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The most common toxinotypes of the bacterium *Clostridium perfringens* in small ruminants and their resistance to antibiotics

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Abstract

Infection with the bacterium *C. perfringens* in sheep and goat herds leads to considerable losses. The most common diseases in small ruminants caused by this bacterium are pulpy kidney disease, enterotoxaemia, yellow lamb disease, and necrotic enteritis. *C. perfringens* is widely distributed in the environment and is part of the intestinal microbiota of humans and animals. When physiological conditions in the body change, it begins to produce toxins that lead to various pathological changes. Depending on the toxins it produces, it is classified into seven types (A–G), defined based on the presence of genes encoding the CPA toxin (all types), CPB toxin (type B and type C), ETX toxin (type B and D), ITX toxin (type E), CPE toxin (type F) and NetB toxin (type G). Strains A and D are most commonly associated with enterotoxaemia in small ruminants. According to the available literature, *C. perfringens* toxinotype A is most frequently isolated in small ruminant herds. In addition to toxin genes, antibiotic resistance genes also play an important role in bacterial survival. Of the genetic determinants of antimicrobial resistance discovered to date, most are associated with the following antimicrobial drugs: bacitracin (*bcrR* gene), tetracyclines (*tet* genes), macrolides (*erm* genes), lincosamides (*erm* and *lnu* genes), streptogramins (*erm* genes) and chloramphenicol (*cat* genes). Due to sudden death after the onset of symptoms and the economic unprofitability, very few results are available on antibiotic resistance of *C. perfringens* in small ruminants. Therefore, it has proven to be more economical and effective to work on prevention than on treatment. Since there is no vaccine against enterotoxaemia in Croatia, prevention of the disease is difficult.

Key words: *Clostridium perfringens; enterotoxaemia; toxinotyping; antimicrobial resistance.*

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Introduction

The genus *Clostridium* comprises about 231 species (Brasca et al., 2022). Clostridia are gram-positive, rod-shaped, anaerobic, sporulating bacteria that are widespread in the environment (soil and water) and are part of the intestinal microbiota of humans and animals (Sepehrifar et al., 2021). Certain species of clostridia produce toxins due to changes in physiological conditions in the body. These toxins lead to enterotoxic, neurotoxic and histotoxic pathological changes in humans and animals (Kalender et al., 2023). The species *Clostridium perfringens* contributes significantly to the development of disease and mortality in domestic animals. This species, which produces toxins categorized into seven types (A–G), causes major losses in livestock. In sheep and lambs, and goats and kids, *C. perfringens* commonly causes pulpy kidney disease, enterotoxaemia, yellow lamb disease, and necrotic enteritis (Sepehrifar et al., 2021).

The aim of this study was to outline the microbiological characteristics of the toxinotypes of *C. perfringens* and its associated toxins, focusing on isolates from sheep and goats. In addition, the characteristic pathoanatomical pictures produced by each type of *C. perfringens* are described with a summary of the data published to date on its prophylaxis and treatment.

Basic microbiological characteristics of *Clostridium perfringens*

Unlike most other *Clostridium* species, *C. perfringens* is non-motile and the only species to have a capsule. It can be isolated on blood agar and, unlike other clostridia, is relatively aerotolerant. After a full day of incubation under anaerobic conditions, colonies of *C. perfringens* on blood agar are round, 3–5 mm in diameter and greyish in colour. In most cases, colonies are surrounded by a double zone of haemolysis. One of the secondary toxins produced by *C. perfringens*, perfringolysin, leads to the formation of an inner, complete haemolysis zone. The outer, incomplete haemolysis zone is formed by the main toxin of *C. perfringens, Clostridium perfringens* Alpha toxin (CPA). A minor part of *C. perfringens* strains do not carry the perfringolysin gene (*pfoA*), so that in rare cases it is possible that the characteristic double haemolysis zone is missing. For this very reason, it is not reliable to rely solely on morphological characteristics when determining the toxinotypes of *C. perfringens* (Habrun, 2014; Uzal et al., 2022).

Microscopically, Gram-stained colonies of *C. perfringens* appear as large, purple rods. If many such rods are found in the intestinal swab of a recently deceased animal, enterotoxaemia can be suspected (Habrun, 2014).

Clostridium perfringens toxinotypes

The virulence of the opportunistic bacterium *C. perfringens* is primarily determined by its capacity to produce more than 20 distinct toxins and extracellular enzymes. Based on toxin production, strains are classified into seven toxinotypes (A–G), which are defined by the presence of genes encoding for: the *Clostridium perfringens* Alpha toxin (CPA), *Clostridium perfringens* Beta toxin (CPB), *Clostridium perfringens* Epsilon toxin (ETX), *Clostridium perfringens* Iota toxin (ITX), *Clostridium perfringens* enterotoxin (CPE), and Necrotic enteritis toxin B-like (NetB). Furthermore, certain strains of each toxin type can produce one or more non-typing toxins: *Clostridium perfringens* Beta2 toxin (CPB2), Perfringolysin O theta toxin (PFO), Net-like toxin F (NetF), Beta-2 enterotoxin (BEC) and others. The coding gene for the CPA toxin (*cpa/plc*) is the only one located in a genetically stable chromosomal region. Therefore, all strains of *C. perfringens* produce this toxin. The genes coding for other typing (lethal) toxins are located on plasmids. Therefore, toxin typing is based on the detection of individual plasmids. The location of the gene may affect the course and outcome of the disease, as plasmid-encoded genes may spread to intestinal-resident *C. perfringens* toxinotype A strains through bacterial mating. In this way, new strains with heightened virulence potential are created. The plasmid contains the clinically relevant toxins *cpb2* and *cpe* and genes for antibiotic resistance, enabling *C. perfringens* to survive under unfavourable conditions and increasing its virulence (Forti et al., 2020; Camargo et al., 2024).

Clostridium perfringens toxinotype A

Clostridium perfringens toxinotype A produces a single lethal toxin, CPA, encoded by the *cpa* and *plc* genes. The CPA toxin consists of 370 amino acids. By acting as a phospholipase C (PLC), this toxin damages the host's cell membranes by hydrolysing phospholipids, enabling clostridia to produce cytolytic, haemolytic and dermonecrotic effects.

It is assumed that the activity of the CPA toxin alone is not sufficient for the outbreak of the disease. It has been shown that isolated strains of *Clostridium perfringens* toxinotype A that produce the non-typing CPB2 toxin develop more severe clinical manifestations. Recent research has demonstrated the synergistic effect of CPA and CPB2 toxins in the development of ruminant enteritis. CPA damages the cell membrane through its enzymatic activity, leading to the degradation of sphingomyelin and phosphatidylcholine, while CPB2 causes haemorrhagic and necrotic changes in the small and large intestinal mucosa, leading to degeneration, necrosis and inflammation of epithelial cells. Little is known about the structure of CPB2 itself, but it is known that its mechanism of action involves the formation of cation-selective channels in lipid layers, resulting in ion imbalance and loss of the intestinal barrier integrity (Kalender et al., 2023; Camargo et al., 2024).

Clostridium perfringens toxinotype A causes enterocolitis in broilers, suckling piglets and horses, haemorrhagic gastroenteritis in dogs, and gas gangrene in sheep (Habrun, 2014). The CPA toxin is the primary virulence factor in clostridial myonecrosis in adult sheep, which is caused by toxinotype A. High concentrations of this toxin cause damage to the plasma membrane of the host cells. Toxin binding to the ganglioside receptor GM1 leads to the degradation of sphingomyelin and phosphatidylcholine in the plasma membrane of the cell. Diacylglycerol (DAG) and ceramide (CER) are formed, and the receptor tropomyosin kinase A (TrKA) is activated, starting a chain reaction inside the cell that leads to the release of interleukin-8 (IL-8). Phosphatidyl inositol triphosphate (IP3) plays a key role by causing calcium to move from the endoplasmic reticulum into the cell, and IL-8 then helps drive an inflammatory response. IL-8 attracts neutrophil granulocytes, whose activation releases enzymes and free radicals that damage the tissue and lead to tissue infection with subsequent crepitations and necrosis (Camargo et al., 2024).

In humans, toxinotype A is associated with food poisoning, causing diarrhoea within 8–20 hours after consumption of contaminated food (Hamza et al., 2018).

Clostridium perfringens toxinotype B

In addition to the CPA toxin, *C. perfringens* toxinotype B also produces the toxins CPB and ETX, which are encoded by the genes *cpb* and *etx* (Forti et al., 2020; Camargo et al., 2024).

Clostridium perfringens toxinotype B commonly causes dysentery in lambs younger than 14 days and haemorrhagic enteritis in calves and foals (Alimolaei and Shamsaddini Bafti, 2023).

CPB is a thermolabile toxin consisting of 336 amino acids (Camargo et al., 2024). It is activated by the action of trypsin inhibitors and leads to cell death and lysis. It creates pores in the cell and leads to ion exchange, with Ca^{2+}, Na^{+} and Cl^{-} entering the cell and K^{+} flowing out. As a result of the described change, cell swelling and necrosis occur (Uzal et al., 2018).

The ETX toxin consists of three domains: the amino-terminal domain involved in receptor binding, the central region domain responsible for membrane channel formation, and the carboxy-terminal domain responsible for proteolysis (Camargo et al., 2024).

The activation of proteases is important for the action of the two main toxins responsible for the virulence of *C. perfringens* toxinotype B. This toxinotype develops a specific clinical state (necrohaemorrhagic enteritis/encephalomalacia), depending on which of the toxins has the main effect (CPB or ETX). If the CPB toxin has a predominant effect, binding to the platelet endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31) leads to pore formation in the vascular endothelial cells of the intestinal mucosa described above, and the resulting haemorrhagic necrotising enteritis (Uzal et al., 2018; Camargo et al., 2024).

Clostridium perfringens toxinotype C

Clostridium perfringens toxinotype C produces the toxins CPA and CPB. In some cases, it also produces the CPE toxin, which is encoded by the *cpe* gene. Among the intestinal infections linked to different toxinotypes of *C. perfringens*, those caused by type C strains are notably common in both humans and animals, whereas other toxinotypes tend to be associated with disease in either humans or animals, but not both (Uzal et al., 2018; Camargo et al., 2024).

CPB is the major virulence factor in lethal intestinal necrosis of piglets and in human necrotic enteritis, better known as porcine enteritis, and is encoded by a specific *cpb2* gene. As CPB is extremely sensitive to chymotrypsin and trypsin, piglets and newborns are under a greater threat of type C disease, due to the inhibitory effect of colostrum on trypsin. Colostrum prevents the proteolytic degradation of immunoglobulins in the neonatal period and thus indirectly "protects" CPB. As described, trypsin inhibitors prevent the intestinal degradation of toxins, and reduced intestinal motility additionally favours the local accumulation of toxins (Uzal et al., 2018).

In humans, cases of necrotic enteritis (Pigbel) have been sporadically observed across several Southeast Asian nations, and less frequently in other regions. The name of the disease itself comes from the English term for abdominal pain after eating pork. The disease is associated with the ritual consumption of infected pork. It usually affects severely malnourished people and diabetics, and is particularly fatal in children. The CPB toxin, regarded as the most important among those produced by *C. perfringens*, plays a key role in intestinal diseases in both humans and pigs. A local infection with *C. perfringens* toxinotype C due to the CPB toxin leads to haemorrhagic necrosis of the epithelium of the intestinal mucosa, which spreads to all layers of the intestinal wall. Progressive inflammation and infection lead to arterial and venous blockage and eventually to necrosis. Most patients were malnourished children, due to protein deficits and the resulting lack of trypsin. This is in accordance with the occurrence of toxinotype C infections in newborns, where the activity of trypsin is inhibited by the effect of colostrum. In more developed countries, the occurrence of the disease has been observed in diabetics, which in turn is related to trypsin, the concentration of which is disturbed due to the dysfunction of the pancreas in diabetics (Wormald et al., 2016; Camargo et al., 2024).

Clostridium perfringens toxinotype D

Clostridium perfringens toxinotype D produces the CPA toxin, which is common to all toxinotypes of *C. perfringens*. In addition to the CPA toxin, type D also produces the ETX toxin. Although both *C. perfringens* toxinotypes B and D produce the ETX toxin, the latter is more commonly associated with diseases caused by *C. perfringens* toxinotype D (Finnie et al., 2020; Camargo et al., 2024).

Of all toxins synthesised by *C. perfringens*, the ETX toxin is thought to have the strongest effect. Its toxicity is evidenced by the fact that it was classified as a biological weapon by the US Department of Agriculture and the Centers for Disease Control and Prevention until 2012. In France, it is still considered a potential biological weapon and laboratories working with it require special permits (Duracova et al., 2019). ETX is secreted into the digestive tract of animals as an inactive protoxin and becomes active through the action of proteolytic enzymes such as trypsin, chymotrypsin, and other proteases. After activation in the intestine, ETX is distributed via the bloodstream to the target organs: brain, heart, kidneys and lungs. After binding to the endothelial cells, ETX increases vascular permeability through its action, leading to oedema in the target organs along with fluid buildup in the body cavities. By targeting endothelial cells in the kidneys, this toxin causes pulpy kidney disease. Accumulation in the kidneys could be a form of defence by the host organism trying to prevent the deadly accumulation of toxins in cerebral tissue. The accumulation of toxins in the kidneys leads to congestion, interstitial haemorrhage, and breakdown of the distal tubule lining. During epsilon enterotoxaemia in lambs, glycogen is released from the liver, leading to glycosuria. When ETX crosses the blood-brain barrier, it binds to neuronal cells and leads to death. Neurons are the most sensitive to ETX toxin, followed by oligodendrocytes and astrocytes. The effect of ETX in the brain is manifested by the formation of oedema, vacuolisation of the cytoplasm, and necrosis due to hypoxia caused by the toxins (Stiles et al., 2013; Uzal et al., 2018; Sepehrifar et al., 2021; Camargo et al., 2024).

The ETX toxin has also been detected in some patients with multiple sclerosis, suggesting that it may contribute to the development of this demyelinating disorder in humans. However, additional research is needed as this disease in humans is distinct from the naturally occurring disease caused by ETX in ruminants. It should also be emphasised that *C. perfringens* toxinotype B has been isolated in humans suffering from multiple sclerosis and not type D, which is associated with neurological symptoms of the disease in animals (Uzal et al., 2018; Finnie et al., 2020).

Clostridium perfringens toxinotype E

In addition to CPA, *C. perfringens* toxinotype E produces the intracellular binary toxin ITX, which consists of an enzyme (Ia) and a binding component (Ib) and is encoded by the genes *iap* and *ibp* (Rood et al., 2018; Uzal et al., 2018; Forti et al., 2020). The enzyme component of ITX binds to the lipolysis-stimulated lipoprotein receptor. After binding to the receptor, the binding components of the toxin are incorporated into the cell membranes, forming channels that allow endocytosis and the entry of the enzymatic component and the movement of ions. In addition, ADP-ribosylation of actin, depolymerisation of actin filaments and an increase in G-actin monomers occur, leading to a change in cell morphology. The cytotoxic effects of ITX include cell swelling, mitochondrial dysfunction and ATP depletion. These alterations result in characteristic changes to the small intestinal epithelial mucosa seen in enteritis. The permeability of the intestinal villi increases, and serous and mucosal haemorrhage occurs. *C. perfringens* toxinotype E is frequently linked to haemorrhagic enteritis in calves and rabbits (Uzal et al., 2018; Camargo et al., 2024).

Clostridium perfringens toxinotypes F and G

In recent years, increased attention has been given to two more recent types of *C. perfringens* - toxinotypes F and G. *C. perfringens* toxinotype F is associated with diarrhoeal disease in humans caused by food poisoning and antibiotics, while it is very rarely isolated in animals. The production of CPE enterotoxin by the bacterium *C. perfringens* is mainly associated with type F, although this toxin can also be associated with strains of types A, C, D and E as a non-typing toxin. CPE is a polypeptide comprised of 319 amino acids. During spore formation it is released into the intestinal tract where it binds to specific receptors located at the tips of the intestinal villi in the small intestine (claudin receptors). By binding to the receptors, CPE forms a complex, which then oligomerises and forms the CH-1 complex. The CH-1 complex increases the permeability of the cytoplasmic membrane by forming pores, leading to an influx of Ca^{2+} and the subsequent activation of apoptotic or oncotic death pathways. The induced death of epithelial cells leads to fluid accumulation in the intestine and diarrhoea (Uzal et al., 2018; Camargo et al., 2024).

C. perfringens toxinotype G is the aetiological agent of necrotic enteritis in poultry. In addition to the standard CPA toxin, this toxinotype also produces a NetB toxin, which is encoded by the *netB* gene. This toxin targets cholesterol-free membrane regions of intestinal epithelial cells, forming hydrophilic pores that increase mucosal permeability by allowing ion influx. This process leads to enterocyte necrosis and, in extreme cases, to coagulation necrosis of the superficial mucosa. The triggering of necrotic enteritis is complex and is related to one or more predisposing factors. The most common predisposing factor for necrotic enteritis in poultry is intestinal infection with coccidia *Eimeria* spp. It has been experimentally demonstrated that feeding birds with high-protein feed also contributes to the development of the disease (Uzal et al., 2018; Camargo et al., 2024).

The most frequently isolated toxinotypes of *C. perfringens* in small ruminants

In veterinary medicine, although clostridial infections affect different animal species, they are most frequently associated with small ruminants, causing significant losses in herds. *C. perfringens* is part of the intestinal microbiota of various animal species, including small ruminants. However, when intestinal peristalsis slows due to intestinal dysbiosis, it produces substantial quantities of toxins, leading to enterotoxaemia. The very name enterotoxaemia indicates the presence of intestinal toxins in the bloodstream (Habrun, 2014; Singh et al., 2018).

In addition to the above predisposing factors, animal age and time of year also influence the development of enterotoxaemia. Previous studies have shown that the isolation rate of *C. perfringens* decreases with animal age. In addition, the number of isolated bacteria was considerably higher in late winter and spring. One possible explanation for the more frequent occurrence of the disease in spring is the fact that the sheep then go out to pasture and eat greedily after a period of being housed in the barn with monotonous feeding. The stomachs of the sheep, which are not used to green fodder, swell up and accumulate gasses. This creates an anaerobic environment that is ideal for the development and multiplication of clostridia (Alimolaei and Shamsaddini Bafti, 2023).

C. perfringens toxinotypes A and D predominate as the most isolated toxinotypes in sheep and lambs, and goats and kids (Singh et al., 2018; Forti et al., 2020; Alimolaei and Shamsaddini Bafti, 2023; Kalender et al., 2023). A lower percentage of toxinotype F was isolated in small ruminants in Iran, Turkey, and Italy (Forti et al., 2020; Alimolaei and Shamsaddini Bafti, 2023; Kalender et al., 2023). A unique toxinotype E strain was isolated from a sheep from Umbria, containing the secondary toxin genes *cpe* and *aty-cpb2* in addition to the main toxin genes *cpa* and *iap-ibp*. The secondary toxin genes *cpe* and *aty-cpb2* encode the non-typing CPE toxin and the non-typing CPB2 toxin. The specific role of the CPB2 toxin remains to be seen, but it is known to act synergistically with the major toxins in the development of necrotic and haemorrhagic enteritis (Forti et al., 2020). Its synergistic effect is reflected in its action with CPA toxin and the development of pathogenicity in type A strains (Kalender et al., 2023). CPB2 is encoded by the *cpb2* gene with two allelic forms: consensus (*cons*) and atypical (*aty*). A study conducted in Italy in 2020 showed that *cons-cpb2* genes dominate over *aty-cpb2* genes (Forti et al., 2020).

Alimolaei and Shamsaddini Bafti (2023) also demonstrated the prevalence of *C. perfringens* toxinotypes A and D, compared to toxinotype C, which was the most commonly isolated in Iran until then (Jabbari et al., 2011; Alimolaei and Shamsaddini Bafti, 2023). In Iran, toxinotype G, which is usually associated with necrotic enteritis in poultry, was isolated from two lambs younger than one month and a 1.5-year-old sheep that died of enterotoxaemia (Alimolaei and Shamsaddini Bafti, 2023). In the study by Yadav et al. (2017), of 29 isolates of *C. perfringens* toxinotype A isolated from goats with diarrhoea, 37.9% were positive for the *cpa* gene only, 51.7% for *cpa* and *cpb2*, 3.4% for *cpa* and *cpe*, 6.9% for all three genes (*cpa*, *cpb2*, *cpe*). *C. perfringens* isolated from the faeces of healthy goats carried only the *cpa* gene in 72.2% of cases, 22.2% were positive for *cpa* and *cpb2*, and 5.6% for *cpa* and *cpe*. The presence of *cpb2* and *cpe* genes in healthy animals is not a reliable indicator of disease, but as carriers, they may transmit the infection to other animals and humans. Since *C. perfringens* is a commensal bacterium of the gastrointestinal tract of humans and animals, the disease can occur under unfavourable environmental conditions and when the individual's immune system is weakened (Yadav et al., 2017).

When comparing sick and healthy individuals, a significantly higher isolation rate of *C. perfringens* was found in sheep with diarrhoea than in healthy and asymptomatic animals (Alimolaei and Shamsaddini Bafti, 2023).

Pathological findings of the most common toxinotypes of *C. perfringens* isolated from small ruminants

Toxinotype A

Clostridium perfringens toxinotype A is the most isolated toxinotype of *C. perfringens* and is frequently isolated from the intestines of healthy humans and animals. Therefore, independent isolation of toxinotype A without characteristic changes observed at necropsy is of little diagnostic value. In toxinotype A, the disease most commonly develops through the synergistic action of CPA toxins and non-type-specific toxins.

Toxinotype A ovine enterotoxaemia, which is often characterised by the pathoanatomical picture and is also known as yellow lamb disease, is manifested by generalised jaundice. In affected animals, the liver and spleen are enlarged and pale, while the small intestine is haemorrhagic with a large amount of gas. Fibrin threads can also be found in the intestinal lumen. Due to haemolysis and haemoglobinuria, the urine may be red. Histopathological changes in toxinotype A include periacinar necrosis of the liver, splenic congestion, necrosis of the small intestinal epithelium with visible bacilli mixed with fibrin, desquamated epithelial cells and leukocytes in the lumen. Pathohistological findings in the kidneys include nephrosis, congestion and oedema in the lungs (Uzal and Songer, 2008). Haemorrhagic abomasitis can also be seen at necropsy in lambs and goats. Haemorrhagic abomasitis is characterised by an enlarged abomasum filled with casein and a large amount of gas. On the serous surface of the stomach, multifocal black foci representing petechial haemorrhages are characteristic. Numerous ulcers and erosions are found on the mucous membrane of the abomasum. Changes in the myocardium are characterised by ecchymoses of the epicardium and endocardium. Pathohistologically, acute necrotising haemorrhagic inflammation of the abomasal mucosa and moderate to severe hyperaemia and congestion with oedema in the mucosa and submucosa due to the effect of the toxin are observed. Ulcers characterised by epithelial degeneration and desquamation are observed in the submucosa, while the connective tissue of the abomasal mucosa is affected by inflammatory infiltration by neutrophils, lymphocytes and macrophages (Kalender et al., 2023).

Among the anamnestic data, sudden weakness, reluctance, inability to suckle, and death within 2–3 hours after the onset of symptoms are most frequently reported. Toxinotype A strain is most frequently isolated from healthy newborn animals that have received a sufficient amount of colostrum. The mortality rate of lambs and goat kids in flocks is 15–20% (Kalender et al., 2023).

Toxinotype D

As described above in the pathogenesis of ETX toxin, its toxic effect begins when the toxin enters the bloodstream from the intestine. In lambs, entry of the toxin into the bloodstream without reaching the target organs can already be fatal. The target organ for ETX toxin in lambs is the kidneys. The changes in the kidneys are known in veterinary medicine as pulpy kidney disease. At autopsy, the kidneys are soft, sticky, irregular and barely recognisable in shape, and the cortex and nucleus are not visible in cross-section (Uzal et al., 2018; Finnie et al., 2020).

In sheep and goats, the subacute or chronic course of the disease is more common, with more pronounced symptoms in relation to the target organs of the ETX toxin. Sheep and goats show neurological symptoms in the form of ataxia, bruxism, opisthotonos, convulsions and blindness. In goats with subacute or chronic intoxication, dark green diarrhoea with blood admixtures in the form of necrohaemorrhagic enteritis may occur, though this is not the case in sheep. Post-mortem examinations of sheep and goats often reveal petechiae in the serosa of the abdominal, peritoneal and thoracic cavities, foam in the trachea, pulmonary oedema, pericardial, abdominal, and pleural effusions, possibly accompanied by fibrin deposits. A large amount of straw-coloured/light pink fluid may be present in the abdominal cavity. There is multifocal haemorrhaging on the epicardium and straw-coloured fluid in the pericardium. In adults, the kidneys show changes characteristic of lambs and young goats. The kidneys are haemorrhagic, soft in consistency, and chocolate-coloured urine may also be observed. Neurological symptoms are more common in sheep and intestinal symptoms in goats (Finnie et al., 2020; Hussain et al., 2022).

The pathohistological changes caused by the ETX toxin also affect the target organs: brain, heart, kidneys and lungs. A characteristic finding in sheep and sporadically in goats poisoned with ETX toxin is focal symmetrical encephalomalacia. Preferred sites for the development of symmetrical foci in the brain are the basal ganglia, the thalamus and the medulla oblongata. Microscopic examination of lung samples from infected sheep and goats may show oedema, emphysematous changes, accumulations of fibrinous exudate in bronchioles, bronchi and alveoli, infiltration of mononuclear cells in alveoli, congestion and haemorrhaging. Microscopically, coagulation necrosis, degeneration of heart cells and severe myocarditis can be observed in the heart muscle. The changes in the kidneys are most pronounced in lambs and goat kids but can occur at any age. In toxin-infected kidneys, histological changes include congestion, interstitial haemorrhage and degeneration of the distal tubules interspersed with erythrocytes. In goats, the intestines are frequently affected by pathohistological changes. Microscopic changes in the intestine include congestion, haemorrhaging and necrosis of the mucosa, submucosa and villi. Infiltration of mononuclear cells occurs in the connective tissue (Stiles et al., 2013; Finnie et al., 2020; Hussain et al., 2022).

Anamnestic data for toxinotype D include the occurrence of watery diarrhoea, acute anorexia and dehydration, animals huddled in a corner, stretching of the body when standing and walking, reluctance to eat and drink. During convulsions, the animal cannot stand and instead lies on the floor with its legs stretched out and the head and neck thrown back (Hussain et al., 2022).

Prevention and treatment of enterotoxaemia in sheep and goats

C. perfingens is found in the soil, faeces and intestines of animals and humans. Improper treatment, changes in eating habits and parasite infestation lead to intestinal dysbiosis, bacterial proliferation and toxin production (Forti et al., 2020).

Like other anaerobic bacteria, *C. perfringens* also shows resistance to aminoglycosides, polymyxins and older generation fluoroquinolones. Resistance to aminoglycosides is to be expected as these antibiotics require oxygen to enter the bacterial cell and reach their target site of action. Conversely, nitroimidazole antibiotics such as metronidazole are generally effective because they lack the oxygen for their intracellular degradation to active antimicrobial metabolites. The existence of thymidine within necrotic tissue can cause trimethoprim-sulphonamide to be less effective. In general, clindamycin, metronidazole or penicillin cover most anaerobic bacteria well, but are not approved for use in animals raised for consumption in the European Union due to genotoxicity. All *Clostridium* species are naturally resistant to trimethoprim as they possess dihydrofolate reductases that are insensitive to trimethoprim (Archambault and Rubin, 2020).

Most available data on the susceptibility of *C. perfringens* to antibiotics relate to isolates from broiler chickens, as necrotic enteritis is an important poultry disease that can be controlled with antibiotics. Avilamycin, bacitracin, lincomycin and virginiamycin are used prophylactically and therapeutically in food against necrotic enteritis in many countries. Recent studies of *C. perfringens* isolates from poultry have shown resistance to tetracycline and lincomycin. There is a limited number of reports on the susceptibility of *C. perfringens* to antimicrobials in other animal species. According to the available data from isolates from pigs, resistance to clindamycin, erythromycin, lincomycin and tetracycline was emphasised, while the isolates showed sensitivity to chloramphenicol and penicillin (Archambault and Rubin, 2020). In a Brazilian study on piglets, *C. perfringens* was susceptible to amoxicillin and ceftiofur and resistant to lincomycin and tetracycline. Of the resistant pig isolates, 82% showed resistance to more than one antibiotic (Salvarani et al., 2012). In research carried out in Canada, isolates of *C. perfringens* from cattle were less sensitive to clindamycin, florfenicol and tetracycline (Slavić et al., 2011). Isolates from dogs with acute diarrhoea were resistant to tetracycline and less sensitive to metronidazole. All dog isolates showed susceptibility to chloramphenicol, clindamycin and all tested beta-lactams (Gobeli et al., 2012). In Costa Rica, 5% of strains of *C. perfringens* of animal origin were multiresistant to chloramphenicol, clindamycin, metronidazole and penicillin (Gamboa-Coronado et al., 2011). Unfortunately, data is sparse on the antibiotic resistance of the *C. perfringens* isolated from small ruminants, which is most likely due to the rapid death of infected animals (Archambault and Rubin, 2020).

Bacterial antimicrobial resistance can be acquired on mobile genetic elements. In addition to acquired antibiotic resistance, bacteria can also adapt and become resistant to antibiotics through the formation of biofilms. Studies have shown that *C. perfringens* forms a biofilm that protects the cells from contact with atmospheric oxygen and high concentrations of penicillin (Adams et al., 2018; Archambault and Rubin, 2020; Ilić, 2021).

As described, the virulence of *C. perfringens* itself is based on highly specific genes, including genes for antibiotic resistance. The most frequently described genetic determinants of antibiotic resistance to date relate to several antimicrobial agents, i.e., bacitracin, chloramphenicol, lincosamides, macrolides, streptogramins and tetracyclines. Bacitracin resistance is associated with the *bcrR* gene, which leads to the alteration of udecaprenol kinase, an enzyme that plays a key role in the biosynthesis of peptidoglycan, which is important for the integrity of the bacterial cell wall. Tetracycline resistance is associated with *tet* genes, first discovered on the conjugative plasmid pCW3, with *tet(A)*, *tet(B)*, and *tet(M)* being the most common genetic determinants. The *tet(A)* gene codes for proteins that remove tetracycline from the bacterial cell through the effect of the proton gradient. In this way, the concentration of the antibiotic in the cell decreases and its effectiveness diminishes. *Tet(M)* genes modify ribosomal RNA sequences through methylation, in particular 16S rRNA, which is the target site of tetracycline. Macrolide resistance is mediated by *erm* genes. These genes lead to dimethylation of a specific adenine residue (A2058) of the 23S rRNA molecule on the 50S subunit of the bacterial ribosome, which is the target binding site of macrolides (erythromycin), lincosamide (clindamycin) and streptogramin. Dimethylation is catalysed by rRNA methyltransferase. This resistance mechanism results in the MLSB resistance phenotype, which confers cross-resistance to macrolides, lincosamides, and streptogramin B antibiotics. To date, the genes *erm(B)*, *erm(F)* and *erm(Q)* have been detected in *C. perfringens* isolates. Lincosamide resistance may also result from enzymatic mechanisms, such as hydroxyl group phosphorylation and nucleotidylation. Lincosamide nucleotidyl transferases are encoded by the *lnu(A)* and *lnu(B)* genes, located like a transposon on the functional transposable genetic element tISCpe8. The resistance of *C. perfringens* to chloramphenicol is mediated by chloramphenicol acetyltransferases (CAT), enzymes encoded by the specific genes *cat(P)* and *cat(Q)* located on the mobilising transposon Tn4451. Mobilising transposons can be transferred but need conjugative elements to do so (Ćupić et al., 2014; Archambault and Rubin, 2020; Ilić, 2021; Dos Santos et al., 2022).

The treatment of enterotoxaemia caused by clostridia is a major challenge. The use of the disk diffusion method in the preparation of an antibiogram is not reliable because *C. perfringens* is an anaerobic microorganism and does not meet the reproducibility requirements in this type of test, due to the varying or reduced growth rates of anaerobes. Additionally, there is a lack of correlation between the disk diffusion test outcomes and the microdilution reference method (Archambault and Rubin, 2020). The most effective means of prevention is vaccination. While the vaccine against enterotoxaemia has not been on the market long in Croatia, in Italy efforts are being made to prevent enterotoxaemia by using biological preparations from specific bacterial strains isolated from herds that had received the vaccine. Studies on the efficacy of the vaccine have shown that the antibody titre in sheep is significantly higher than in goats, especially after vaccination. It has also been found that the overall duration of humoral immunity is longer in sheep than in goats. It should be noted that for successful immunisation of goats, vaccination should be given every three to four months (Forti et al., 2020; Kostelić, 2022; Asadi et al., 2023).

Conclusions

The bacterium *C. perfringes* is an enteropathogenic clostridium found in the soil, intestines and faeces of animals and humans. As a result of intestinal dysbiosis, the bacteria multiply in the intestine and produce toxins, which are used to classify the species into seven toxinotypes (A–G). Most toxins (CPB, ETX, ITX, CPE and NetB) begin their pathophysiological action with pore formation in the cell, leading to swelling. Studies worldwide have shown that strains A and D are most frequently associated with enterotoxaemia in small ruminants. *Clostridium perfringens* toxinotype A, which is considered part of the physiological microflora, is most frequently isolated from sheep and goats. The type A toxin is present in the environment and in the gut of clinically healthy humans and animals, which reduces its diagnostic value. The pathogenicity and increase in virulence are due to the synergistic effect of the typing CPA toxin and the non-typing CPB2 and CPE toxins. In sheep and goats, toxinotype A is usually associated with gas gangrene and yellow lamb disease. *C. perfringens* toxinotype D is the second most common type isolated from sheep and goats. In addition to the CPA toxin, which is produced by all strains, the type D strain also secretes one of the most pathogenic toxins of *C. perfringens* – the ETX toxin, which targets the kidneys and brain. It is associated with pulpy kidney disease and enterotoxaemia in sheep and goats (Singh et al., 2018; Uzal et al., 2018; Forti et al., 2020; Sepehrifar et al., 2021; Alimolaei and Shamsaddini Bafti, 2023; Kalender et al., 2023; Camargo et al., 2024). When the A or D strain is isolated, characteristic clinical pictures develop that include pathological changes in the intestine (type A), kidneys and brain (type D) (Uzal and Songer, 2008; Finnie et al., 2020; Hussain et al., 2022).

The virulence and pathogenicity of *C. perfringens* is related to genes, plasmids and chromosomes. All toxins are encoded by genes, and in addition to toxin genes, antibiotic resistance genes are also a major factor in bacterial survival. Due to the large number of antibiotic resistance genes identified to date, it is not surprising that treatment is very complex and often unsuccessful. As *C. perfringens* requires anaerobic conditions for growth, the diagnosis of antibiotic resistance itself is not easy to carry out. The disk diffusion technique may not be the preferred choice for accurate antibiogram results. Microdilution has proven to be more accurate and effective in determining sensitivity to antibiotics. A major problem with the treatment itself is also the fact that the data available to date on the susceptibility of *C. perfringens* to antibiotics mostly comes from human isolates. In animals, most studies on antibiotic resistance have been carried out on isolates from poultry. Few results are available on the antimicrobial susceptibility of isolates from small ruminants, either due to the sudden onset of death or due to economic unprofitability. It has been shown that in small ruminants, it is more profitable and better to work on prevention than on treatment. While vaccines from strains characteristic of a particular herd are already used in Italy, there is no commercial vaccine on the market in Croatia (Adams et al., 2018; Archambault and Rubin, 2020; Forti et al., 2020; Kostelić, 2022).

In the future, the prevalence of toxins and strains isolated from small ruminants in Croatia and their antibiotic resistance and humoral immunity should be investigated in order to reduce economic losses and the spread of the disease on farms. Since transmission of strains of *C. perfringens* from animals, their secretions and the soil to humans has been detected on dairy farms, its zoonotic potential should not be ignored (Yadav et al., 2017).

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> Najčešće izdvojeni toksinotipovi bakterije *Clostridium perfringens* u malih preživača i njihova otpornost na antibiotike

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Infekcija bakterijom *Clostridium (C.) perfringens* u stadima ovaca i koza dovodi do znatnih gubitaka. Najčešće bolesti malih preživača prouzročene ovom bakterijom su bolest kašastih bubrega, enterotoksemija, yellow lamb disease i nekrotični enteritis. Bakterija *C. perfringens* je rasprostranjena u okolišu i dio je crijevne mikrobiote ljudi i životinja, ali prilikom promjene fizioloških uvjeta u organizmu počinje proizvodnju toksina koji dovode do različitih patoloških promjena. S obzirom na toksine koje proizvodi dijeli se u sedam tipova (A – G). Toksinotipovi su definirani na temelju prisutnosti gena koji kodiraju za CPA (svi tipovi), CPB (tip B i tip C), ETX (tipovi B i D), ITX (tip E), CPE (tip F) i NetB (tip G) toksin. Uz enterotoksemije malih preživača najčešće se vezuju sojevi A i D. Tip A, prema dostupnoj literaturi, ujedno predstavlja i najčešće izdvojen toksinotip bakterije *C. perfringens* u stadima malih preživača. Osim toksinskih gena važnu ulogu u preživljavanju bakterije imaju i geni za otpornost na antibiotike. Od do sada otkrivenih genetskih determinanti mikrobne rezistencije većina je povezana sa sljedećim antimikrobnim lijekovima: bacitracinom (*bcrR* gen), tetraciklinima (*tet* geni), makrolidima (*erm* geni), linkozamidima (*erm* i *lnu* geni), streptograminima (*erm* geni) i kloramfenikolom (*cat* geni). Zbog naglog nastupa smrti nakon pojave simptoma i ekonomske neisplativosti vrlo je malo dostupnih rezultata o rezistenciji izolata bakterije *C. perfringens* iz malih preživača. Posljedično navedenom pokazalo se isplativijim i boljim raditi na prevenciji, nego na samom liječenju. Međutim, u Hrvatskoj je zbog nedostupnosti cjepiva protiv enterotoksemije i prevencija same bolesti otežana.

Ključne riječi: *Clostridium perfringens, enterotoksemija, toksinotipizacija, antimikrobna rezistencija.*