Int J Enteric Pathog. 2015 November; 3(4): e28979. doi: 10.17795/ijep28979

Published online 2015 November 2. **Review Article**

Isolation, Specification, Molecular Biology Assessment and Vaccine Development of *Clostridium* in Iran: A Review

Reza Pilehchian Langroudi[1,*]

[1]Department of Anaerobic Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Karaj, IR Iran

*Corresponding author: Reza Pilehchian Langroudi, Department of Anaerobic Vaccine Research and Production, Razi Vaccine and Serum Research Institute, P. O. Box: 31975148, Karaj, IR Iran. Tel: +98-2634502720, Fax: +98-2634552194, E-mail: r.pilehchian@rvsri.ac.ir

Received 2015 April 8; **Revised** 2015 June 9; **Accepted** 2015 June 18

Abstract

Context: The genus *Clostridium*, which consists of spore-forming anaerobes, can cause different diseases in domestic animals and human and some of them are serious and fatal. According to the increasing economic value of the meat and milk-producing animals, the importance of a certain number of such diseases in Iran is unquestionable.

Evidence Acquisition: In Iran, and probably in other Near East countries, much attention was formerly paid to control more serious contagious diseases, such as rinderpest, anthrax, etc. resulting in the negligence of diseases such as enterotoxaemia. The epizootiological position has now changed whereby some of the contagious diseases are eradicated or are being methodically controlled.

Now it is time to care about the other problems such as clostridial diseases, which threaten the health of the sheep and cattle. It is impossible to eradicate these infectious microorganisms, since they are normally found in the soil and the intestinal contents of apparently healthy animals. Therefore, it is necessary to resort to vaccination which in some cases has given encouraging results. To avoid the losses from such infections it is necessary to have the best possible vaccination information, methodically and regularity of the susceptible animals.

Conclusions: This review refers to the veterinary aspects of the anaerobic clostridial diseases and vaccine development concerning the works carried out in Iran and especially at the Razi Serum and Vaccine Research Institute in the last eight decades.

Keywords: Vaccines, Veterinary Aspects, Isolation, Specification, *Clostridium* spp.

1. Context

Clostridia are large, anaerobic, rod-shaped, and Gram-positive spore forming bacteria of highly polyphyletic class of Firmicutes. These bacteria are found either as vegetative forms or dormant spores. Soil and intestinal tract of animals, and human are their natural habitats. Dormant spores of several *Clostridium* species are found in healthy muscular tissue of horses and cows. Differentiation of the various pathogenic and related species is based on diagnostic characteristics of biochemical reactions, morphology of spore shape and position, and also the antigenic specificity of toxins or surface antigens (1). Pathogenic strains or their toxins may be acquired by susceptible animals either by wound contamination or ingestion. In many parts of the world, clostridial diseases are a constant threat to successful livestock production. The genus Clostridia is divided to histotoxic and neurotoxic Clostridia (2). The members of histotoxic Clostridia are invasive and cause extensive destruction of muscle and connective tissue and are characterized by the formation of gas, including *Clostridium chavoei*, *C. colinum*, *C. hemolyticum*, *C. novyi*, *C. perfringens*, *C. septicum* and *C. sordellii*. The neurotoxic Clostridia including *C. botulinum* and *C. tetani*, *C. difficile* and *C. spiroforme* are noninvasive and produce neurotoxin (3).

In Iran, clostridial infections are among the most important diseases of cattle and sheep. Among the various diseases caused by these groups of bacteria, lamb dysentery, struck, pulpy kidney and black disease in sheep and blackleg in cattle are often observed in the country. Strains of *C. perfringens* types B, C and D, *C. oedematiens* types B and D, *C. chauvoei* and *C. septicum* from the specimens of infected animals are isolated at the Razi Serum and Vaccine Research Institute in Iran (4).

2. Evidence Acquisition

2.1. Genus Clostridium and Associated Diseases

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic, spore-forming, and heat-resistant bacterium of genus *Clostridium* that has capsules, and the ability to produce heat resistant spores under improper environmental conditions, and is also a secondary pathogen in diseases such as necrotic enteritis (5, 6). *Clostridium per-*

fringens has 118 species and is classified into five isotypes (A, B, C, D, and E) based on producing four major toxins, iota (iA), alpha (cpa), beta (cpb) and epsilon (etx) (7, 8). *Clostridium botulinum*, *C. difficile*, *C. perfringens*, and *C. spiroforme*, can cause enteric problems in animals as well as humans. These diseases, which are often fatal, are partly attributed to binary protein toxins that follow a classic AB paradigm. All clostridial binary toxins destroy filamentous actin via mono-ADP-ribosylation of globular actin by a component within a targeted cell (9). Complete genome sequence of *C. perfringens* was reported in 2002 (10). Table 1 shows different types of *C. perfringens* and their major toxins.

Clostridium perfringens toxin type A strain that produce alpha toxin (CPA) is the most common type of *C. perfringens* and is a member of the normal intestinal flora of warm-blooded animals. This microorganism causes gas gangrene, food poisoning, and gastrointestinal illness in humans, necrotic enteritis in chickens, yellow lamb disease in sheep, enteritis, abomasitis (abomasal bloat) and enterotoxaemia in goats, cattle, pigs and horses (11, 12).

Clostridium perfringens type D is the causal agent of enterotoxaemia of sheep. It was first described by Lucey and Hutchins (13). This organism produces several major and minor toxins. Epsilon toxin is a major lethal toxin produced by *C. perfringens* types B and D (14, 15). In 1933 it was named Epsilon (16). It is responsible for a rapidly fatal enterotoxaemia in economically important livestock (17). In the small intestine of the infected animals, the toxin is produced in the form of prototoxin activated by proteolytic enzymes as well as other proteolytic enzymes in vitro in the small intestine (18, 19). Prototoxin activation by trypsin is due to cleavage and removal of a small 14-amino acid peptide from the amino terminal (20). There is a tryptophan residue and a histidyl residue in the structure of epsilon toxin which are respectively important and essential for its lethal activity (21). Recently, *Clostridium perfringens* type D epsilon toxin gene was cloned and expressed in *E. coli* and its immunization response was tested *in vivo*. Results showed good protection against native epsilon toxin (22, 23).

Clostridium perfringens type B belongs to enterotoxaemia is a major problem of veterinary sciences. Beta toxin produced by *C. perfringens* types B and C, is the major toxin of these types and causes inflammation and bloody necrotic enteritis and fatal diseases originating in the intestines of humans or live-stock (24, 25). It is known to aid in the lysis of endothelial cells by forming pores in the cell membrane (26). This function is necessary both for necrotizing enteritis and lethal enterotoxaemia caused by type C isolates (27, 28). A 17-protein exotoxin is produced by this bacterium which four of them are major (alpha, beta, epsilon and Utah), and the others (sigma, theta, kappa, lambda, mu, nu, neuraminidase and enterotoxin) are minor toxins (29). In 2012, in Brazil a vaccine was produced in *E. coli* (rBT) based on a beta toxoid of *C. perfringens* type C. The non-toxic rBT was innocuous for mice and induced 14 IU/mL of beta antitoxin in rabbits, which was complying with the European Pharmacopeia and CFR9-USDA guidelines (30).

The enteric toxins of *C. perfringens* showed two general characteristics 1st, beta and epsilon toxin are pore-forming toxins, and 2nd, iota and TpeL (31) modify an intracellular target. These enteric toxins are involved in the pathogenesis of avian enteric necrosis disease (24).

Clostridium septicum is a large anaerobic, Gram-positive, rod-shaped and fermentative bacterium of genus *Clostridium*. Terminal spore gives the bacterium a drumstick-like shape while holding and peritrichous flagella enable the bacterium to be motile. *C. septicum* is a member of the normal gut flora in humans and other animals; therefore, it can cause different diseases both in humans and animals. *Clostridium septicum* can produce and secrete a number of toxic proteins such as alpha, beta, gamma and delta. Alpha toxin that is the lethal cytolytic and the major virulence factor appears to be its immunodominant extracellular antigen (32, 33).

Clostridium chauvoei, (*C. feseri*) is an anaerobic, spore forming, motile, and polymorph bacteria, which its size varies from 0.5 - 1 to 3 - 8 micron and could be observed as individual bacterium, diplococcus, and rarely *streptococcus* (34). Blackleg is a fatal disease of young cattle. It produces an acute local infection, and the resulting blood poisoning leads to rapid death. *Clostridium chauvoei* and, less frequently, *C. septicum* are most commonly the responsible organisms. Vaccination is the only effective means to control blackleg disease. Several kinds of vaccine are commercially available (4).

Table 1. Different Types of *Clostridium perfringens* and Their Major Toxins

Clostridium Perfringens Type	Alpha Toxin	Beta Toxin	Epsilon Toxin	Iota Toxin
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Black disease or infectious necrotic hepatitis is an acute toxemia of sheep, cattle and occasionally pigs caused by the alpha toxin of *C. oedematiens* type B (*C. novyi*), which is a Gram-positive, endospore-forming, obligate anaerobic bacteria of the class Clostridia. It is ubiquitous, being found in the soil and faeces. This bacteria is pathogenic, causing a wide variety of diseases in man and animals. *C. novyi* alpha-toxin belongs to the family of large clostridial cytotoxins, which act on cells through the modification of small GTP-binding proteins. The distribution of the disease is worldwide and is always fatal in sheep, cattle and pigs (35).

Clostridium difficile, one of the most important anaerobic of genus *Clostridium*, is a prevalent factor that can lead to antibiotic associated diarrheas. This bacterium is the causative agent of pseudo membrane colitis. The role of this bacterium along with the overuse of antibiotics is proved to result in colitis. Toxins A and B are the major virulence factors of these bacteria (36).

2.2. Isolation and Specification of Different Species of Genus Clostridium in Iran

In Iran, the clostridial infections among domestic animals were reported first in 1938 during an outbreak of blackleg of cattle. The disease was found in many parts of the country especially on wet bottom lands, low hilly and sandy areas. Several sporadic outbreaks so far had been reported from different parts of the country; however, the most severe one occurred in August 1968 among herds of cattle in Southern Iran (34). Fifteen strains of *C. chauvoei* were isolated from the specimens received for laboratory diagnosis at anaerobic vaccine research and production department.

Further studies proved that the clostridial infections were widespread all over the country. The first strain of *C. Perfringens* type D was isolated from cases of enterotoxaemia of lamb and sheep in 1954 (37). Later on, the infections caused by *C. Perfringens* Iranian variant type B was isolated in 1954 from intestinal contents of enterotoxaemia of sheep and goats. Three strains of *C. welchii* type B were isolated which were different from the classical type B strains in the production of (kappa) and non-production of (lambda) and hyaluronidase toxins. Two of the strains were isolated from young goats and the other one from an adult sheep (38). *Clostridium perfringens* type C was isolated in 1971 from cases of necrotic enteritis of piglets (39) and enterotoxaemia of sheep. The first strain of *C. septicum* from cases of gas gangrene (malignant edema) in cattle was isolated in 1971.

Black disease is an acute and fatal disease of sheep and goats in Iran. Fifty one strains of *C. oedematiens* types A, B, and D were isolated and typed from liver lesions received from different parts of the country. Isolation and rapid identification by fluorescent labeled antibodies, typing, sugar fermentation, toxicity and hemolytic activity of the isolated strains were used for more than 330 suspected livers received to diagnose black disease from different parts of the country. Results showed that 187 cases were positive (40). *Clostridium oedematiens* type B strain, causative agent of black disease of sheep, was first isolated in 1969 and *C. oedematiens* type D was also isolated from cases of liver necrosis in sheep (41).

In 1988, a putrefied carcass of a dairy cow was submitted to Razi Institute. A necropsy was performed, but the internal organs were decomposed. Smears prepared from liver tissue and the Gram staining showed many Gram-positive rod-shaped bacilli, some of them with ovoid or elongated sub-terminal spores (42). In 1997, seventeen *C. perfringens* strains isolated by post mortem from sheep and goats, were examined by biochemical tests and enzyme immunoassay (EIA). Seven of these strains belonged to type B, eight strains were type D, one strain was type A, and one strain was untypable. To identify the Iran subtype of *C. perfringens* type B, the isolated strains were examined for Minor Toxin Lambda (proteinase). The results were compared to the characteristics of *C. perfringens* reference strains (43).

In 1999 specimens of pathological tissues of cattle were examined to identify the malignant edema causal agents. Nineteen specimens out of thirty-eight were positive and fluorescent antibody analyzing showed three strains of *C. septicum*, four strains of *C. chauvoei*, and one strain of *C. oedematiens*. Fermentation tests showed that eleven of the isolated strains were *C. perfringens* and they were all type A (44). Several suspected cases of anaerobic diseases from different parts of Iran were studied and intestine contents of fish, cattle and sheep, were examined for different types of *C. perfringens*. Results showed that 104 isolates out of 110 specimens were identified as *C. perfringens* type A (45).

2.3. Vaccination Against Clostridial Diseases

Vaccination is frequently practiced to protect animals against clostridial diseases and seems to be the most effective way to control *C. perfringens* diseases. However, the industrial production of clostridial toxins is laborious. Wide varieties of vaccines are available, in the form of bacterins, toxoids, or mixtures of bacterins-toxoids. Single vaccination with most clostridial vaccines does not provide adequate levels of protection and a booster dose within three to six weeks is needed. Since young animal vaccination dose is not adequate for protective immunity until at least until the age of one to two months, therefore, most vaccination strategies target the pregnant dam therefor the maximal immunity is transferred to the neonate in colostrum. The clostridial vaccines often cause tissue reactions and swelling and should be administered in the neck and by the Subcutaneously rather than the intramuscular (IM) route. Most commercial vaccines are inactivated and may contain two to eight combinations of clostridial bacterins/toxoids. These should be optimally timed for provision of maximal protection at the most likely age of susceptibility (46).

2.3.1. Clostridial Vaccine Production for Veterinary Use in Razi Institute

In 1976, large-scale production and the standardization

of polyvalent *C. perfringens* vaccine in Iran was started. Based on this report over 20 million doses of this vaccine was produced and utilized in Iran every year. Certain modifications were made to the culture media used in the animal anaerobic bacterial vaccine Department of Razi Institute to produce this vaccine at low cost. The prepared vaccine was highly immunogenic as determined by the laboratory examination on the quality of the vaccine according to British Veterinary Codex and the field reports (4) and European pharmacopoeia (47).

A report in 1988 revealed that two types of clostridial vaccines had been prepared and tested in rabbits and sheep according to the british pharmacopoeia (BP) and field reports; one including *C. perfringens* types, B, C, D and *C. oedematiens* and the other one including *C. chauvoei*. Both vaccines had developed adequate antibody titer in the injected animals (48).

In 1992, attempts were made to produce and formulate the ingredients of a culture medium suitable to obtain a highly immunogenic *C. oedematiens* type B vaccine to immunize sheep and goats against black disease. Large-scale production of *C. oedematiens* toxin was achieved in a special culture medium. The prepared vaccine was diluted to concentrations of 20%, 40%, 60% and 80% of antigens, and precipitated using adjuvant. The potency test of the prepared vaccine was determined according to the BP. Maximum titer was obtained at 80% with the level of 33 units per milliliter of alpha antitoxin in rabbit pooled serum (RPS). The obtained alpha antitoxin was 20, 16 and 8 units per milliliter for 60%, 40% and 20% of diluted antigen in RPS, respectively. Sheep were vaccinated in the areas affected by black disease. Reports on the field indicated that black disease in sheep had been effectively controlled by this vaccine in Iran (49).

Clostridium chauvoei and, less frequently, *C. septicum* are most commonly the responsible organisms for blackleg disease. Vaccination is the only effective means to control blackleg disease. Several kinds of vaccine are available commercially. It was that blackleg vaccine was produced in the traditional manner in Razi Institute for four decades until 2005; then, because of the enhanced demand of the country, it was decided to improve the production procedure of this vaccine using a large-scale fermenter; therefore, *C. chauvoei* was adapted for growth and proliferation in the fermenter to prepare a potent vaccine. Accordingly, attempts were made to prepare and formulate the ingredients in order to obtain high yield of *C. chauvoei* in the culture medium by the fermenter. Results showed high yield of *C. chauvoei* suspension in the fermenter after 10 hours, using enriched culture medium (more than 1,480,000,000 organisms/mL), but no significant changes were obtained in the condition of glass bottles compared to that of the fermenter. The safety and potency of the prepared vaccine was satisfactory in sheep and guinea pigs according to British Pharmacopoeia (veterinary). Since this research was successfully conducted in Razi Research Institute, and enriched culture medium fermenter is currently used to produce the mono-valent inactivated blackleg vaccine to immunize cattle in Iran (50).

Concentrated blackleg vaccine was prepared according to the method described by food and agriculture organization (FAO). The medium (modified medium to produce experimental *C. chauvoei* vaccine by fermenter) including peptone, glucose, sodium chloride, cysteine hydrochloride and yeast extract was prepared by fermenter and inoculated by *C. chauvoei* strain to prepare blackleg vaccine. Aluminum hydroxide gel was used as adjuvant to the high yield vaccine. The vaccine was also concentrated by precipitation method. None of the tested animals showed any local or general adverse reactions. All of the vaccinated guinea pigs resisted the challenge with 4four minimum lethal dose (MLD) of virulent *C. chauvoei* (51). Table 2 shows the clostridial vaccines manufactured for veterinary use in Razi institute.

The effects of enterotoxaemia vaccine, manufactured by Razi Institute, on reducing isolates of intestinal Clostridia genus specifically *C. perfringens* were studied. Sheep dung samples were randomly collected from 10 areas in Kerman, Iran. Following processing and culture the samples were processed and cultured, and colonies were identified; *C. perfringens* were isolated from (54.0%) 27 out of 50 unvaccinated sheep and (2.2%) 2 out of 90 vaccinated sheep; isolates were analyzed by multiplex Polymerase Chain reaction (PCR). Genotyping of two strains, isolated from the vaccinated sheep, indicated that the strains were type D, while the strains isolated from the unvaccinated sheep were types A, B, C and D; 14.8%, 22.2%, 40.7%, 22.2%, respectively. No isolates contained iota gene (type E). Results showed that vaccination against enterotoxaemia had a significant effect ($P < 0.01$) on reducing *C. perfringens* isolates. Occurrence of the disease in the vaccinated and unvaccinated groups was 3.3% and 64.0% ($P < 0.01$), respectively (52).

Table 2. The Clostridial Vaccines Manufactured in Razi Institute for Veterinary Use

Vaccine Name	Microorganism(s)	Disease(s) Protected	Million Dose of Production, y
Enterotoxemia tetra-valent	*C. perfringens* type B (Iran variant)	Lamb dysentery	120
	C. perfringens type C	Struck in sheep	120
	C. perfringens type D	Enterotoxaemia	120
	C. septicum	Braxy	120
Blackleg mono-valent	*C. chauvoei*	Blackleg	30
Black disease mono-valent	*C. oedematiens* (*C. novyi* type B)	Black disease	2.5

2.3.2. Molecular Biology Studies on Clostridium to Produce Vaccine in Iran

A genetic construct containing *C. perfringens* epsilon and beta toxin genes was produced in 2013. Epsilon and beta toxin genes were fused using a small linker sequence. And the fusion gene was expressed as a soluble protein in *E. coli* and its immunogenicity was studied in mouse. The recombinant cell lysate was used for immunization studies in mouse. Potency of the toxin (as an antigen) induced 6 and 10 IU/mL of epsilon and beta anti-toxin in rabbit, respectively. In conclusion, *E. coli* is a suitable expression host for immunogenic epsilon-beta fusion toxin of *C. perfringens* (53). *C. perfringens* type A alpha toxin gene cloned in *E. coli* as a candidate for recombinant vaccine production was described in 2014. High molecular weight genomic DNA of *C. perfringens* type A was isolated and cpa was amplified using one pair of primers. The 1,094 base pair gene was ligated into 2,974 bp pJET1.2 blunt recombinant vector and 4,068 bp pJETAα recombinant vector was produced. After extraction of pJETAα recombinant cloning vector, Nucleotide sequence and a 364-amino acid protein sequence was deposited into the GenBank. In silico analysis of these sequences showed several putative conserved domains (46).

In 2014, molecular cloning and sequencing of *C. septicum* vaccine strain alpha toxin gene was studied. After extraction of genomic DNA and amplification of the target gene through polymerase chain reaction by specific primers, pJETαsep cloning vector was produced and *E. coli*/TOP10 competent cells were transformed. Then pJETαsep recombinant plasmid was purified and sequenced using universal primers. Sequencing and BLAST analysis of csa showed over 99% identity to other previously deposited csa in the GenBank. The csa sequence was deposited into the GenBank under access number JN793989. *E. coli*/TOP10/pJETαsep as a recombinant bacterium could be used to purify the recombinant *csa* gene and its expression in the suitable prokaryotic hosts (54).

In 2012 in Iran, a single PCR assay was developed and used to detect *cpb2* gene and identify the Beta2 harboring isolates among different types of *C. perfringens* isolated from animal enteric diseases. The obtained results showed that *cpb2* presents among *C. perfringens* isolates types A, B, C and D with 54.5% (6/11), 62% (13/21), 42.8% (6/14), and 69.25% (9/13), respectively. Totally, 34 of 59 (56.7%) isolates screened by PCR were *cpb2*-positive (55).

The effects of *C. perfringens* type D prototoxin and toxin on the mouse body weight was evaluated. After preparation of the filtrated and freeze dried crude prototoxin, three series of experiments were set up. First, MLD/mL was determined and according to MLD, 50% end point (LD_{50}) was determined. Finally, different concentrations of activated freeze dried prototoxin were injected to the mice with 18 to 20 g body weight. Body weight decrease was observed two days after injection while no body weight decrease was observed in the control group. The results indicated that the activated *C. perfringens* culture filtrate temporarily inhibits mouse general metabolism. Since the secreted prototoxin is activated in the small intestine of the infected animals, vaccination of the domestic animals at the right time and using the right vaccine could prevent these effects (56).

Since 1892 that William Welch discovered *Bacillus aerogenes* capsulatus, Nov. Spec. (a gas-producing *Bacillus*) (57), and described its distribution (58) and after that Morton in 1928 reported phlegmonous gastritis that was originated by this bacterium (59), scientists and researchers are working on eradication of this bacterium, since it is normally found in the soil and the intestinal contents of apparently healthy animals. Therefore, it is necessary to resort to vaccination which in some cases has given encouraging results.

Razi Vaccine and Serum Research Institute was funded in 1925 and started vaccine production against Rinderpest. In Iran, enterotoxaemia was recognized in sheep in 1938. For the first time, the disease was found in the imported merinos, but, later, it was found in the fat-tailed sheep in the country. The 1st report on anaerobic clostridial whole culture formalized vaccine in Iran was published in 1961. In the last five decades, conventional anaerobic vaccine is produced to control clostridial diseases. Therefore, up to now it is about eight decades that anaerobic diseases are studied and anaerobic vaccines are produced in Razi Institute. Hence, inducing immunity against *Clostridium* species has been achieved in Iran. Furthermore, research and development on these vaccines and also in the field of molecular biology of different *Clostridium* vaccine strains are continually done and anaerobic toxoid vaccines are produced in Razi Institute.

3. Conclusions

At the present time there are no licensed clostridial vaccines which protect against either gas gangrene or epsilon-toxin in humans. However, it seems that the vaccines developed for animals, have the potential to be developed for humans (60).

Tetanus toxoid is commonly used as a single vaccine in horses but often a common combination of tetanus toxoid plus *C. perfringens* types C and D is used in sheep, goats, and cattle. However, tetanus toxoid is not used for animals in Iran. In cattle, a frequently used combination in feedlots is a 4-way vaccine that consists of killed cultures of *C. chauvoei*, *C. septicum*, *C. novyi*, and *C. sordellii* to protect against blackleg and malignant edema. In Iran, blackleg vaccine against *C. chauvoei* is used for cattle. A more complex clostridial vaccine that contains *C. perfringens* types C and D plus Iranian variant type B (34) in addition to the *C. septicum* vaccine (braxy vaccine) is used to protect sheep and goats against enterotoxaemia as well. The addition of *C. haemolyticum* extends the protection to include infectious necrotic hepatitis but it is not used in Iran.

Acknowledgments

6th January 2016 is the 90th anniversary of Razi Institute. The author wishes to express his warm thanks to all managers of Razi institute during last nine decades and the present general director, Dr. Hamid Kohram and all of the scientific members and staffs of anaerobic vaccine production department of Razi Vaccine and Serum Research Institute including Dr. Mahmood Ardehali, Dr. Mohsen Moosawi Shoshtari and Dr. Ahmad Reza Jabbary.

References

1. Cordoba MG, Aranda E, Medina LM, Jordano R, Cordoba JJ. Differentiation of Clostridium perfringens and Clostridium botulinum from non-toxigenic clostridia, isolated from prepared and frozen foods by PCR-DAN based methods. *Nahrung.* 2001;**45**(2):125–8. doi: 10.1002/1521-3803(20010401)45:2<125::AID-FOOD125>3.0.CO;2-8. [PubMed: 11379285]
2. Finsterer J, Hess B. Neuromuscular and central nervous system manifestations of Clostridium perfringens infections. *Infection.* 2007;**35**(6):396–405. doi: 10.1007/s15010-007-6345-z. [PubMed: 18034207]
3. Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, et al. Towards an understanding of the role of Clostridium perfringens toxins in human and animal disease. *Future Microbiol.* 2014;**9**(3):361–77. doi: 10.2217/fmb.13.168. [PubMed: 24762309]
4. Ardehali M, Darakhshan H. Production and standardization of polyvalent Clostridium perfringens vaccine in Iran. *Dev Biol Stand.* 1976;**32**:31–4. [PubMed: 187509]
5. Rood JI, Cole ST. Molecular genetics and pathogenesis of Clostridium perfringens. *Microbiol Rev.* 1991;**55**(4):621–48. [PubMed: 1779929]
6. Ochi S, Oda M, Matsuda H, Ikari S, Sakurai J. Clostridium perfringens alpha-toxin activates the sphingomyelin metabolism system in sheep erythrocytes. *J Biol Chem.* 2004;**279**(13):12181–9. doi: 10.1074/jbc.M307046200. [PubMed: 14702348]
7. Sheedy SA, Ingham AB, Rood JI, Moore RJ. Highly conserved alpha-toxin sequences of avian isolates of Clostridium perfringens. *J Clin Microbiol.* 2004;**42**(3):1345–7. [PubMed: 15004115]
8. Uzal FA, Vidal JE, McClane BA, Gurjar AA. Toxins Involved in Mammalian Veterinary Diseases. *Open Toxinology J.* 2010;**2**:24–42. [PubMed: 24511335]
9. Stiles BG, Wigelsworth DJ, Popoff MR, Barth H. Clostridial binary toxins: iota and C2 family portraits. *Front Cell Infect Microbiol.* 2011;**1**:11. doi: 10.3389/fcimb.2011.00011. [PubMed: 22919577]
10. Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, et al. Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. *Proc Natl Acad Sci U S A.* 2002;**99**(2):996–1001. doi: 10.1073/pnas.022493799. [PubMed: 11792842]
11. Petit L, Gibert M, Popoff MR. Clostridium perfringens: toxinotype and genotype. *Trends Microbiol.* 1999;**7**(3):104–10. [PubMed: 10203838]
12. Sawires YS, Songer JG. Clostridium perfringens: insight into virulence evolution and population structure. *Anaerobe.* 2006;**12**(1):23–43. doi: 10.1016/j.anaerobe.2005.10.002. [PubMed: 16701609]
13. Lucey BP, Hutchins GM. William H. Welch, MD, and the discovery of Bacillus welchii. *Arch Pathol Lab Med.* 2004;**128**(10):1193–5. doi: 10.1043/1543-2165(2004)128<1193:WHWMAT>2.0.CO;2. [PubMed: 15387691]
14. Batty I, Bullen JJ. The effect of Clostridium welchii type D culture filtrates on the permeability of the mouse intestine. *J Pathol Bacteriol.* 1956;**71**(2):311–23. [PubMed: 13398877]
15. Alves GG, Machado de Avila RA, Chavez-Olortegui CD, Lobato FC. Clostridium perfringens epsilon toxin: the third most potent bacterial toxin known. *Anaerobe.* 2014;**30**:102–7. doi: 10.1016/j.anaerobe.2014.08.016. [PubMed: 25234332]
16. Batty I, Glenny AT. Titration of Cl. welchii epsilon toxin and antitoxin. *Br J Exp Pathol.* 1947;**28**(2):110–26. [PubMed: 20252714]
17. Silva RO, Lobato FC. Clostridium perfringens: A review of enteric diseases in dogs, cats and wild animals. *Anaerobe.* 2015;**33**:14–7. doi: 10.1016/j.anaerobe.2015.01.006. [PubMed: 25644183]
18. Bosworth TJ, Glover RE. A Differential Character of Clostridium welchii Type D: (Section of Comparative Medicine). *Proc R Soc Med.* 1935;**28**(8):1004–6. [PubMed: 19990317]
19. Turner AW, Rodwell AW. The Epsilon Toxin of Cl. Welchii Type D. *Australian J Exp Biol Med Sci.* 1943;**21**(1):27–36. doi: 10.1038/icb.1943.5.
20. Bhown AS, Habeerb AF. Structural studies on epsilon-prototoxin of Clostridium perfringens type D. Localization of the site of tryptic scission necessary for activation to epsilon-toxin. *Biochem Biophys Res Commun.* 1977;**78**(3):889–96. [PubMed: 199192]
21. Sakurai J, Nagahama M. Role of one tryptophan residue in the lethal activity of Clostridium perfringens epsilon toxin. *Biochem Biophys Res Commun.* 1985;**128**(2):760–6. [PubMed: 2859854]
22. Souza AM, Reis JK, Assis RA, Horta CC, Siqueira FF, Facchin S, et al. Molecular cloning and expression of epsilon toxin from Clostridium perfringens type D and tests of animal immunization. *Genet Mol Res.* 2010;**9**(1):266–76. doi: 10.4238/vol9-1gmr711. [PubMed: 20198582]
23. Lobato FC, Lima CG, Assis RA, Pires PS, Silva RO, Salvarani FM, et al. Potency against enterotoxemia of a recombinant Clostridium perfringens type D epsilon toxoid in ruminants. *Vaccine.* 2010;**28**(38):6125–7. doi: 10.1016/j.vaccine.2010.07.046. [PubMed: 20670910]
24. Songer JG. Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev.* 1996;**9**(2):216–34. [PubMed: 8964036]
25. Sakurai J, Duncan CL. Some properties of beta-toxin produced by Clostridium perfringens type C. *Infect Immun.* 1978;**21**(2):678–80. [PubMed: 211090]
26. Steinthorsdottir V, Halldorsson H, Andresson OS. Clostridium perfringens beta-toxin forms multimeric transmembrane pores in human endothelial cells. *Microb Pathog.* 2000;**28**(1):45–50. doi: 10.1006/mpat.1999.0323. [PubMed: 10623563]
27. Sayeed S, Uzal FA, Fisher DJ, Saputo J, Vidal JE, Chen Y, et al. Beta toxin is essential for the intestinal virulence of Clostridium perfringens type C disease isolate CN3685 in a rabbit ileal loop model. *Mol Microbiol.* 2008;**67**(1):15–30. doi: 10.1111/j.1365-2958.2007.06007.x. [PubMed: 18078439]
28. Uzal FA, Saputo J, Sayeed S, Vidal JE, Fisher DJ, Poon R, et al. Development and application of new mouse models to study the pathogenesis of Clostridium perfringens type C Enterotoxemias. *Infect Immun.* 2009;**77**(12):5291–9. doi: 10.1128/IAI.00825-09. [PubMed: 19805537]
29. Pilehchian Langroudi R. Molecular biology of Clostridium perfringens focusing on epsilon and beta toxin genes. *J Vet Lab Res.* 2013;**5**:5–19.
30. Milach A, de los Santos JR, Turnes CG, Moreira AN, de Assis RA, Salvarani FM, et al. Production and characterization of Clostridium perfringens recombinant beta toxoid. *Anaerobe.* 2012;**18**(3):363–5. doi: 10.1016/j.anaerobe.2012.01.004. [PubMed: 22306065]
31. Chen J, McClane BA. Characterization of Clostridium perfringens TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. *Infect Immun.* 2015;**83**(6):2369–81. doi: 10.1128/IAI.03136-14. [PubMed: 25824828]
32. Liechti ME, Schob O, Kacl GM, Caduff B. Clostridium septicum aortitis in a patient with colon carcinoma. *Eur J Clin Microbiol Infect Dis.* 2003;**22**(10):632–4. doi: 10.1007/s10096-003-1009-0. [PubMed: 14513404]
33. Smith-Slatas CL, Bourque M, Salazar JC. Clostridium septicum infections in children: a case report and review of the literature. *Pediatrics.* 2006;**117**(4):e796–805. doi: 10.1542/peds.2005-1074. [PubMed: 16567392]
34. Ardehali M, Khalili K, Dowran H. Characterization of Clostridium chauvoei strains isolated from an outbreak of blackleg in Iran. *Archiv Razi Inst.* 1971;**23**:119–23.
35. Busch C, Schomig K, Hofmann F, Aktories K. Characterization of the catalytic domain of Clostridium novyi alpha-toxin. *Infect Immun.* 2000;**68**(11):6378–83. [PubMed: 11035748]
36. Imani Fooladi AA, Rahmati S, Falah Mehr Abadi J, Halabian R, Sedighian H, Soltanpour MJ, et al. Isolation of Clostridium difficile

and detection of A and B toxins encoding genes. *Int J Entric Pathog.* 2014;**2**(1):e15238.
37. Ardehali M, Dowran H. Isolation of Clostridium septicum from cases of malignant oedema of cattle in Iran. *Arch Razi Ins.* 1969;**21**:67–72.
38. Brooks ME, Entessar F. Anomalous Clostridium welchii type B strains isolated in Iran. *Br Vet J.* 1957;**113**:506–8.
39. Baharsefat M, Ardehali M, Amjadi AR, Ahourai P, Darakhshan H, Dowran H. First report on infectious necrotic enteritis in piglets in Iran. *Arch Inst Razi.* 1974;**26**:31–8.
40. Ardehali M, Moosawi M, Pilehchian Langroudi R. Characterization of Costridium oedematiens Strains isolated from cases of black disease of sheep In Iran. *Arch Razi Ins.* 1989;**40**(37-45).
41. Ardehali M, Darakhshan H. Isolation and typing of Clostridium oedematiens (Cl. novyi) from cases of black disease of sheep in Iran. *Comp Immunol Microbiol Infect Dis.* 1979;**2**(1):107–11. [PubMed: 544165]
42. Ahourai P, Ardehali M, Ezzi A, Gholami MR, Moosavi M. Bovine bacillary hemoglobinuria (Clostridium haemolyticum) in Iran. *J Vet Diagn Invest.* 1990;**2**(2):143–4. [PubMed: 2094437]
43. Ghazi F, Younan M, Ardehalli M, Muller W. Differentiation of proteinase (minor toxin lambda) in Clostridium perfringens strains from sheep and goats in Iran. *Dtsch Tierarztl Wochenschr.* 1997;**104**(10):443–5. [PubMed: 9394541]
44. Moosawi M, Ardehali M, Farzan A, Pilehchian Langroudi R. Isolation and Identification of Clostridium Strains from Cattle Malignant Edema Cases. *Arch Razi Ins.* 1999;**50**:65–70.
45. Pilehchian Langroudi R, Khaki P. Nucleotide Sequencing and Putative Conserved Domains Analysis of Clostridium perfringens Type A Alpha Toxin Gene Strain Isolated in Iran. *Asian J Pharm Technol Innov.* 2014;**2**(4):1–10.
46. Nijland R, Lindner C, van Hartskamp M, Hamoen LW, Kuipers OP. Heterologous production and secretion of Clostridium perfringens beta-toxoid in closely related Gram-positive hosts. *J Biotechnol.* 2007;**127**(3):361–72. doi: 10.1016/j.jbiotec.2006.07.014. [PubMed: 16959352]
47. European Directorate for the Quality of Medicines . Clostridium perfringens vaccines for veterinary use. Strasbourg, France; European pharmacopoeia. 2008.
48. Moosawi M. Production and standardizatiqn of two types of clostridial vaccines, for sheep and cattle. *Arch Razi Ins.* 1988;(38-39):83–8.
49. Ardehali M, Darakhshan H, Moosawi M. Mass production and standardization of Clostridium oedematiens vaccine against black disease (infectious necrotic hepatitis) of sheep. *Dev Biol Stand.* 1986;**64**:137–40. [PubMed: 3792643]
50. Langroudi RP, Jabbari AR, Shoshtari MM. Large scale production of blackleg vaccine by fermenter and enriched culture medium in Iran. *Arch Razi Ins.* 2012;**67**(1):43–9.
51. Mossawi Shoshtary M, Pilehchian Langroudi R, Abdolmohammadi L, Jabbari A. Preparation of concentrated blackleg vaccine. *Arch Razi Ins.* 2007;**62**(3):165–9.
52. Ahsani MR, Bafti MS, Esmailizadeh AK, Mohammadabadi MR. Genotyping of isolates of Clostridium perfringens from vaccinated and unvaccinated sheep. *Small Ruminant Res.* 2011;**95**(1):65–9.
53. Pilehchian Langroudi R, Shamsara M, Aghaiypour K. Expression of Clostridium perfringens epsilon-beta fusion toxin gene in E. coli and its immunologic studies in mouse. *Vaccine.* 2013;**31**(32):3295–9. doi: 10.1016/j.vaccine.2013.04.061. [PubMed: 23707696]
54. Bozorgkhoo Z, Pilehchian Langroudi R, Khaki P, Jabbari AR, Moradi Bidhendi S, Moosawi Shoshtari M. Molecular cloning of Clostridium septicum vaccine strain alpha toxin gene in E. coli. *Arch Razi Ins.* 2014;**69**(1):15–20.
55. Jabbari AR, Tekyei F, Esmaeilizad M, Pilehchian Langroudi R. Occurrence of Beta2 toxigenic Clostridium perfringens isolates with different toxin types in Iran. *Archives of Razi.* 2012;**67**(2):133–7.
56. Pilehchian Langroudi R. Clostridium perfringens Type D Epsilon Prototoxin and Toxin Effects on the Mouse Body Weight. *Int J Enteric Pathog.* 2014;**3**(2):e18632.
57. Welch WH, Nuttall GHF. A gas-producing bacillus (Bacillus aerogenes capsulatus, Nov. Spec.) capable of rapid development in the blood-vessels after death. *Bull Johns Hopkins Hosp.* 1892;**3**(81-91).
58. Welch WH. Distribution of Bacillus Aerogenes Capsulatus. (Bacillus Welchi, Migula.). *J Boston Soc Med Sci.* 1901;**5**(7):369–70. [PubMed: 19971366]
59. Morton JJ, Stabins SJ. Phlegmonous Gastritis: Of Bacillus Aerogenes Capsulatus (B. Welchii) Origin. *Ann Surg.* 1928;**87**(6):848–54. [PubMed: 17865897]
60. Titball RW. Clostridium perfringens vaccines. *Vaccine.* 2009;**27 Suppl 4**:D44–7. doi: 10.1016/j.vaccine.2009.07.047. [PubMed: 19837285]