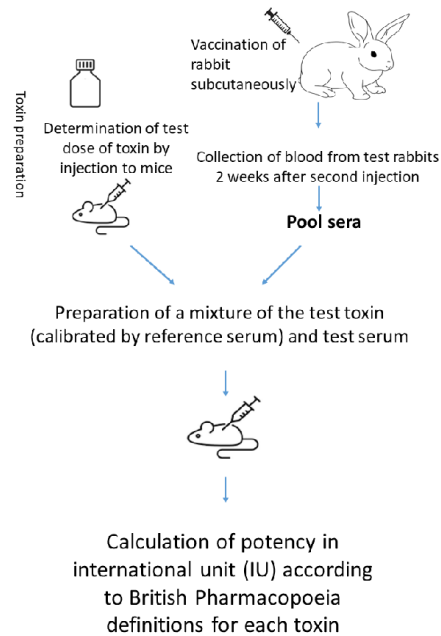
LD50 is defined the dose that is lethal for 50% of mice tested and is usually given as milligrams of toxin per kilogram

of body mass, and sometimes as nanograms, micrograms, or grams per kilogram. This test was introduced by J. W. Trevan in 1927 and used for determination of L+/10. Several studies have reported lethal activity of clostridial toxins in mice. Zayerzadeh *et al.* (2014) purified beta toxin of *C. perfringens* type C vaccine strain by DEAE-CL6B Sepharose column and estimated LD50 as 2.21 µg/kg although, Sakurai & Duncan (1978) mentioned 1.87 µg/kg and the lethal amount of this toxin was <400 ng/kg for adult mice (Worthington & Mülders, 1975). In another study, epsilon toxin of *C. perfringens* type D vaccine strain also purified by cation exchange chromatography (CM-Sepharose) and gel filtration (Sephadex G-100) estimated LD50 of 90 ng/kg for mice (Zayerzadeh *et al.*, 2015). LD50 was estimated to be 70 ng/kg and 100 ng/kg by Li *et al.* (2013) however, Pilehchian Langroudi (2016) determined LD50 of 0.000215 mg/mL or 0.0107 mg/kg for adult mice.

L+ is defined the smallest amount of mixture of toxin along with 1 IU antitoxin that will kill 80–100% mice after intravenous injection. L+/10 is also defined as the minimum amount of mixture of toxin along with 0.1 IU antitoxin that will kill 80–100% mice after injection. A study with mixture of 0.01 mg/mL semi purified epsilon toxin of *C. perfringens* type D strain along with 0.1 IU antitoxin showed that L+/10 will kill 80-100% mice after intravenous injection (Abdolmohammadi Khiav *et al.*, 2022). Although the *in vivo* assay is known as a standard method and mentioned in international guidelines, LD<sub>50</sub> results may be misleading due to variation between mice groups.

### 3) Potency (serum neutralisation assay; SN)

The gold standard technique for assaying the efficacy of toxoids is SN (European Pharmacopoeia, 2019). SN is an *in vivo* assay based on the neutralisation of pooled antibodies from immune rabbits with clostridial toxins. For vaccines, especially combined vaccines, the definition of potency is more complicated than for medications. It is the desired immune response induced by administration of vaccine to prevent infection in target host (Taffs, 2001). The potency of a final blended vaccine consisting of alpha, beta, or epsilon toxoids (either all or some) are assessed according to British Pharmacopoeia monographs (0363). It is recommended to use not less than ten healthy 3–6 months old rabbits, and after two injections of vaccine and pooling sera, the international unit of vaccine is calculated by SN methods. Several dilutions of the pooled sera are prepared, mixed with the clostridial toxin, incubated, and the mixtures injected intravenously into mice (Abdolmohammadi Khiav *et al.*, 2022) (Fig. 2). The international unit for each toxin should be equal to or greater than the standard value (Table 2). Many researches have been done on *C. perfringens* vaccines up to now. Laboto *et al.* (2010) reported that the maximum antibody titre for recombinant *C. perfringens* type D vaccine was on day 56 in target hosts. The average titres on day 56 were 14.3 IU/mL, 26 IU/mL, and 13.1 IU/mL in goats, sheep, and cattle, respectively. However the maximum antibody titre range for epsilon toxoids vaccine was reported between 6.90–11.47 IU/mL in cattle and 1.11 to 4.40 IU/mL in goats in the study of de Castro Oliveira *et al.* (2021).



**Fig. 2.** A schematic presentation of potency assay. Potency test consists of two stages: vaccination of rabbits and titration of antibody by serum neutralisation in mice and calculation according to international standard.

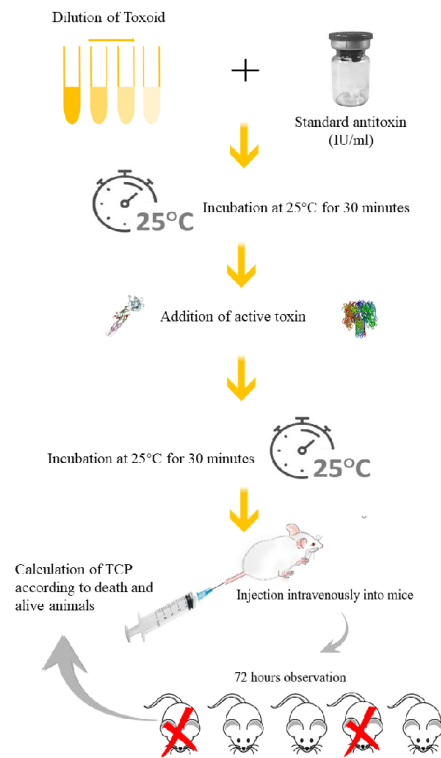
The antitoxin titre for recombinant *Clostridium perfringens* toxoids  $\alpha$  and  $\beta$  vaccine in rabbit sera was 9.6 IU/mL, and 20.4 IU/mL for alpha and beta antitoxin, respectively, and the average  $\alpha$  and  $\beta$  antitoxin titres in sows' sera:  $6.0 \pm 0.9$  IU/mL and  $14.5 \pm 2.2$  IU/mL (Salvarani *et al.*, 2013). Kelneric *et al.* (1996) also reported beta antitoxins for commercial bacterin-toxoid in pig sera of 9.0 to 26.0 IU/mL (average 14.16 IU/mL). The level of antibodies titre for trivalent recombinant vaccine in rabbit sera was 9.6, 24.4, and 25.0 IU/mL against alpha, beta, and epsilon antitoxin, respectively, although respective antibodies levels for commercial vaccine against alpha, beta, and epsilon toxins were 6.9, 22.4, and 12 IU/mL, e.g. higher than the mentioned levels in inter-

national guideline especially for trivalent recombinant vaccine with its unique formulation (Moreira *et al.*, 2016). In this research, the trivalent recombinant vaccine was able to induce titres of 13.71 IU/mL against beta toxin, and 12.74, 7.66, and 8.91 IU/mL against epsilon toxin of *C. perfringens* for cattle, sheep, and goat hosts, respectively. The level of epsilon and beta antitoxin for epsilon-beta recombinant protein vaccine in rabbits was 6 and 10 IU/mL, respectively (Pilehchian Langroudi *et al.*, 2013). Evaluation of neutralising antibodies levels was also reported for other clostridial vaccines such as *C. botulinum* vaccine. Although the *in vivo* assay, known as a gold standard, is highly sensitive for determining *C. perfringens* toxins, it is time-consuming, and requires a large number of mice (Sobrinho *et al.*, 2010). Special regulations are also required at a manufacturing site include supply appropriate biosafety level for prevention risk of cross-contamination, with good ventilation systems, and temperature and humidity conditions that makes some challenges for good monitoring. Furthermore, results may be misleading due to variation between mice groups (Henderson, 1984).

#### 4) Total combining power method (TCP)

To validate the determination of the quality of inactivated antigens in the final product, the TCP test was introduced to the clostridial vaccine industry by manufacturers. This test involves partial neutralisation of antitoxin with serial dilutions of toxoids. The antitoxin in the reaction is then mixed with a fixed dose of active toxin and injected intravenously into mice (Fig. 3) (Jafari Khoshanabadi *et al.*, 2021). The TCP test is based on the binding ability of toxoid to antibody, similar to

L+ assay. This method uses mice as indicator. TCP results show a linear correlation between dilutions ranges up to 1:256 (Sobrinho *et al.*, 2014; Brandi *et al.*, 2016). The correlation coefficient for epsilon toxoid was 0.9999 (Sobrinho *et al.*, 2014). It has high specificity and precision for epsilon toxoid assay. TCP is a robust tool that can be replaced with toxin assay methods, although it also requires a large number of animals (Brandi *et al.*, 2016).



**Fig. 3.** Calculation of total combining power (TCP).

#### 5) Challenge test

Mice (weighing 18–20 g) are injected into the lateral tail vein with 0.5 mL clostridial vaccine. A second injection is car-

ried out as the first injection at 2–4 weeks interval. After immunisation, vaccinated and unvaccinated groups are challenged with 0.5 mL virulent *C. perfringens* strain. If unvaccinated animals die and vaccinated animals survive for five days, the vaccine is considered as a potent product (Pilehchian Langroudi *et al.*, 2013). For the challenge, *C. perfringens* type A wild strain appears fully suitable in the chicken model (Peng *et al.*, 2020). The predictive value for vaccine efficacy depends on the animal challenge model, the type of microorganism, and route of experimental infection.

As mentioned, the efficacy testing of *C. perfringens* vaccine is mainly based on an immunisation-neutralisation in laboratory animal models. Many attempts have been developed as alternative test models in order to replace, reduce, and refine (3R) the laboratory animals used in efficacy tests of clostridial vaccines (Kumar *et al.*, 2018). Nowadays, *in vitro* assay has used for the evaluation of vaccine quality instead of animal-based methods, including limit of flocculation (LF) assay, enzyme-linked immunosorbent assay (ELISA), toxin binding inhibition (ToBI) test, cell culture, haemolysin assay, and lecithinase activity.

#### 6) Limit of flocculation (LF)

Limit of flocculation (LF) is an *in vivo* assay based on the antigen-antibody reaction that aggregates and form floccules. The assay was first developed for the evaluation of tetanus antitoxin and then epsilon antitoxin in the immunised laboratory and target animals and comparing its sensitivity to serum neutralisation test and TCP assay. The results showed that the correlation coefficient between TCP and LF was 99.97%. However, LF was not very sensitive compared to SN due to the

result read by eye. Nevertheless, it was suggested for the evaluation of *C. perfringens* toxin, instead of TCP (Sobrinho *et al.*, 2009). Saadh (2020) developed this test for determination of *C. perfringens* beta toxin. The LF assay is easy, straightforward, rapid, time-saving, and economical without using animals (Spaun & Lyng, 1970) although reading should be done by trained technicians (Sobrinho *et al.*, 2009).

#### 7) Enzyme-linked immunosorbent assay (ELISA)

ELISA is a quantitative method for calculating toxin and toxoid concentration in the biological samples such as vaccines. ELISA the most commonly used for determination of efficiency of clostridial vaccine (Uzal *et al.*, 2003; Redhead *et al.*, 2011) although the sensitivity depends on the type of used ELISA. Sandwich ELISA, capture-ELISA, and competitive ELISA can be applied to quantify beta and epsilon toxins in fermenter supernatants. Uzal *et al.* (2003) showed higher sensitivity of polyclonal capture-ELISA than that of monoclonal capture ELISA for epsilon toxin assay. Nagahama *et al.* (1991) showed that ELISA can detect up to 1.0 mg/mL for beta toxins and 0.1 ng/mL of *C. perfringens* epsilon toxin (Nagahama *et al.*, 1991) and El Idrissi & Ward (1992a,b) reported that sandwich ELISA could detect 2 ng/mL with linear correlation in the range 7.8–125 ng/mL. Based on their results, the sensitivity and specificity of ELISA were 90.5% and 89.2% for the beta toxin and 97.4% and 95.6% for the *C. perfringens* epsilon toxin. Sandwich ELISA has high specificity and sensitivity but finding two antibodies against the same target can be challenging. Roskopf-Streicher *et al.* (2004b) confirmed the reproducibility of competi-

tive ELISA for antibodies against vaccine's epsilon toxin. Another ELISA was used to calculate the antibodies and it was concluded that the method could be used as an alternative for the *in vivo* assay. As mentioned, in animal-based assay, the manufacturer QC unit has to use numerous mice as animal indicator for quantification of active toxin and to determine the international unit separately for each toxin in the combined vaccine (Redhead *et al.*, 2011). It should be noted that potency tests for veterinary clostridial vaccines consist of two stages: vaccination of rabbits with the specified dose for laboratory animals (twice) and titration of antibody against vaccine elements by toxin neutralisation assay in mice, although ELISA consists of a single stage. According to European Directorate for the Quality of Medicines & Healthcare (EDQM), for the antibody titration, sufficient information supports our movement from animal to *in vitro* assay. Monographs 0361, 0362, 0363, and 0364 have mentioned that permit to manufacturer for use immunochemical methods such as ELISA for quality control of the *C. perfringens* vaccine (Lucken *et al.*, 2002). The correlation between mouse neutralisation test (MNT) or SN and ELISA results has been proved in the researches. In a study, a competitive ELISA for epsilon antibody assay was developed by monoclonal antibody and it was compared with mouse neutralisation test (MNT). Their results showed a good correlation between two *in vivo* and *in vitro* assays (Sojka *et al.*, 1989). Uzal *et al.* (1997) reported a correlation coefficient of 0.96 between indirect ELISA and competitive ELISA for epsilon antibody assay. In indirect ELISA, several secondary antibodies can bind to the primary antibody. However, it is more time-consuming compared to direct ELISA and

has cross-reaction from the secondary antibody, but in competitive ELISA depends on the base ELISA selected. Ebert *et al.* (1999) showed correlation coefficients of 0.88 and 0.41 for the capture ELISA and competitive ELISA, respectively. The ELISAs are time-saving and simple, able to quantify and qualify toxins and toxoids; the drawback of ELISA is detecting only total antibody titres, so it is unable to detect neutralising antibody titres (Weddell & Worthington, 1985). Furthermore, purification of monoclonal antibody is challenging and non-specific reactions are prominent problem (Arslan & Erbaş, 2023). In addition to the ELISA developed by researchers, commercial kits are nowadays available for the detection of *C. perfringens* alpha, beta, epsilon, and theta toxin in biological fluids. Commercial kits use monoclonal antibody to obtain high specificity and reliable results. The application of the ELISA kits is user-friendly and flexible.

#### 8) Toxin binding inhibition (ToBI)

The toxin binding inhibition test is based on the inhibition of toxin binding to antitoxins adsorbed onto a microplate (Sobrinho *et al.*, 2010). The ToBI test was first developed for antitoxin assay against tetanus and diphtheria toxins (Hendriksen *et al.*, 1988). Several reports have assessed the efficiency of the ToBI test in clostridial vaccines. Fayez *et al.* (2005) reported a good correlation (96%) between MNT as a golden standard and this test. Sobrinho *et al.* (2014) reported correlation coefficients above 97% for epsilon antitoxin assay that can detect precisely neutralising antibody titres. The same results were obtained in other studies for epsilon, diphtheria antitoxin, and tetanus toxoid assay (Sobrinho *et al.*, 2010). The ToBI test is time-saving, reproducible,

precise, and invaluable as it can detect exactly neutralising antibody titers.

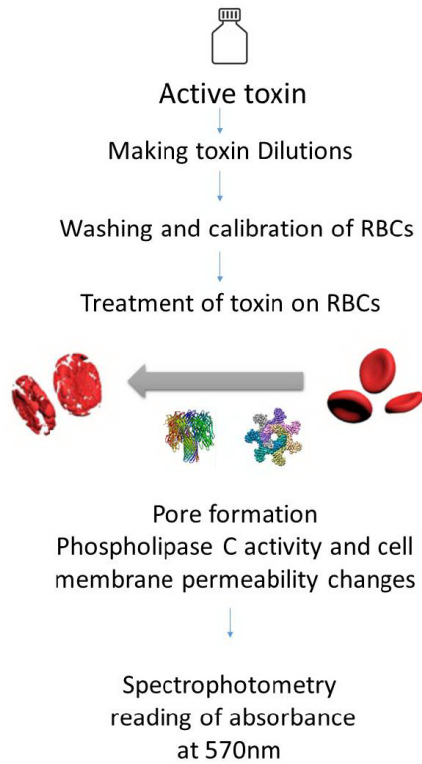
#### 9) Cell culture assay

Knight *et al.* (1986) examined the cytopathic effect of clostridia on cell culture and found that *C. septicum* and *C. novyi* filtrates affected the most cell culture. Knight *et al.* (1990) were the first to successfully develop the cell culture assay for alpha toxin assay. The cytopathic effect of *C. perfringens* type D epsilon toxin was observed on MDCK cells (Payne *et al.*, 1994; Lindsay, 1996). Borrmann (2006) developed the cell culture assay for the evaluation of *C. perfringens* epsilon antitoxin and *C. novyi* alpha antitoxin in rabbit sera and compared it with SN assay. The results showed a good correlation between both tests (Borrmann *et al.*, 2006). Salvarani *et al.* (2010) also successfully developed the cell culture assay for *C. septicum* alpha antitoxin assay and compared its sensitivity to SN test. They reported antibody titres with a correlation coefficient of 99.12% between *in vivo* and *in vitro* assay. Similar results were also reported for *C. septicum* and *C. novyi* type B alpha-toxin by Lima *et al.* (2020). Cytotoxicity assay can be substituted *in vivo* mouse neutralisation (Borrmann *et al.*, 2006; Salvarani *et al.*, 2010) which has advantages over the animal-based method. The cell culture assay does not require monoclonal antibodies, which is a prerequisite for ELISA systems (Ebert *et al.*, 1999; Roskopf-Streicher *et al.*, 2004a). It is more sensitive than titration in animal models. The use of standard reagents is decreased in cell culture (Salvarani *et al.*, 2010). It is a specific, reproducible, and reliable method for toxin assay (Lima *et al.*, 2020). The drawback of cell culture is cells storagee in liquid nitrogen with a cryopreservation medium, including

DMSO. Maintenance of asepsis is also complex; expertise and experience are urgent in working.

#### 10) Haemolysin assay

The haemolysin test is applied to assay toxin activity in fermenter culture supernatants. The test can be done to determine of the minimum effective dose (M.E.D) or minimum haemolytic dose (M.H.D). It is the smallest amount of toxin that can be detected by haemolytic activity. The choice of red blood cells (RBCs) for haemolysin assay depends on the susceptibility of red cells to a specific toxin. For example, *C. perfringens* alpha toxin cannot affect horse RBCs. RBCs of sheep and rabbits are the most recommended red cells that can be used. For performing this test, it is essential to know that efficient washing and careful calibration of RBCs is crucial for ensuring reliable and reproducible results (Batty, 1971). Similar to MLD assay, a serial dilution of toxin is prepared. Washed RBCs are added to tubes, mixed, and incubated at 37 °C for 20 min. The mixture was centrifuged at 10,000 rpm, and the absorbance of the supernatants was measured at 570 nm (Fig. 4). The smallest amount of toxin that can lyse the cell suspension, is defined the minimum haemolytic dose of the toxin (Suzaki *et al.*, 2021). The alpha-toxin of *C. septicum* had haemolytic activity on five types of red blood cells. However, the haemolysis rate in rats and rabbits red blood cells was the most and least hemolytic activity of all, respectively (Najafi *et al.*, 2019). In another study, the haemolysin test was performed for *C. perfringens* type A alpha-toxin, and the purification steps were monitored (Fathi Najafi, 2020), similar to induction of *C. septicum* alpha-toxin production (Fathi Najafi *et al.*, 2020a).



**Fig. 4.** A schematic presentation of haemolysin assay. Changing the red cells' permeability by degradation from alpha and epsilon toxins of *C. perfringens*. Pore formation and phospholipase C activity on phospholipids are the main causes of cell membrane degradation.

A similar study was performed by Jafari *et al.* (2021) for beta toxin purification steps monitoring (Jafari Khoshanabadi *et al.*, 2021). Using RBCs as indicator simplifies the monitoring of toxin production in fermenter and purification steps. Haemolysin assay is cheap, accessible, and simple without the utilisation of laboratory animals to perform.

#### 11) Lecithinase activity

Lecithinase is the phospholipase that act on lecithin. Lecithinase activity of *C.*

*perfringens* alpha toxin (based on Nagler reaction) can be used as an indicator for monitoring of fermenter alpha toxin content. Egg yolks are washed in 0.85% saline, the albumin removed, then clarified with paper and sterilised. It should be remembered that the shelf life of this reagent is short, and after one month the results may be unreliable (Batty, 1971). Lecithin dissolved in Tris-HCl buffer is added to samples and incubated at 37 °C for 30 min. The absorbance of the samples was measured at 640 nm. Fathi Najafi (2020) used lecithinase assay for *C. perfringens* alpha toxin detection and as an indicator in purification steps of clostridial species.

#### DISCUSSION

*C. perfringens* is the cause of enterotoxaemia, which is a fatal enteric disease. Enterotoxaemia affects domestic animals resulting in sudden death. *C. perfringens* produces major toxins named alpha, beta, epsilon, and theta categorised into A-E types. Vaccination and good management of farms are crucial for the prevention of disease. The quality control assay including sterility, safety, purity, and efficacy, is used for the evaluation of clostridial vaccines. The efficacy is the most critical assay employed in vaccine manufacture. So, it should be designed and optimised to monitor the concentration of major *C. perfringens* toxins during production and estimate the impact on the final product on eliciting an immunological response. Veterinary clostridial vaccines commonly contain more than one species to induce animal immunity. This complex nature of combined vaccines makes in-process QC and final product QC more complicated, and the manufacturer may be required to use a combination of validated and not

validated tests for assessment of toxin concentration and final product toxoid concentration (Taffs, 2001). So many factors influence the efficacy of a clostridial vaccine, including the toxins of other bacterial species, the detoxification process, and the presence of impurities, adjuvant, and excipients (Metz *et al.*, 2007). For example, the aluminum salt adjuvant stimulates Th2 cells and results in increased antibody production but it is unable to stimulate Th1 cells. MF59 results not only in increased Th2 cells, but also stimulates effectively Th1 cells (Araghi *et al.*, 2023). Another adjuvant, ISA206, is based on water/oil/water emulsion which slowly releases the antigen, so induces a strong long-term immunity (Araghi *et al.*, 2023). The advantage of using mineral adjuvant especially  $Al(OH)_3$ , for the formulation stage of clostridial vaccines is the low cost compared to an oil adjuvant. Several studies carried out on chitosan adjuvants in vaccines showed a significant increase in humoral and cellular immunity. Chitosan is considered a suitable adjuvant due to its permeable, stable, antibacterial, antifungal, and antioxidant activities (Ahmed & Aljaeid, 2016). Chitosan can also regulate the release of the antigen, and regulate the response of humoral immunity and cell-mediated immunity after the vaccination schedule (Fathi Najafi *et al.*, 2020). On the contrary, de Castro Oliveira *et al.* (2021) reported that purified toxoid vaccine along with aluminum hydroxide adjuvant induces humoral immunity more than adjuvant chitosan after the vaccination in cattle and goats. On the other hand, treatment of *C. perfringens* bacteria or toxoids with preservatives such as formaldehyde makes the condition more complicated because these reagents have a significant effect on the structure of antigens, epitopes, and stabil-

ity of proteins. In many cases, formaldehyde causes loss of some epitopes and forming new epitopes. So, the results of active and inactive substances may be different. The result of toxin inactivation is a heterogeneous product with many proteins and amino acids, and epitope modifications. In neutral or slightly acidic conditions, formaldehyde can act and add to the protein group by methylene bridges. Formaldehyde is recognised as a cross-linking agent that participates in protein structure and creates covalent bonds through intermolecular and intramolecular linkage (Metz, 2004). Being a low-molecular-weight molecule, formaldehyde could be easily moved between the protein structures. Thimerosal breaks down to ethylmercury and thiosalicylate in the body so does not stay in the body for a long time and cleans quickly than some preservatives and has no neurologic toxicity effects on body. Therefore, it is very safe for use (Hurley *et al.*, 2010). Action of thimerosal is along with prevention of growth of bacteria and fungi with minor side effect (Barrett, 2005).

There are different techniques to control of the efficacy of clostridial vaccines. Most of these tests are designed and validated using laboratory animals, requiring a large number of mice. In veterinary vaccine manufacture, there is a strong interest in finding and developing methods for intermediate products antigen, and final product antigen measurement to reduce animal use, improve the reproducibility of products' biological features, formulations, finished product efficacy consistency, and shorten testing timelines (Kubiak, 2012). ELISA and MLD, haemolysis, lecithinase assays and cell culture test can be applied to calculate alpha, beta, and epsilon toxins in fermenter culture content before inactivation. These

tests are categorised as an in-process control to measure of quantify fermenter toxin content, and monitor the reduction of using of toxic substances in the detoxification process. After formulation and blending of clostridial species, the toxoid concentration can be calculated by ELISA, TCP, LF, ToBi, challenge, and SN assay. Each of these quality control assays has advantages and some limitation that have been used in upstream and downstream step of vaccine manufacture. The clostridial species are commonly blended based on the proper toxin concentration in final product under good manufacture practices. Consideration for quality control assay should be based on multiple factors including active or inactive form of major toxins, biological reference standards, supplies and facility of production department. Special regulations are also required at a manufacturing site that makes some challenges for good monitoring. All quality control parameters applied for clostridial vaccine should be validated based on biological reference standards at a manufacturing unit. Furthermore, all the methods applied in the quality control of vaccines should be well done by trained personnel, in order to gain reliable results with high precision, and reproducibility.

## CONCLUSION

This study presents a literature overview of the evaluation of toxin and toxoids by *in vivo* and *in vitro* methods. Comparison and knowing the nature of each QC method is also very crucial for manufacturers and QC organisations. Laboratory animals are used extensively for evaluation of efficacy but *in vivo* assays are not always possible to perform. So, alternative tests can be occasionally used. Although the *in vitro* methods have some advan-

tages, yet there is no validated method for complete replacement of *in vivo* methods.

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