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REVIEWS

Vaccination against pathogenic clostridia in animals: a review

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Abstract

Clostridium is a Gram-positive, rod-shaped, anaerobic, and spore-forming bacterium, which is found in the surrounding environments throughout the world. *Clostridium* species cause botulism, tetanus, enterotoxaemia, gas gangrene, necrotic enteritis, pseudomembranous colitis, blackleg, and black disease. *Clostridium* infection causes severe economic losses in livestock and poultry industries. Vaccination seems to be an effective way to control *Clostridial* diseases. This review discusses the toxins and vaccine development of the most common pathogenic *Clostridium* species in animals, including *Clostridium perfringens*, *Clostridium novyi*, *Clostridium chauvoei*, and *Clostridium septicum*. In this comprehensive study, we will review different kinds of clostridial toxins and the vaccines that are experimentally or practically available and will give a short description on each vaccine focusing on its applications, advantages, and disadvantages.

Keywords *Clostridium* · Toxin · Vaccine · Immunogenic · Veterinary

Introduction

Clostridium is a Gram-positive, rod-shaped, anaerobic, and spore-forming bacterium. *Clostridium* consists of around 250 species, which can be found in different environments in the world. Some cases of *Clostridium* infections have been reported since years ago. *Clostridium* infection causes severe economic losses in livestock and poultry industries and wild life. Some *Clostridium* species are the cause of disease in humans due to the release of exotoxins (e.g. tetanus, botulism, food poisoning, gas gangrene). Also, some *Clostridium* species are important in animals as causative agents of enterotoxaemia, gas gangrene, necrotic enteritis, blackleg, and black disease. *Clostridium* exotoxins cause mild to severe damage in gastrointestinal tract, soft-tissues, and nervous system (Carter et al. 2014). Practices with improper hygiene control and the lack of vaccination are important causes of clostridial disease in human and animals. Hence, regular vaccination is an effective way to control clostridial infection in the world.

In this review, the clostridial important toxins are listed and their 3D structures (accessed by PHYRE2 and SWISS-MODEL servers) as well as their genetic origins are presented (Fig. 1). Also, the main clostridial species used in vaccine research and industry are described and different types of available commercial and experimental vaccines are explained.

Clostridium perfringens

Clostridium perfringens is a straight or slightly curved rod-shaped and non-motile bacterium, with blunt ends, which is arranged singly or in pairs, with 0.6–2.4 μm width and 1.3–19.0 μm length. On sheep blood agar, large flat and rough-edged or smooth and domed colonies can be observed with large zones of partial haemolysis out of narrow zones of complete haemolysis. They are very pleomorphic and sensitive to oxygen pressure (Brazier et al. 2002). The *C. perfringens* species is classified into six isotypes including A, B, C, D, E, and F based on their major toxins, iota (*ia*), alpha (*cpa*), beta (*cpb*), and epsilon (*etx*) (Kiu and Hall 2018). Only types A, C, and F have been determined to cause disease in humans, whereas all of the types have been demonstrated to cause disease in

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animals (Li et al. 2016). *C. perfringens* also produces other toxins such as enterotoxin (cytotoxin), necrotic enteritis beta-like toxin, delta (hemolysin), teta or perfringolysin (hemolysin (O_2 labile), cytolysin), kappa (collagenase, gelatinase), lambda (protease), mu (hyaluronidase), nu (deoxyribonuclease), gamma, eta, and neuraminidase (sialidase) (McDonel 1980). Structures of clostridial major and minor toxins are presented in Fig. 1. A brief description of some of its toxins is defined below:

Alpha toxin or phospholipase C

Chromosome-encoded alpha toxin (43 kDa) is produced by all types of *C. perfringens*. Alpha toxin has local effects on the cell membrane and, finally, leads to endocytosis and cell death (Oda et al. 2015). *C. perfringens* type A causes traumatic clostridial myonecrosis in both humans and animals (Stevens et al. 2012), food poisoning and gas gangrene in humans, enterotoxaemia in lambs (yellow lamb disease), and

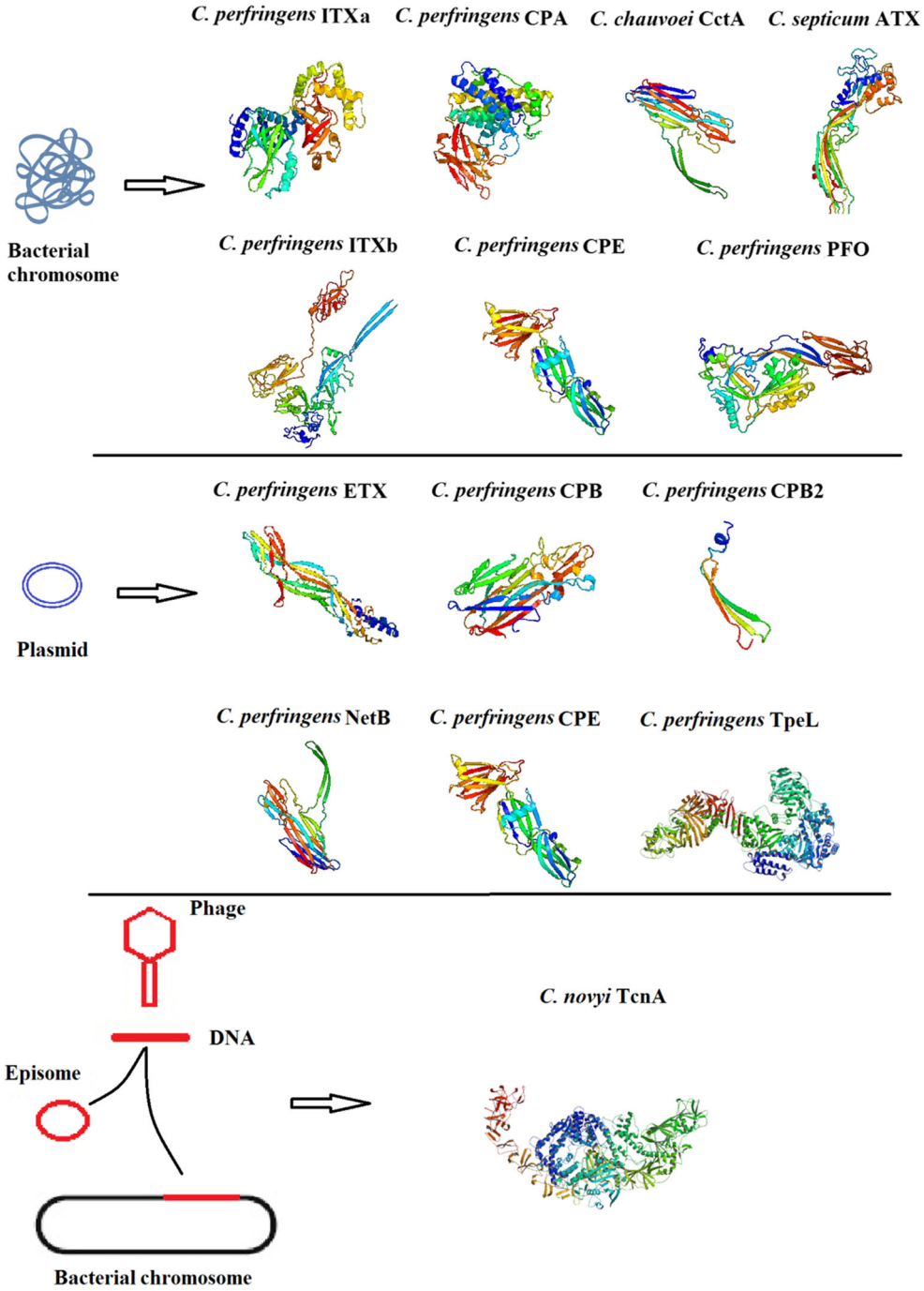

Fig. 1 3D structure of clostridial major toxins and their genetic origins

enteritis or enterotoxaemia in cattle, pigs, horses, and goats. However, the role of alpha toxin for intestinal disease is controversial (Goossens et al. 2014). This toxin is responsible for necrotic enteritis in chickens (Sawires and Songer 2006), but its role is also controversial (Keyburn et al. 2008).

Epsilon toxin

Plasmid-borne epsilon toxin (ETX) is produced by *C. perfringens* type D and B strains, which cause fatal enterotoxaemia (pulpy kidney) in sheep and goats (Uzal et al. 2014). This toxin is associated with sudden change and large consumption of carbohydrates, which allow the rapid growth of *C. perfringens* strains (Songer 1996). The accumulation of the active toxin (29 kDa) in the intestine increases permeability of the animal intestinal epithelium, and then, the toxin is absorbed into the bloodstream and spreads to other organs, such as the brain, lungs, and kidneys (Popoff 2011).

Beta toxin

Plasmid-borne *C. perfringens* beta toxin (CPB) is produced by *C. perfringens* type B and C strains. *C. perfringens* type B causes lamb dysentery, and *C. perfringens* type C causes enterotoxaemia and necrotic enteritis in domestic animal newborns. Beta toxin forms an oligomeric complex that channels into the cell membrane, enters into the bloodstream (Nagahama et al. 2015), and causes necrosis of the small intestine, toxaemia, and shock (Uzal et al. 2014).

Beta2 toxin

The CPB2 is produced by all types of *C. perfringens*. The *cpa*-encoding gene has been characterized in strains of *C. perfringens* types A–E. *C. perfringens* type D *cpb*2 gene is encoded by 48–110-kb plasmids. In *C. perfringens* type E isolates, *cpb*2 gene is located on 70–90-kb plasmids. Also, *C. perfringens* type B *cpb*2 gene is located on a 65-kb plasmid that also carries the *etx* gene (Uzal et al. 2010). Beta2 is a pore-forming toxin, which causes enteritis in neonatal pigs and diarrhoea in horses (El-sify 2015).

ITX

Iota toxin is a binary toxin including Ia (an enzymatic component) (45 kDa) and Ib (a binding component) (100 kDa). The ITX components are encoded by *iap* (~1160 nt) and *iab* (~2630 nt) genes. Ib section binds to a surface receptor on the target cells and then heptamerizes to form channels, allowing the enzymatic component (Ia) to translocate into the cytosol disrupting the cell cytoskeleton (Takehara et al. 2017). Iota toxin causes increased vascular permeability, which is associated with hemorrhagic enteritis (Uzal et al. 2010).

CPE

The gene (*cpe*) encoding *C. perfringens* enterotoxin (CPE) is encoded either on the chromosome or on the plasmid. CPE is a pore-forming toxin (~34 kD) and has one free sulfhydryl group (Granum et al. 1981), which is produced by *C. perfringens* types A and E and some isolates of types C and D. *C. perfringens* type A isolates are divided to two *cpe* plasmid families: a ~75-kb plasmid family that carries the *beta2* toxin gene and a ~70-kb plasmid family lacking the *beta2* toxin gene (Uzal et al. 2010). The toxin binds to claudin receptors on the target cells, forming pores in the cell membrane, allowing an unregulated influx of calcium, which leads to necrosis (Freedman et al. 2016). CPE is responsible for food poisoning, sporadic diarrhoea, antibiotic-associated diarrhoea, and sudden infant death syndrome in humans (Lindström et al. 2011); however, the role of *cpe* gene for gastrointestinal disease in animals is not clear (Uzal et al. 2010).

NetB

Plasmid-borne necrotic enteritis beta-like toxin (NetB) is produced by *C. perfringens* type A isolates (Keyburn et al. 2010). NetB is a β-pore-forming toxin (33 kDa) with 323 amino acids, which is an important virulence factor involved in necrotic enteritis in birds (Keyburn et al. 2008). Seven NetB monomers make a heptameric structure that forms channels in phospholipid bilayers (Rood et al. 2016) allowing cations to enter the cell, resulting in cell rounding and lysis (Yan et al. 2013).

C. perfringens large cytotoxin (*TpeL*)

TpeL is produced by *C. perfringens* type A, B, and C strains (Amimoto et al. 2007). *C. perfringens* type B and type C TpeL toxins are encoded by plasmids ranging from 60–65 kb to ~ 90 kb. There is no information about TpeL toxin of *C. perfringens* type A isolates (Sayeed et al. 2010). TpeL toxin is the largest *C. perfringens* toxin. TpeL toxin binds to LDL receptor-related protein 1 in host cells and enters the target cell by endocytosis. Then, the toxin enters to endosomal membrane and releasing the DXD fragment (glycosylating domain, which is located in the N-terminal region) into the cytosol. DXD fragment modifies the regulatory GTPase (including Rac1, Ras, Rap and Ral) and disrupts the regulation of the actin cytoskeleton leading to disaggregation of the intercellular connections and then cell death (Nagahama et al. 2012). The role of TpeL is not obviously known, but it has been mentioned that TpeL might have been associated with avian necrotic enteritis (Coursodon et al. 2012).

Teta toxin or PFO

The gene (*pfo*A) encoding perfringolysin O (PFO) is located on the chromosomal DNA near the origin of replication (Shimizu et al. 2002). PFO is produced by all types of *C. perfringens*. The molecular weight of the PFO is 54 kDa and consists of four domains and a 27-amino acid signal peptide (Tweten 1988). The C-terminal domain binds to cholesterol and causes conformational changes in other domains and insertion in membrane (Shepard et al. 2000). PFO has been associated with gas gangrene and bovine necrohemorrhagic enteritis (Awad et al. 2001; Verherstraeten et al. 2013).

Clostridium novyi

C. novyi or *C. oedematiens* is a Gram-positive rod-shape and motile bacterium with the dimensions of 1.1–2.5 × 3.3–22.5 μm. *C. novyi* is a fastidious bacterium due to its obligate anaerobic conditions (Moore 1968). On blood agar, colonies are small, flat, rough or rhizoidal, translucent and β-haemolytic for 48–72 h (Brazier et al. 2002). *C. novyi* is classified into four different types, including A, B, C and D. *C. novyi* type B and A produce alpha toxin (TcnA) (Fig. 1), which has glycosyltransferase activity that modifies small GTP-binding proteins and causes is responsible for cell rounding (Selzer et al. 1996). The gene encoding TcnA is located on the prophage genome in *C. novyi* strains (Skarin and Segerman 2014). This bacteriophage is responsible to regulate toxin production (Fortier 2017). Also, *C. novyi* type B produces beta toxin, that has phospholipase activity (Hauer et al. 2004). *C. novyi* type B causes black disease, which is an acute toxaemia especially in sheep and cattle (Navarro and Uzal 2016).

Clostridium chauvoei

Clostridium chauvoei is a straight rod-shape bacterium with rounded ends and 0.6 × 3–8 μm dimensions, which is arranged singularly or in short chains of bacteria and is motile by peritrichous flagella. On the sheep blood agar, it forms large (5 mm in diameter) colonies, with hemolysis. *Clostridium chauvoei* toxin A (CctA) is the major virulence factor (Frey et al. 2012) with molecular mass of 32.2 kDa, which causes cell lysis by increase in permeability of the animal tissue (Nicholson et al. 2019). Other virulence factors are associated with the pathogenesis including neuraminidase/sialidase NanA, beta toxin-DNAse, oxygen-labile hemolysin D (or hemolysin III), hyaluronidase Nag (previously called γ-toxin), and flagella. *C. chauvoei* is responsible for blackleg in cattle and rarely in small ruminants, which spreads in a short time and kills the animals (Frey et al. 2012).

Clostridium septicum

Clostridium septicum is Gram-positive, rod-shape bacteria with numerous sub-terminal spores. On blood agar, they usually produce a thick haemolytic swarming growth (Brazier et al. 2002). *C. septicum* produces four toxins, namely, alpha, beta, gamma, and delta. Alpha toxin (ATX) (Fig. 1) is the major virulence factor, encoded by the *csa* gene (Kennedy et al. 2009). Alpha toxin has lethal, haemolytic, and necrotizing activities. Beta toxin (desoxyribonuclease) has enzymatic activity. Gamma toxin is also an enzyme. Delta toxin has necrotizing activity, which can be demonstrated by intracutaneous inoculation of guinea pigs. *C. septicum* is responsible for myonecrosis in humans and braxy and malignant oedema (gas gangrene) in ruminants and clostridial dermatitis in poultry (Zaragoza et al. 2019). *C. septicum* causes severe economic losses in livestock and poultry industries.

Vaccine production

Vaccination is one of the most effective methods to prevent diseases. A vaccine helps the body's immune system identify and eradicate the life-threatening pathogens such as *Clostridium* species. Clostridial vaccines have been proven to be effective in domestic animals including cattle, sheep, and goats (Springer and Selbitz 1999) and have been noted to be available since the 1950s (Sargison 2009). These vaccines are effective for the most common clostridial diseases, including pulpy kidney, black disease, and malignant oedema. However, vaccination against *C. chauvoei* has been reported as a common protective method since 1930, especially in cattle herds. A summary of commercial and experimental vaccines available in the world and Iran is described in Table 1.

Generally, clostridial vaccines (except *C. chauvoei*) contain one or more exotoxins produced by *C. perfringens*, *C. novyi*, and *C. septicum*. Commercial *C. chauvoei* vaccines consist of whole formalin-inactivated bacterial cultures, which are presented as monovalent or in combination with other clostridial agents (Zaragoza et al. 2019).

Evaluation of economic losses caused by *C. septicum* is hard due to the lack of data. However, a severe damage can be estimated because of high mortality rate (Baldassi et al. 1985). The control of *C. septicum* is based on preventive measurements and vaccination of the herd (Thachil et al. 2013). For black disease, the economic losses can be inhibited by administration of *C. novyi vaccines* in animals; however, control of fascioliasis is very important in the controlling program of black disease.

Clostridial vaccines have been produced in different methods with different efficiencies and advantages or disadvantages. Different types of vaccines available for each

Table 1 Comparison of commercial and experimental vaccines in the world and Iran.

Strain	Commercial vaccine in the world	Commercial vaccine in Iran	Experimental vaccines in the world	Experimental vaccines in Iran	Target animals
			recombinant CPA(Nagahama et al. 2013)	recombinant CPA, NetB and tpeL (Rostami et al. 2016)	
C. perfringens type A	toxoid vaccine (Zaragoza et al. 2019)	Not produced	recombinant CPA, CPB, ETX, and NetB (Zaragoza et al. 2019)	Recombinant *cpe* C-terminal (Taherian Fard et al. 2010)	Cattle and polutry
			Recombinant NetB (Keyburn et al. 2013)		
C. perfringens type B	toxoid vaccine (Moreira et al. 2016) or bacterin-toxoid vaccine	bacterin-toxoid vaccine (Ardehali and Darakhshan 1976, Ardehali	recombinant ETX (Zaragoza et al. 2019)	recombinant CPB (Bakhshi et al. 2016)	Domestic animals
	(Zaragoza et al. 2019)	and Darakhshan 1977)	recombinant CPB (Zaragoza et al. 2019)	recombinant TpeL (Mamandi et al. 2019)	
			recombinant CPA, CPB, ETX (Jiang et al. 2014)		
			recombinant fused ETX–CPB (Zaragoza et al. 2019)		
C. perfringens type C	toxoid vaccine (Springer and Selbitz 1999) or bacterin-toxoid vaccine (Zaragoza et al. 2019)	bacterin-toxoid vaccine (Ardehali and Darakhshan 1976, Ardehali and Darakhshan 1977)	recombinant CPB (Milach et al. 2012)	Not done	Domestic animals
			Recombinant CPA, CPB (Salvarani et al. 2013)		
			Recombinant fused CPA, CPB, CPB2 (Zeng et al. 2011)		
C. perfringens type D	toxoid vaccine (Kennedy et al. 1977, Moreira et al. 2016) or bacterin-toxoid vaccine (Zaragoza et al.	bacterin-toxoid vaccine (Ardehali and Darakhshan 1976, Ardehali and Darakhshan 1977)	recombinant ETX (Lobato, et al. 2010)	recombinant ETX (Aziminia et al. 2016)	Domestic animals
			ETX non-toxic Mutant vaccine	recombinant fused ETX–CPB (Langroudi et	

	2019)		(Alimolaei et al. 2016)	al. 2013)	
C. perfringens type E	Not produced	Not produced	Recombinant ITX (Das et al. 2016)	Recombinant ITX (Seyed Sayyah et al. 2018)	-
C. perfringens type F	Not produced	Not produced	Recombinant CPE (C-terminal) with Shiga toxin subunit (Hosomi et al. 2019)	Not done	-
C. perfringens type G	Not produced	Not produced	Recombinant NetB (Zaragoza et al. 2019)	Not done	-
C. novyi	toxoid vaccine (Amimoto et al. 1998, Roberts 2000, Robertson and Keppie 1943, Tytell and Logan 1947)	*C. novyi* with *C. perfringens* bacterin-toxoid vaccine (previously) (Moosawi 1988) Monovalent bacterin-toxoid vaccine (Ardehali et al. 1984)	nano vaccine (Felix et al. 2019)	Recombinant TcnA (Daryaei et al. 2017, Fathi Nahafi et al. 2014, Noshahri et al. 2016)	Sheep and cattle
C. chauvoei	bacterin-toxoid vaccine (Zaragoza et al. 2019)	Bivalent vaccine (previously) (Jabbari et al. 2008, Sori et al. 2018) Monovalent bacterin-toxoid vaccine (Pilehchian et al. 2002)	recombinant CctA (Frey et al. 2012)	Not done	Cattle and sheep
C. septicum	toxoid vaccine (Amimoto et al. 2002) or bacterin-toxoid vaccine (Thachil et al. 2013)	Monovalent bacterin-toxoid vaccine (previously) (unpublished data) *C. septicum* with *C. perfringens* bacterin-toxoid vaccine (Langroudi and Jabbari 2013)	recombinant ATX (Lancto et al. 2014)	recombinant ATX (Bozorgkhoo et al. 2014)	Domestic animals

clostridial disease mentioned in this review are presented as follows:

Whole formalin–inactivated vaccines

The production of the vaccine is achieved by culture of *C. chauvoei* strains in a fermenter using complex media containing casein hydrolysate, peptone, tryptone, yeast extract, and meat extract, NaOH, L-cysteine hydrochloride, glucose plus trace element, and vitamin for approximately 9 h at 37 °C and then inactivation by formaldehyde for approximately 6 days at 37 °C (Langroudi et al. 2012; Pilehchian et al. 2002). The efficacy of the blackleg vaccine has been surveyed in challenged vaccinated and unvaccinated cattle (control group). This study has shown that no cases of disease were found in the vaccinated cattle, while some unvaccinated cattle died or became ill. A significant difference between vaccinated and unvaccinated animals has been observed (Haghroosta et al. 2014).

Bacterin-toxoid vaccines

Bacterin-toxoid vaccine is produced by culture of *C. perfringens* type D, B, and C strains separately in fermenter using complex media containing peptone, yeast extract, glucose, or dextrin, vitamins, and trace element in anaerobic conditions for approximately 4–5 h. Finally, the whole culture is chemically inactivated using formaldehyde treatment (Ardehali and Darakhshan 1976).

For black disease vaccine, bacterin-toxoid vaccine has been produced by culture of *C. novyi* strains in glass bottles using complex media containing peptone or other protein sources, L-cysteine, maltose, and NaOH for approximately 72 h at 37 °C and inactivation of TcnA by formaldehyde at 37 °C for 3 days (Ardehali et al. 1984; Ardehali et al. 1986). Nowadays, production of the bacterin-toxoid vaccine is achieved by growing vaccine strain in fermenters containing peptone, L-cysteine, maltose, NaOH, vitamins and trace element, Tween 80, and glycerol in anaerobic conditions for approximately 24 h at 37 °C and inactivation by formaldehyde at 37 °C (Fathi Najafi et al. 1398).

For production of vaccine against *C. septicum*, the stains are cultured in anaerobic conditions in a fermenter using complex media such as meat peptones, tryptone, yeast extract, casein hydrolysate, glucose, trace elements and vitamins, L-cysteine hydrochloride, and NaOH at 37 °C with pH of 7.2–7.4 for approximately 9 h and inactivation by formaldehyde at 37 °C (Langroudi and Jabbari 2013).

Bacterin-toxoid vaccines have good efficiency due to the bacterial cell compartments leading to a high immune response against all the infection-associated antigens. Some of these vaccines are available without any additional inducing agents or adjuvants. However, some antibodies will be produced against some non-important parts of the pathogen with no specific function related to the disease and so impose extra burden to the immune system. This may decrease the efficiency of the adaptive immunity in specifically triggering the pathogenic factors. Killed vaccines usually require adjuvant and several injections (Baxter 2007).

Toxoid vaccines

Formaldehyde-inactivated *C. perfringens* supernatant (crude toxoid) has been produced by Lovland and his colleagues and they have proven that *C. perfringens* type A or C or combined type A and C crude toxoids are significantly successful in protection against infection (Lovland et al. 2004).

The ETX and CPB toxoid vaccine has been prepared from the inactivated cultures using ammonium sulfate to control enterotoxaemia in animals throughout the world (Fisher et al. 2006). Also, NetB toxoid vaccine has been able to protect against necrotic enteritis (da Costa et al. 2013). Additionally, alpha toxin (CPA) toxoid vaccine (NETVAX®, Schering-Plough) has been also prepared to protect against necrotic enteritis in birds (Fernandes da Costa et al. 2016). Different research have proved that the control of clostridial disease by administration of a *C. perfringens* toxoid vaccine has been effective in animals including piglets, cattle, lambs, sheep, and goats (Kennedy et al. 1977; Springer and Selbitz 1999).

Crude alpha-toxoid vaccines have been prepared using inactivated culture supernatant of *Clostridium* strains (Amimoto et al. 1998). *C. novyi* type B alpha-toxin was purified using column chromatography, inactivated by formaldehyde, mixed with aluminium phosphate (as gel adjuvant), and then administered to guinea pigs. Alpha antitoxin protective efficiency was estimated by challenge with *C. novyi* type B spores. Results showed that *C. novyi* vaccine (toxoid) was protective against challenge in guinea pigs (Amimoto et al. 1998).

In another effort, the inactivated culture was concentrated and purified by salting out or molecular filtration. Then, the residual agent was neutralized using sodium bisulfite solution. Finally, a multicomponent toxoid vaccine was prepared by mixing seven strains of *Clostridium* and saponin as gel adjuvant. The prepared vaccine was administered to cattle intramuscularly or subcutaneously. Also, the formulated vaccine was evaluated by potency tests in rabbits and guinea pigs. The result showed that the multicomponent toxoid vaccine was effective and safe without local reactions and had the potential to be used as a commercial vaccine (Roberts 2000). There are other studies that have proven the effectivity of toxoid vaccines in animals (Amimoto et al. 2002; Ardehali and Darakhshan 1976; Robertson and Keppie 1943).

C. septicum alpha toxin has been harvested, concentrated, purified, and inactivated by formalin, and the prepared alpha

toxoid has been shown to protect guinea pigs against spores of *C. septicum* (Amimoto et al. 2002).

Since toxoid vaccines are free of any additional antigens such as proteins bound to the bacterial cell wall, and they are low molecular weight proteins, they are not highly immunogenic on their own unless they are inoculated in large amounts or multiple doses. Hence, they need addition of adjuvants to induce a sufficient adaptive immune response in the hosts. These pure toxoids are being demanded by the community, because of no extra burden on the host immune system and being safe due to not including any bacterial cells or spores and no chance of conversion to virulence. Also, toxoid vaccines are expected to be more stable than whole-culture bacterial vaccines, as they are less susceptible to changes in storage conditions such as light temperature and humidity (Baxter 2007).

Genetically engineered vaccines

NetB mutant vaccine as a non-formaldehyde genetically produced toxoid has been reported to decrease cytotoxicity and haemolytic activity, purposing the mutant vaccine as a candidate potent vaccine (da Costa et al. 2013). Furthermore, ETX non-toxic mutant vaccine (toxoid) has been synthesized and expressed in *Lactobacillus casei* cells. The bacterial suspension expressing the recombinant protein was used for immunization in mice. The results showed that this vaccine could be an effective candidate vaccine against enterotoxaemia (Alimolaei et al. 2016).

In recent years, research has been focused on experimental recombinant *C. perfringens* vaccines. The *ETX* and *cpb* gene regions of the *C. perfringens* have been used for design of the new generation vaccines, separately (Aziminia et al. 2016; Bakhshi et al. 2016). Recombinant vaccines expressed in *E. coli* have resulted in high immunity in laboratory animals (Aziminia et al. 2016; Lobato et al. 2010). Also, monovalent recombinant CPA has been prepared and evaluated. The result of study showed that the recombinant vaccine seems to be protective in mice (Nagahama et al. 2013). Experimental monovalent recombinant Iota and TpeL toxins have been expressed separately in *E. coli* (Mamandi et al. 2019; Seyed Sayyah et al. 2018). Monovalent recombinant vaccines against *cpe* C-terminal region from *C. perfringens* type A strain did not show any systemic effect on BALB/c mice and guinea pigs (Taherian Fard et al. 2010). Moreover, it has been reported that recombinant NetB vaccine is effective against NetB infection in birds (Keyburn et al. 2013). Some recombinant multivalent vaccines have been prepared and compared to the traditional monovalent or bivalent vaccines (Goossens et al. 2016; Moreira et al. 2016; Salvarani et al. 2013). Pilehchian et al. proved that bivalent recombinant fused ETX–CPB vaccine seems to be protective in mice and rabbit (Langroudi et al. 2013). Furthermore, a study on bivalent recombinant CPE (C-terminal region) from *C. perfringens* and the subunit of the Shiga toxin (*Escherichia coli*) have shown that they were protective in mice (Hosomi et al. 2019). On the other hand, experimental trivalent recombinant CPA, NetB, and TpeL resulted in high antibody titre suggesting them as a candidate vaccine against clostridial disease (Rostami et al. 2016). Also, successful experimental tetravalent recombinant CPA, CPB, ETX, and NetB have been reported (Zaragoza et al. 2019). These research have shown that multivalent recombinant vaccines could be effective in comparison with traditional vaccines in immunization of animals (Goossens et al. 2016).

Bioinformatics and molecular biology studies have been conducted on the alpha toxin of *C. novyi* for identification of the most antigenic regions of the toxin. The result of this study has shown that recognition of a small fragment of alpha toxin with high affinity can produce higher immune responses than whole antigen (Fathi Nahafi et al. 2014). So, these experiments suggested that bioinformatics tools can increase the chances of production of highly antigenic vaccines (Fathi Nahafi et al. 2014).

Also, several experimental recombinant vaccines of *C. novyi* alpha toxin have been prepared and evaluated using immunological methods. Results have shown that antibody produced against recombinant proteins was more effective than that of normal toxin (Daryaei et al. 2017; Noshahri et al. 2016).

Soluble antigens are considered as immunogenic components associated with blackleg disease. CctA is the major virulence factor; hence, some experimental recombinant CctA vaccines have been developed and evaluated for the immune response against blackleg. Recombinant CctA vaccine has shown protective against challenge with virulent *C. chauvoei* strain. So, this toxin seems a valuable candidate for the design of a suitable vaccine (Frey et al. 2012). Also, a recombinant *nanA* containing the sialic acid-binding domain (CBM40) vaccine has been efficient against the *C. chauvoei* infection (Vilei et al. 2011). On the other hand, somatic antigens are considered as immunogenic components associated with the protection against *C. chauvoei* disease. Therefore, experimental recombinant vaccine has been developed and evaluated for protection against disease in mice. The results showed that poor immunity induced. So, a conformation-dependent epitope is important in immune response against blackleg (Kojima et al. 2000).

A recombinant alpha toxin (noncytolytic ATX) experimental vaccine has been made against *C. septicum* disease (Bozorgkhoo et al. 2014). The recombinant vaccine has been also compared to the traditional vaccine. ELISA analysis and challenge results showed higher antibody concentrations than native vaccine. The recombinant vaccine is inexpensive compared with traditional vaccines; so, it is a suitable candidate for new generation vaccine (Lancto et al. 2014).

Genetically engineered vaccines seem to be promising candidates due to the decrease in the laborious works and multiple steps in the culture and production of bacterial vaccines. Production of recombinant vaccines against clostridial infections has been considered as a non-toxic, high-yielding process with superior stability and biosafety (Salvarani et al. 2013). However, the need for adjuvants for induction of host immune system remains for intramuscular inoculation of purified recombinant toxoids. Another disadvantage of purified recombinant proteins is that the production process for insoluble proteins is laborious and time-consuming with multiple steps of solubilization, refolding, and purification. Although immunization of animals with non-purified recombinant antigens (e.g. inclusion bodies or supernatant lysate) has been proposed as a good alternative (Moreira et al. 2016; Lobato et al. 2010), there is still a doubt for replacing these kinds of vaccines because of the presence of bacterial contents such as lipopolysaccharides (LPS) (Terpe 2006).

Nanovaccines

In recent years, much attention has been focused on nanovaccines using nanoparticles as inducing adjuvants for enhancing antigen presentation and stability in the host. Also, one of the important problems in production of vaccines consisting of pore-forming toxins is to convert toxins to non-toxic toxoids without significant changes in the protein structure and antigenic epitopes. Current methods used for detoxification such as chemical- and heat-mediated methods can change the toxin structure and reduce immunogenicity and vaccine potency (Parish and Cannon 1960; Metz et al. 2004; Cryz Jr et al. 1982). A red blood cell membrane-coated nanoparticle system has been used to inactivate pore-forming toxins without protein denaturation, by neutralizing the membrane-damaging activity of toxins. Mice vaccinated with this nanoparticle-detained toxin showed higher immune response than with heat-denatured toxin (Hu et al. 2013).

Water-oil nanoemulsion containing concentrated and formalin-inactivated *C. novyi* type B α-toxin has been prepared and evaluated by alpha-toxin challenge in immunized mice. Necropsies of the liver, spleen, and other part of the body were also examined to determine the safety of the vaccine. Data suggested that the nanovaccine was efficient for promoting antigen delivery and protection against lethal doses of bacteria in immunized mice (Felix et al. 2019). The effect of chitosan nanoparticles has been studied on the efficiency of clostridial vaccines. The vaccine containing chitosan could stimulate humoral immunity 2–3 times higher than the non-chitosan toxoid vaccinated rabbits (Fathi Najafi et al. 2020) or non-immunized chickens (Ramadan et al. 2020). The results of nanovaccines should be compared with other vaccines currently used and their protective effects need to be evaluated in the field, to confirm their applicability. Possibly, it would be expected to use nanovaccines with no need to addition of adjuvants and yet obtain the desirable results after vaccination.

Polyvalent vaccines

Blackleg and hemorrhagic septicaemia vaccine have been produced in a traditional manner with anaphylactic shock or local inflammation for last years in Iran (unpublished data). Later, efforts were made to prepare a modified blackleg and hemorrhagic septicaemia vaccine, which showed no anaphylactic shock or local inflammation in laboratory animals or target animals. (Jabbari et al. 2008; Sori et al. 2018).

Different polyvalent clostridial vaccines are commercially or locally available including bacterin and/or toxoids of *C. perfringens*, *C. novyi*, *C. septicum*, *C. chauvoei*, *C. sordellii*, and also *C. tetani.* A study on two 9-valent clostridial vaccines has shown that the antibody titres were observed up to 90 days after second immunization and then decreased until becoming non-detectable at 6 months. Also toxoids of *C. tetani* and *C. novyi* type B were the most immuno-dominant antigens and *C. septicum* induced the lowest antibody response. Variable antibody response and shorter time period of antibody existence have led to the suggestion of antigenic competition in application of polyvalent vaccines (Rossi et al. 2018). That might be due to the suboptimal antigen presentation because of insufficient MHC-II binding of specific antigens, lower number of available T helper cells, and the presence of antigens in the vaccine that occupy the immune system (Dhungyel et al. 2014).

There has been no further information on the occurrence of this phenomenon while using polyvalent clostridial vaccines. Hence, it is worth focusing on the comparative studies on mono- and polyvalent clostridial vaccines to find the best number/volume/interval of doses for immunization or the optimum unit of each antigen in the polyvalent vaccine production.

Conclusion

Throughout the world, a wide variety of clostridial toxoid or bacterin-toxoid or whole culture vaccines are used to protect animals against diseases. Some of these vaccines have not been used at all in our country. For example, tetanus toxoid is not used as a monovalent vaccine in horses or as a polyvalent vaccine for immunization of goats and sheep in Iran. Also, *C. haemolyticum* has been used in parts of the world for protection against hepatitis infections of the liver, but it has not been used in Iran. In some other countries, a polyvalent vaccine consisting of *C. perfringens* types B, C, and D, *C. septicum*, *C. novyi*, *C. chauvoei*, and *C. sordellii* has been used to immunize animals against the related diseases. In Iran, a monovalent blackleg vaccine, monovalent black disease,

and polyvalent enterotoxaemia are available. The large-scale productions of clostridial vaccines have remained unchanged since the 1900s in the world. Nevertheless, it has been proven that the nanovaccines and recombinant vaccines are effective and can be potential alternatives to conventional vaccines in laboratory animals such as mice, rabbits or genie pigs; however, these vaccines are not commercially available yet, and their production has still several disadvantages compared with traditional vaccines. A major challenge in the development of an ideal vaccine lies in evoking immune system in the host. Perhaps, recent developments to improve clostridial vaccines include generation of mutants by recognition of clostridial target genes, as well as generation of DNA vaccines. Also, new vaccine formulations with appropriate adjuvants especially for toxoid vaccines are useful for augmentation of animal immune system. For new generation vaccines, it is important to consider purification and inactivation steps, antigen presentation and concentration, vaccine delivery and stimulation of specific immune responses especially to the polyvalent vaccines.

Author contribution LAK had the idea for the article, performed the literature search and prepared the original draft. AZ participated in literature search and critically revised the work.

Data availability Not applicable

Declarations

Ethics approval The manuscript does not contain clinical studies or patient data.

Consent to participate All the authors approved the final manuscript.

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