Alpha-toxin of *Clostridium septicum:* Mechanisms of pathogenesis and prospects for vaccine development

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Abstract

Clostridium septicum alpha-toxin (ATX) is the primary virulence factor responsible for lethal gas gangrene and associated systemic infections. This comprehensive narrative review synthesizes four decades of research on ATX molecular structure, activation mechanisms, pathogenic pathways, and vaccine development strategies. By integrating genomic, structural, and immunological evidence, this review provides a unified scientific understanding of ATX biology and highlights recent advances in recombinant and toxoid-based vaccine approaches. *Clostridium septicum* is a Gram-positive, obligately anaerobic bacillus and a highly invasive pathogen that causes fulminant myonecrosis and a broad spectrum of other infections in humans and animals, with ATX recognized as the principal contributor to its pathogenesis and disease severity. Its alpha-toxin (ATX) is a β-pore–forming toxin that rapidly disrupts cellular integrity by creating

transmembrane pores, leading to cell lysis, tissue necrosis, and systemic disease progression. Despite extensive investigation over several decades, the literature on ATX has historically been fragmented across diverse disciplines, including structural biology, immunology, and veterinary vaccinology. This comprehensive narrative review consolidates the existing knowledge base into a coherent scientific framework, identifies critical knowledge gaps, and proposes future research directions to accelerate the development of effective therapeutic and preventive interventions against *C. septicum* infections.

Key words

Alpha-toxin, Clostridial diseases, Clostridium septicum, Pore-forming toxin, Vaccine development

1. Context

Clostridium septicum, first isolated by Pasteur and Joubert in 1877 [1], is a pathogenic anaerobe whose lethal and hemolytic properties were later characterized in murine models [2]. Subsequent investigations established pore formation in host cell membranes as a central mechanism of its virulence [3].

The bacterium is naturally present in soil, the gastrointestinal flora of healthy ruminants, and has occasionally been isolated from the human appendix [4]. Unlike other gastrointestinal *Clostridium* species, the prevalence and clinical significance of *C. septicum* colonization in humans remain low and uncertain [5]. Tissue microenvironments with acidic pH and reduced oxygen tension, such as those found in tumors or infected wounds, promote anaerobic glycolysis and facilitate spore germination into vegetative cells [6]. Current evidence suggests that *C. septicum* lacks well-characterized adhesins for host-cell attachment [7]. *C. septicum* is a Gram-positive, motile, spore-forming anaerobic bacillus, measuring 3–5 μm in length and approximately 0.5 μm in diameter[8]. Motility is mediated by peritrichous flagella. The organism accounts for approximately 1.3% of all clostridial infections [9]. Its carbohydrate-fermentation profile is useful in laboratory identification [5].

C. septicum is characterized by positive reactions for esculin and gelatin hydrolysis, glucose, lactose, and salicin fermentation, and milk digestion, whereas it is negative for arabinose, cellobiose, and sucrose fermentation, as well as lipase, lecithinase, indole, urease, and nitrate reduction [4, 10]. *C.septicum* produces oval, subterminal spores resistant to heat, alcohol, and acidic pH [10, 11]. Germination requires an anaerobic environment with adequate nutrients and temperature. Sporulation enhances the organism's survival and environmental persistence[12]. Spores also resist common cleaning and disinfection procedures [13].The organism grows readily on blood agar [10]. Morphology ranges from irregular and fringed to smooth and round depending on the strain [10]. Its chromosome is 3.27 Mbp (G+C 27.5%) [14, 15], and its single 32-kb plasmid lacks known toxin genes [14]. The genome encodes 3125 ORFs [15].

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Clostridium septicum produces four primary exotoxins—alpha-, beta-, gamma-, and delta-toxin [16]—along with a diverse array of additional virulence determinants, including fibrinolysin [2], leucocidin, collagenase, internalin [17], proteases, and neuraminidases (sialidases) [18]. In addition, *C.septicum* exhibits pronounced pleomorphism, which influences cytotoxicity, adhesion, and epithelial invasion. During logarithmic growth, bacteria display coordinated swarming with low hemolytic activity. As the culture approaches late log phase, cells transition to shorter rods and secrete maximal hemolytic toxin levels [16]. Hemolytic activity declines as the bacteria enter the stationery and death phases (Figure 1). Adhesin expression also varies during the life cycle [7], with swarming forms showing reduced adherence and cytotoxicity. Swarming facilitates tissue invasion, while differential virulence-factor expression supports colonization and infection [5]. Despite substantial progress in elucidating the structural and functional properties of ATX, no licensed vaccine is currently available against C. septicum infections in humans [14]. The development of an effective vaccine is hindered by several factors, including the extreme potency of the toxin, its rapid systemic dissemination, and the lack of well-defined correlates of protective immunity. Conventional toxoid-based vaccines, although widely employed in veterinary medicine, suffer from inherent drawbacks such as batch-to-batch variability, residual formalin toxicity, and suboptimal immunogenicity [11, 14]. These limitations have motivated the exploration of recombinant subunit vaccines based on non-cytolytic ATX derivatives, which offer the advantages of defined antigen composition, enhanced safety, and scalability [14, 19].

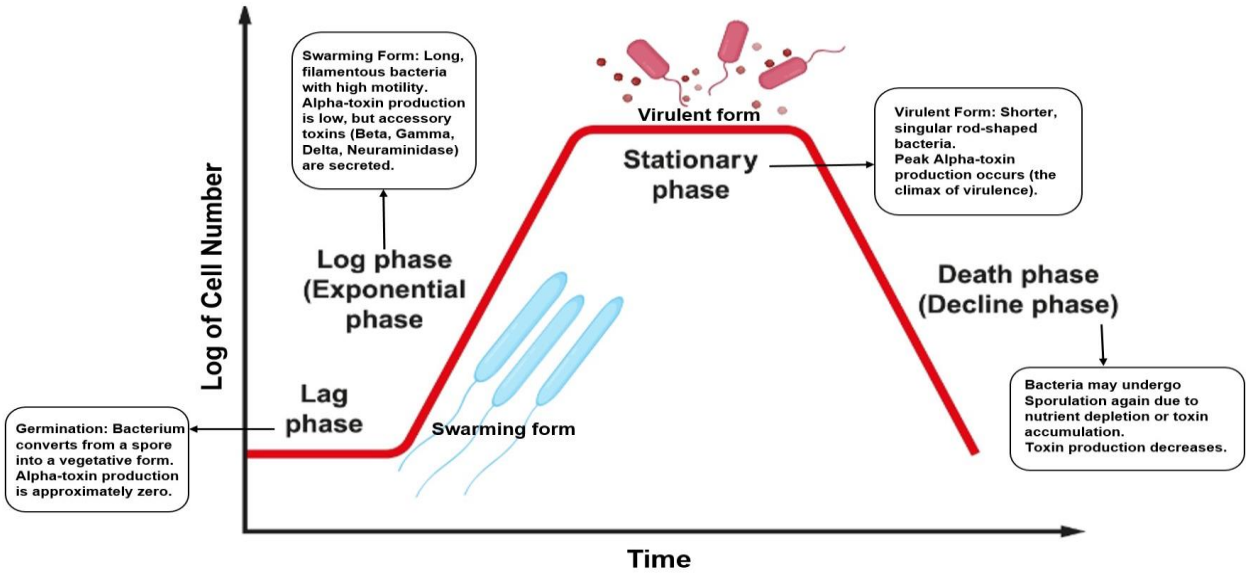


Figure 1. Regulation of Virulence and Morphology across the *C. septicum* Growth Curve. The graph integrates phenotypic and virulence data, demonstrating the transition from motile, low-toxin swarming forms during the logarithmic phase to highly hemolytic, short-rod morphotypes in the stationary phase, followed by sporulation in the death phase. This phase-dependent regulation underscores the adaptive virulence strategy of *C. septicum*. (Created by the authors using BioRender.com). Data synthesized from [7, 16].

2. Data Acquisition

A comprehensive literature search was performed to identify and analyze existing studies on *Clostridium septicum* alpha-toxin (ATX), with a focus on its molecular structure, pathogenic mechanisms, and vaccine development strategies. The review was designed to capture the most recent information about ATX biology, host immune responses, and translational approaches for vaccine design. It was conducted up to August 2026.

2.1. Search Strategy and Study Selection

A comprehensive literature search was performed using PubMed, Scopus, and Web of Science, Embase, and Google Scholar databases. The search strategy employed the following Boolean combination of keywords, adapted for each database :("*Clostridium septicum*" OR "*C. septicum*") AND ("alpha toxin" OR "alpha-toxin" OR "ATX" OR "pore-forming toxin" OR "cytolysin") AND (virulence OR pathogenicity OR infection OR "myonecrosis" OR "gas gangrene" OR vaccine OR toxoid OR immunization OR genetics OR genomics).These basic terms were supplemented with specific research field keywords such as "receptor binding," "pore formation," "immune evasion," "MAPK signaling," "inflammasome activation," "recombinant vaccine," and "chimeric antigen" to identify studies focused on ATX biological and immunological aspects. In addition, the search approach was supplemented with a manual review of reference lists from relevant publications discovered during the original search.

2.2. Inclusion and Exclusion Criteria

2.2.1. Inclusion Criteria:

Studies were selected based on their direct relevance to ATX biology, mechanisms of action, host–pathogen interactions, and vaccine development strategies. Preference was given to original research articles, comprehensive reviews, and authoritative book chapters. Studies investigating ATX molecular structure, activation mechanisms, receptor binding, pore formation, cytotoxic pathways, and host immune responses were included. Laboratory studies on vaccine efficacy, including recombinant subunit vaccines, toxoid formulations, and novel delivery platforms, were also considered. Experimental, observational, genomic, and immunological studies involving humans, animals, cell lines, or recombinant protein systems were included. Supporting contextual studies on clostridial toxin biology and comparative analyses with related pore-forming toxins (e.g., aerolysin) were incorporated to provide scientific background.

2.2.2. Exclusion Criteria:

Studies that did not specifically address C. septicum ATX or lacked scientific objectivity were excluded. Non-original articles such as editorials, commentaries, and conference abstracts without original data were omitted. Duplicate publications reporting overlapping datasets were identified and removed.

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2.3. Data Extraction and Synthesis

Given the narrative scope of this review, which aims to integrate multidisciplinary knowledge into a cohesive conceptual framework, the narrative synthesis methodology was used to combine findings from various study designs and methodologies, resulting in a thorough summary of existing knowledge about ATX biology. The included literature was critically evaluated through qualitative synthesis, with emphasis placed on methodological rigor, reproducibility, and translational relevance. The search strategy yielded a total of 52 relevant publications that met the inclusion criteria and were included in this review.

3. Results

3.1. Diseases Caused by *Clostridium septicum*

Clostridium septicum is a well-recognized pathogen responsible for a wide spectrum of clinical syndromes in both animals and humans. However, the pathological manifestations of *C. septicum* infection in animals are not as thoroughly characterized as those reported in humans [20]. In humans, the organism is a causative agent of both traumatic myonecrosis and non-traumatic myonecrosis—also referred to as gas gangrene. Notably, the mortality associated with non-traumatic myonecrosis is nearly twice that of traumatic cases, likely due to its insidious and rapidly progressive onset. The highly lethal *C. septicum* infection is initiated via two main routes: 1. Traumatic Gas Gangrene (Exogenous), involving entry through an external wound/trauma from a contaminated environment; and 2. Non-Traumatic Gas Gangrene (Endogenous), arising from an internal focus (e.g., tumor) causing septicemia. Both routes lead to systemic toxicity, characterized by acute muscle destruction (alpha-toxin effect) and culminating in rapid shock and death (Figure 2A) [15, 21, 22].

Beyond myonecrosis, *C. septicum* has been implicated in multiple additional conditions, including necrotizing enterocolitis and pericarditis [12], neutropenic enterocolitis [20], and several important veterinary diseases. These include purulent and necrotizing abomasitis (Braxy/Bradsot) in ruminants, omphalitis in lambs, and potentially some forms of enterotoxemia. The bacterium also contributes to malignant edema across various mammalian species—including cattle, sheep, and horses—post-parturient malignant edema in cows, and clostridial (gangrenous) dermatitis in poultry [10-13, 17] . More recently, *C. septicum* has also been linked to secondary infections in hemolytic uremic syndrome [23], symptomatic anthrax-like disease in pigs [8], and occasional cases of arthritis and aortitis [15] (Figure 2B).

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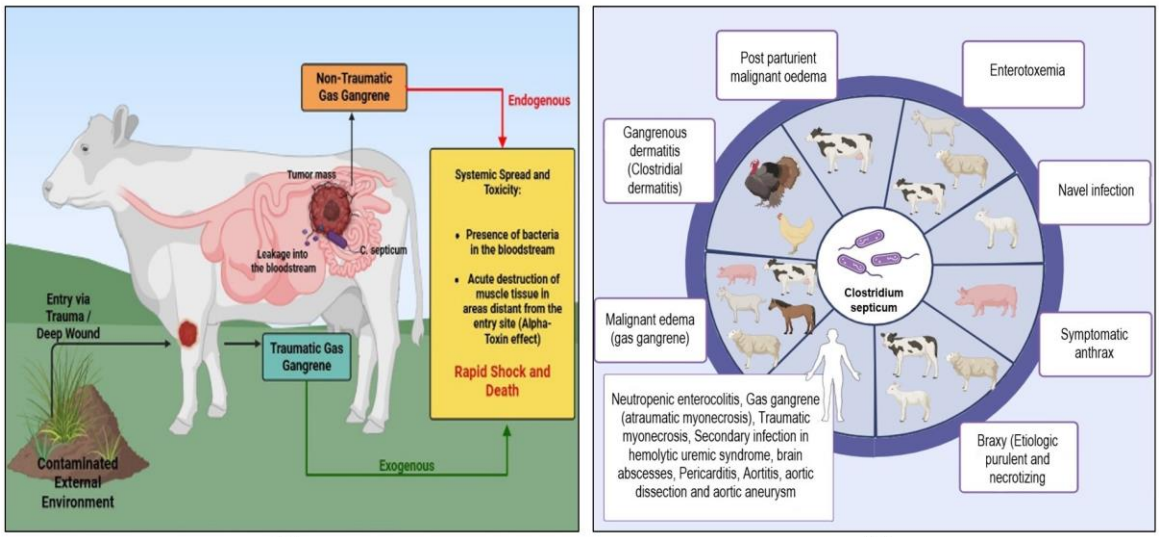


Figure 2. Pathogenesis and Clinical Spectrum of *C. septicum* Infection. **(A)** Schematic representation of the two primary routes of infection—traumatic (exogenous) and non-traumatic (endogenous)—leading to systemic toxicity and gas gangrene. (Created by the authors using BioRender.com). Data compiled from [15, 21, 22]. **(B)** Overview of diseases associated with *C. septicum* in humans and animals, including myonecrosis, necrotizing enterocolitis, braxy, and gangrenous dermatitis. (Created by the authors using BioRender.com). Data compiled from [8, 10-13, 15, 17, 20, 23].

3.2. Diagnosis and Treatment of Infections Caused by *Clostridium septicum*

The diagnosis of *Clostridium septicum* infections typically relies on a combination of clinical presentation, necropsy findings, Gram staining, serological assays, and bacterial culture [12]. Additional confirmatory methods, including PCR and immunohistochemistry, may also be utilized to enhance diagnostic accuracy [12]. Management of *C. septicum* infections generally requires both surgical intervention and antimicrobial therapy, with the most favorable outcomes achieved when these approaches are used concurrently [5].

3.3 Comparison of *Clostridium septicum* with Other *Clostridium* Species

Multiple studies highlight the substantial similarities between *C. septicum* and *C. chauvoei*, including the presence of shared virulence factors. However, *C. septicum* carries the alpha-toxin gene, which is absent in *C. chauvoei*, making it the more dangerous pathogen [17]. In addition, *C. septicum* contains an *agrD* homolog similar to that of *C. chauvoei*, which contributes to the regulation of virulence [24]. Compared with *C. chauvoei*, *C. septicum* exhibits greater diversity in prophages, CRISPR loci, and accessory genes, suggesting enhanced genomic flexibility for environmental adaptation and disease causation. Both organisms contain CRISPR elements and a small plasmid, although the genome of *C. septicum* is slightly larger [17].

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In comparison to *C. perfringens* alpha-toxin, which is a phospholipase C that hydrolyzes membrane lipids, *C. septicum* ATX is a pore-forming cytolysin lacking such enzymatic activity [18, 25]. Moreover, structural similarities between ATX and *C. perfringens* epsilon toxin suggest conserved pore-forming mechanisms among clostridial toxins [3, 25]. Accessory genetic elements in *C. septicum* show G+C contents similar to those of *C. perfringens*, and both species exhibit notable diversity in restriction-modification (RM) systems. Although their genome sizes are comparable, CRISPR elements are absent in some *C. perfringens* strains but consistently present in *C. septicum* [17].

C. septicum alpha-toxin shares substantial structural and functional similarity with aerolysin, the pore-forming toxin of *Aeromonas hydrophila* [12]. Both toxins are believed to have arisen from a common evolutionary ancestor [26]. However, differences in the G+C content of their genes suggest an early divergence in their evolutionary trajectories [2]. Although the primary structure of *C. septicum* alpha-toxin resembles the major large domain of aerolysin, it lacks the smaller ~83-residue lobe found in aerolysin [27]. Approximately 27% of their amino acids are identical, yielding an overall structural similarity of about 72%. Both toxins contain the conserved WDW_W motif, which is essential for receptor binding [28, 29].

3.4. Investigation of alpha-toxin

C. septicum is highly virulent, utilizing an extensive arsenal of toxins and hydrolytic enzymes [16-18]. Alpha-toxin accounts for more than 95% of the organism's total hemolytic activity [16] and is unequivocally recognized as the primary determinant of pathogenicity [30]. It represents the most potent toxin produced by the bacterium. It exhibits lethal, hemolytic, and necrotizing activities [18], functions as an immunodominant extracellular antigen [16], and is a robust, oxygen-resistant β-barrel pore-forming cytotoxin [13, 31]. Alpha-toxin is unique to *C. septicum* [2] and displays cytotoxicity toward multiple cell types, including *Toxoplasma gondii*, for which its effects are best characterized [31]. It shares substantial amino acid homology with aerolysin from *Aeromonas hydrophila* and enterolobin from *Enterolobium contortisiliquum* [32], belongs to the β-PFT family, and inserts into membranes via β-sheet structures [14]. Other members of this toxin family include PE, DT, STx, and PA [12].

Alpha-toxin is synthesized as an inactive precursor (protoxin), which prolongs its functional persistence in the host. As a result, toxic effects may continue even after effective antimicrobial therapy, leading to severe sequelae or death [26]. The protoxin is encoded by the *CSA* gene and secreted through the type II secretion pathway, with a molecular mass of 46.55 kDa and an N-terminal 31-amino-acid signal peptide [1, 2]. Proteolytic activation converts the protoxin to its functional pore-forming form. ATX is activated by furin in vivo [2] and by trypsin or proteinase K in vitro [26], with proteases targeting basic residues being the most efficient. Additionally, an unidentified *C. septicum*–derived activation factor is suspected to play a role [2]. The active toxin is highly unstable in solution, rapidly oligomerizing and aggregating into inactive complexes [2], which diminishes its activity [33].

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A hallmark of α-toxin activity is the rapid efflux of intracellular potassium prior to cell lysis [2]. Pore formation permits uncontrolled ion and water flux, leading to cell swelling and rupture [33]. The resultant dysregulation of ion homeostasis, including calcium influx, culminates in colloid-osmotic lysis [5]. injuring vascular endothelial cells, alpha-toxin markedly reduces tissue perfusion, oxygenation, and nutrient supply; these effects correlate with toxin concentration and drive ischemic necrosis in affected tissues [5, 14].

Alpha-toxin has a broad host range [34]. Variability in species susceptibility and differences in toxin-binding protein mass suggest that different GPI-anchored proteins may function as receptors, contributing to interspecies differences in hemolytic sensitivity [35]. Environmental factors modulate toxin activity pH significantly alters toxin conformation and pore-forming capacity: acidic pH enhances hemolytic activity, whereas alkaline conditions diminish it. Anaerobic growth of *C. septicum* produces an acidic environment conducive to increased toxin function [22]. Temperature and protease exposure also influence activity [35]. Genetic variation in alpha-toxin genes among *C. septicum* strains results in subtle structural and functional differences. These sequence variations alter isoelectric points, although immunization with one toxin variant typically confers cross-protective immunity [36]. Ballard reported an LD_{50} of approximately 10 μg/kg in mice, a hemolytic activity of 2×10^7 hemolytic units/mg, and a pI of ~8.4 [2].

3.5. Structure of Alpha-Toxin

Because of the well-established structural homology between *C. septicum* alpha-toxin and aerolysin—and the absence of an experimentally resolved alpha-toxin structure—its three-dimensional conformation has been inferred through molecular modeling approaches [2] (Figure 3A). A defining distinction is that alpha-toxin lacks the lectin-binding D1 domain characteristic of aerolysin, while retaining regions homologous to the remaining three aerolysin domains [37]. Alpha-toxin is composed of 443 amino acids and includes an N-terminal 31-residue signal peptide required for secretion. This signal peptide is cleaved between Ala32 and Leu33 following export [28]. Aside from this signal sequence, the protein contains no substantially hydrophobic regions [28].

The protoxin (ATpro) and active toxin (ATact) share an identical C-terminal sequence, and activation does not modify the N-terminus [33] nor alter overall hydrophobicity [2]. Proteolytic activation occurs approximately 45 residues upstream of the C-terminus, within a region enriched in basic amino acids (PLPDKKRRGRKRSVD) that includes the canonical furin recognition motif RGKR [26, 34]. The high density of lysine and arginine makes this region exceptionally susceptible to proteases targeting basic residues [28]. Its structural flexibility allows efficient enzymatic access. In vitro, trypsin activates ATpro by cleaving at the amino-terminal side of the SerValAsp sequence [28]. Based on protein sequence analysis, activation occurs between Arg367—adjacent to the upstream RK-rich region—and Ser368, releasing the mature toxin [36].

3.5.1. Domain Organization

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Domain 1 contains five antiparallel β-strands, seven α-helices, and six lobes [27]. It mediates receptor interactions and participates in the transition from the prepore to the mature transmembrane pore complex [3]. This domain also contributes to oligomerization [27]. A receptor-interacting loop regulates β-barrel insertion into the membrane [12], and the Cys-86 residue is essential for receptor binding [37]. Alpha-toxin contains a characteristic WDW_W motif [12] located within an 11-residue tryptophan-rich segment (302 NGYSEWDWKWV 312), critical for binding to GPI-anchored receptors and for cytotoxicity. Tryptophan residues at positions 307 and 311 are particularly important; mutation of either reduces activity. Substituting Asp308 with a basic residue similarly decreases toxicity, indicating the necessity of a negative charge at this position. The residue at position 309 must be aromatic and hydrophobic to maintain full activity [29].

Domain 2 contains the beta-motif involved in pore formation [3]. An amphipathic β-hairpin (residues 203–232) forms the membrane-spanning segment. Upon conformational rearrangement, this region associates with other monomers to assemble the β-barrel inserted into the host membrane[1, 12]. This β-hairpin constitutes the transmembrane domain (TMD). Formation of a disulfide bond within the region restricts β-hairpin movement and prevents pore formation [23]. For pore insertion, the loop must disengage from the toxin backbone, allowing membrane penetration [23]. Deletion or mutation of this domain abolishes pore-forming ability and eliminates toxicity [3], though receptor binding and oligomerization remain intact [23]. Domain 3 primarily governs oligomerization [3]. Although it does not directly participate in pore formation, it interfaces with the membrane [23]. This domain contains the activation region for ATX as well as the chaperone-like propeptide [14] (Figure 3B).

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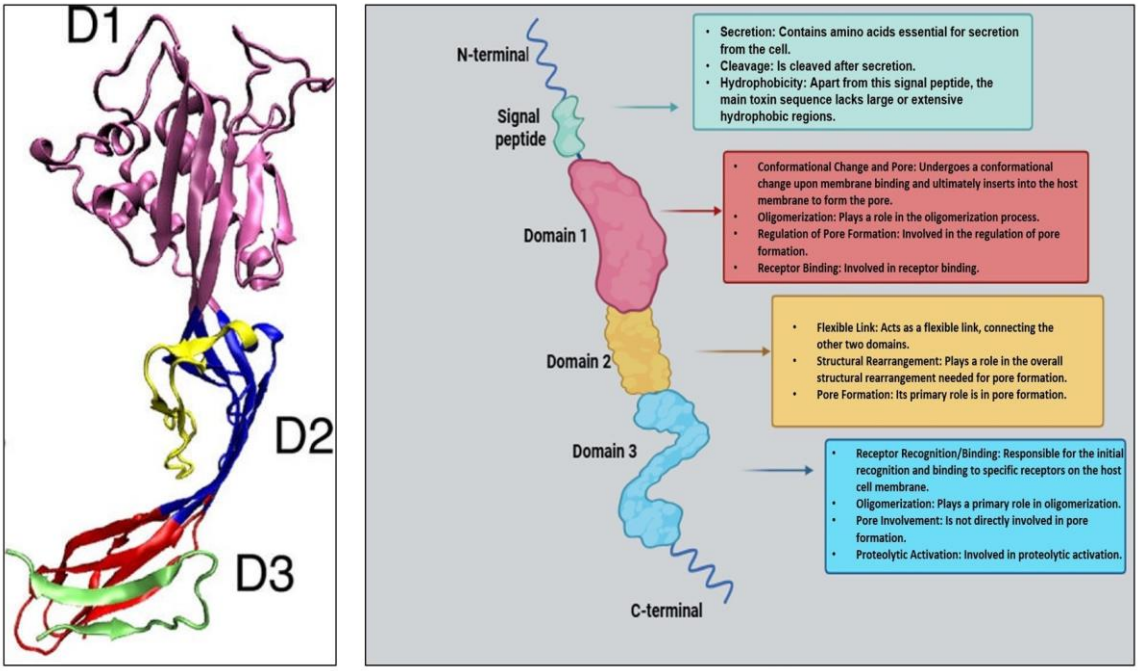


Figure 3. Structural organization of *Clostridium septicum* alpha-toxin (ATX). **(A)** Molecular model of ATX, with domains color-coded: Domain 1 (purple), Domain 2 (blue), Domain 3 (red), Transmembrane Domain (TMD; yellow), and Propeptide (green). (Reprinted with permission from Melton-Witt et al.[27], Copyright 2006, American Chemical Society). **(B)** Schematic linear representation of ATX functional domains, highlighting the receptor-binding region, the amphipathic β-hairpin (TMD), the oligomerization domain, and the proteolytic activation site. This diagram illustrates the key structural domains of the alpha-toxin and their roles in pore formation and host cell lysis. (Created by the authors using BioRender.com). Based on structural data from [14, 23, 27, 31].

The core genome of this bacterium encodes key metabolic pathways— including those for energy production, carbohydrate utilization, amino acids, nucleotides, and vitamins reflecting its ability to carry out essential physiological processes required for survival. The genome also contains a small 5.2-kb plasmid that carries genes for a viroplasm-like protein family and the teicoplanin resistance protein *vanZ*. *Clostridium septicum* additionally harbors four distinct prophages and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) elements. The coexistence of multiple prophages with CRISPR loci suggests that these CRISPR systems likely possess limited functionality in phage inhibition. The bacterium encodes methyltransferases responsible for m6A methylation but lacks mechanisms for m5C methylation. Its restriction–modification (RM) systems are diverse and exhibit notable variability among strains. Moreover, *C. septicum* displays an open pan-genome, indicating a broad and dynamic set of accessory genes that differ significantly between isolates [17].

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3.6. Mechanism of Action of Alpha-Toxin

The cytocidal mechanism of *Clostridium septicum* alpha-toxin is complex and involves several coordinated steps [34]. The toxin is secreted as an inactive monomeric protoxin [31] that first binds to target cells through glycosylphosphatidylinositol (GPI)-anchored receptors, which are not linked to the cytoskeleton. This receptor engagement promotes local toxin concentration and facilitates monomer association into oligomeric complexes. Binding commonly occurs in detergent-resistant microdomains of the plasma membrane[12]. The affinity of the toxin for GPI-anchored receptors strongly correlates with its cytotoxic activity [29]. Known receptors include the human folate receptor (hFR), the neuronal glycoprotein Thy-1, and specific protein components on *Toxoplasma gondii* [27].

Following receptor binding, proteolytic cleavage near the carboxyl terminus is required for activation. This cleavage releases a 45-amino acid, ~5-kDa propeptide [32]. The propeptide functions as an intracellular molecular chaperone, stabilizing the protoxin and preventing premature oligomerization or misfolding in solution [1, 2]. It also contributes to the correct assembly of oligomers on membranes [12]. However, it is absent from the final pore complex and is not required for cytolytic activity; once activation occurs, the propeptide dissociates during oligomerization [38]. After cleavage, the toxin oligomerizes into a seven-monomer complex [28], forming a prepore structure. The oligomer subsequently undergoes a conformational transition to form an amphipathic β-barrel in which hydrophilic residues line the pore interior while hydrophobic residues face outward, enabling membrane insertion [2, 28]. The resulting pores measure 1.3–1.6 nm in diameter [2], exhibit high conductance, remain primarily open, and do not require specific membrane lipids for formation [1]. They are slightly anion-selective [1, 2].

As noted, pore formation proceeds through a temperature-dependent step situated between oligomerization and full pore insertion. While binding, oligomerization, and prepore assembly occur similarly across temperatures, hemolytic activity varies dramatically with temperature [39]. Membrane pores disrupt intracellular ionic equilibrium, causing rapid potassium efflux and concomitant sodium and chloride influx, followed by elevated intracellular calcium. Alpha-toxin–induced cytotoxicity and cell death depend on pore formation and calcium elevation and may be either Ras–c-Raf dependent or independent [12].

Increased intracellular calcium activates calpain proteases, which degrade structural proteins and destabilize lysosomes, releasing cathepsins. These enzymes damage the cytoskeleton and impair cellular integrity. Concurrently, calcium overload injures mitochondria, promoting the generation of reactive oxygen species (ROS), which further compromise mitochondria and other cellular targets, reducing ATP production. ROS-mediated DNA damage activates PARP, which consumes ATP during repair. PARP activation also releases HMGB1, a danger-associated molecular pattern that contributes to necrosis and can precipitate sepsis [5, 31, 34]. Thus, the calpain–cathepsin cascade and mitochondrial dysfunction cooperate to drive rapid necrotic cell death [31].

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Alpha-toxin can also upregulate BAX (Bcl-2–associated X protein), but it does not activate classical apoptotic pathways. The toxin causes severe ATP depletion without directly impairing mitochondrial respiration, suggesting that disruption of cellular energy balance pushes cells from apoptosis toward rapid necrosis [1]. Consequently, although toxin-treated cells may display early features of both apoptosis and necrosis, they ultimately progress to necrosis and then oncosis (cell swelling) [34]. Calcium influx alone does not induce apoptosis in this context [31].

Pore formation also activates innate immune signaling. Potassium efflux triggers inflammasome activation [14]. In parallel, alpha-toxin induces dose-dependent activation of all major mitogen-activated protein kinase (MAPK) pathways—ERK1/2, JNK1/2, and p38—regulating the expression of numerous genes. These pathways contribute to tumor necrosis factor-alpha (TNF-α) release, a key mediator of systemic inflammation. MAPK activation depends on receptor engagement, pore formation, calcium influx, and tyrosine/threonine phosphorylation. ERK1/2 activation is Ras–c-Raf dependent, whereas JNK and p38 activation occur independently of this pathway (Figure 4).

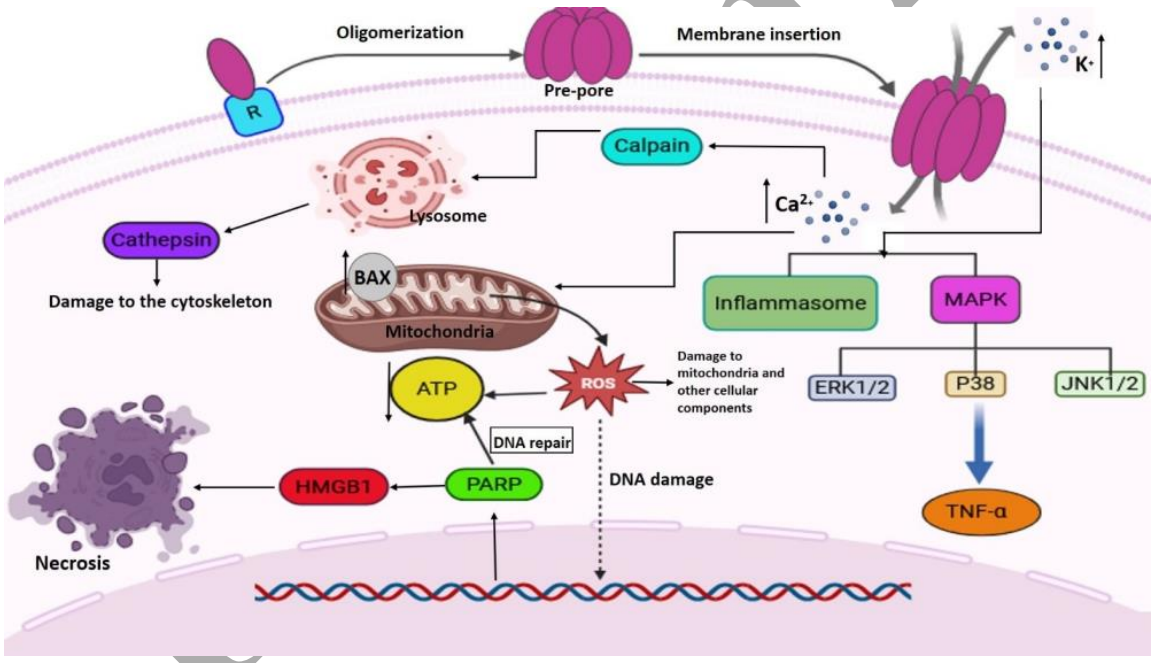


Figure 4. Mechanistic Pathway of ATX-Induced Cell Death and Inflammatory Signaling. Following receptor binding and pore formation, ATX triggers rapid potassium efflux and calcium influx, activating calpain proteases, mitochondrial dysfunction, and ATP depletion. This cascade leads to necrosis and oncosis. Concurrently, potassium efflux activates the inflammasome, while calcium influx and pore formation stimulate MAPK pathways (ERK1/2, JNK1/2, p38), culminating in TNF-α release. The diagram also highlights the immunosuppressive effects, including neutrophil suppression and immune evasion. (Created by the authors using BioRender.com). Pathway synthesized from [31, 34].

Ras, a small GTP-binding protein, localizes to the membrane via farnesylation and activates Raf kinases, which in turn phosphorylate MAP kinase kinases and subsequently ERK. Importantly, MAPK activation during systemic *C. septicum* infection does not always correlate with proportional cytokine responses, due to extensive crosstalk with other signaling networks. Thus, cytokine patterns likely reflect MAPK modulation of multiple interconnected pathways [34]. Although other microbial components (e.g., peptidoglycan, lipoteichoic acid) can activate MAPK pathways, MAPK activation and TNF-α release in distant organs such as the spleen are specifically attributable to circulating alpha-toxin. This supports hematogenous spread of the toxin and its ability to mediate systemic pathology [12]. Consequently, alpha-toxin concentrations vary significantly between the primary infection site and distal tissues [34].

A defining feature of *C. septicum* infections is the marked paucity of inflammatory cells at lesion sites [21]. Alpha-toxin plays a central role in suppressing neutrophil recruitment [37] by inducing neutrophil apoptosis, thereby dampening the host inflammatory response. This immunosuppressive effect extends to the tumor microenvironment, contributing to the limited presence of immune cells within infected tissues. Because neutrophils can exert either anti-tumor or pro-tumor effects depending on local conditions, alpha-toxin–mediated suppression likely compromises their anti-tumor functions.Alpha-toxin also induces hemolysis and thrombocytopenia and can enter the systemic circulation through tumor-associated hemorrhage [6, 14]. In addition, the toxin can kill macrophages by forming pores in both their plasma membranes and phagosomes, thereby facilitating macrophage death. Through the induction of inflammation, autophagy, and apoptosis, the toxin enhances bacterial evasion of host immunity and promotes infection establishment [40].

3.7. Studies on the Production and Development of Vaccines against *Clostridium septicum*

Vaccination remains the most effective strategy for preventing *Clostridium septicum* infections, and extensive efforts have been dedicated to developing toxin-based immunization approaches [12]. A summary of key studies on ATX-based vaccine candidates is presented in Table 1. In a seminal study, Ballard and colleagues purified the 48-kDa alpha-toxin using a multistep chromatographic protocol—anion-exchange, cation-exchange, gel filtration, and high-resolution cation-exchange chromatography. Based on recovered hemolytic activity, the overall purification yield was consistently around 50%. Over time, the purified toxin underwent proteolytic processing, generating a slightly smaller 44-kDa species. At high concentrations, this 44-kDa form further aggregated into ~230-kDa oligomeric structures that were highly resistant to SDS and heat denaturation [41].

These findings led to the important recognition that premature conversion of alpha-toxin into its oligomeric, active state—before encountering its cellular receptor—abolishes receptor binding and thereby eliminates biological activity [3].

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Ballard's group also evaluated the immunogenicity of purified alpha-toxin in vivo. Mice immunized with the toxin exhibited significantly prolonged survival following challenge with viable *C. septicum* compared with non-immunized controls [41].

The same study produced rabbit polyclonal antibodies against the purified toxin. These antibodies were highly specific, recognizing only the alpha-toxin of *C. septicum*—including the derived 44-kDa and 230-kDa forms generated from the original 48-kDa protein—while demonstrating no cross-reactivity with proteins from other closely related clostridial species [41]. Optimization of culture conditions and purification steps is essential for cost-effective production of toxoid vaccines. Studies have shown that temperature and agitation speed can significantly increase toxin yield, while reducing purification steps can shorten production time without compromising quality. These findings highlight the value of process optimization in vaccine manufacturing and suggest similar strategies could improve production of *C. septicum* toxoid and recombinant vaccines [42].

Additional work on alpha-toxin purification was conducted by Hang and colleagues, whose method closely resembled Ballard's but included an initial ammonium sulfate precipitation step at 60% saturation [43]. In another study, Vázquez-Iglesias and Estefanell-Ucha employed an electroelution-based purification method. The purified toxin was subsequently used to generate polyclonal antibodies in rabbits, which specifically recognized *C. septicum* alpha-toxin. The researchers also resolved extracellular proteins secreted by *C. septicum* via SDS-PAGE and identified them through mass spectrometry. As illustrated in their publication *"A simple electroelution method for rapid protein purification: isolation and antibody production of alpha toxin from Clostridium septicum"*, the SDS-PAGE results and mass spectrometry profiles (Figure 5A) confirmed that all detected proteins belonged to *C. septicum*, including three distinct alpha-toxin species. Although rapid, this electroelution technique produced relatively small quantities of toxin [20].

Najafi and Hemmati also employed a three-step purification strategy for alpha-toxin consisting of ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration—an approach noted for its cost-effectiveness and rapid execution [43]. Similarly, Rostamian and Hemmati generated polyclonal antibodies against *Clostridium septicum* alpha-toxin by immunizing rabbits with purified toxin. The resulting antibody response produced a broad spectrum of IgG molecules targeting the antigen, which were subsequently purified using the same three-step protocol. The final IgG preparation demonstrated potential diagnostic value for diseases mediated by *C. septicum*, particularly those driven by alpha-toxin activity [44].

In the production of bacterin-toxoid vaccines, critical variables such as culture timing and vaccine volume significantly influence vaccine efficacy. Effectiveness varies because the performance of *C. septicum* vaccines is affected by the bacterial growth phase during production, culture medium pH during formalin inactivation, and inherent properties of alpha-toxin [13]. Additional challenges include instability during manufacturing, in which complex media produce fluctuating toxin yields and inconsistent product quality. The use of formalin introduces risks of residual chemical contamination and can diminish antigenicity [24].

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Moreover, traditional vaccines often provide only partial protection and frequently fail against polymicrobial infections or genetically diverse strains [17]. Hemolytic activity and antibody levels alone are insufficient predictors of vaccine performance. Other variables—including vaccine dose, the presence of additional toxins [13], and cell-mediated immune responses—play pivotal roles [45].

To address these shortcomings, recent work has focused on recombinant subunit vaccines incorporating non-toxic fragments of alpha-toxin. These genetically engineered constructs—commonly expressed in E. coli—permit the production of purified, inherently safe antigens without requiring chemical detoxification [19]. One such development, the NCAT vaccine, was created by deleting the toxic domain of alpha-toxin. Studies showed that NCAT increased the survival of turkeys challenged with virulent strains, suggesting its utility for preventing gangrenous dermatitis in poultry. This vaccine is advantageous due to its lower cost and improved safety profile [46].

Additional work identified two non-toxic subunits, ntATX-D1 (lacking the proteolytic activation site) and ntATX-D2 (lacking both the signal peptide and pore-forming domain). Although both elicited protective immunity, ntATX-D2 demonstrated superior performance, preventing mortality and moderating disease severity. This fragment also modulated cellular responses and downregulated pro-inflammatory genes [19]. A recent study by Gaghan et al. evaluated the efficacy of ntATX-D2 in broiler chickens. Subcutaneous prime-boost immunization with ntATX-D2 adjuvanted with AddaVax provided significant protection against experimental *C. septicum* challenge, as evidenced by increased body weight gain, reduced gross and histopathological lesions, and modulation of local and systemic inflammatory responses [47].

Despite these successes, single-component vaccines targeting only alpha-toxin have not provided comprehensive protection across diverse strains. Researchers therefore emphasize the need for next-generation vaccines capable of overcoming antigenic competition [46]. This includes integrating complementary antigens such as septicolysin (CstA), which can act synergistically with alpha-toxin. Notably, vaccines focused solely on alpha-toxin may fail against strains with elevated CstA expression [17, 19]. To address this issue, a promising strategy involves engineering key antigens—such as alpha-toxin and septicolysin—into a single chimeric or fusion protein to present a consolidated immunological target [11, 17]. Nanoparticle-based adjuvants have also been proposed to stabilize antigens and enhance long-term immune responses [13].

Aligned with these developments, Thachil et al. evaluated an experimental bivalent *C. perfringens–C. septicum* toxoid vaccine in turkeys for cellulitis control. Their findings demonstrated that the bivalent formulation effectively reduced disease incidence in both laboratory and commercial settings and may decrease reliance on antibiotics within the poultry industry [48]. Polyvalent clostridial vaccines, composed of multiple toxoids, remain widely used and highly effective for managing clostridial diseases in cattle and sheep. Although immune responses vary among components, these vaccines collectively produce substantial reductions in disease incidence [45]. Nonetheless, no licensed vaccine is currently available for human *C.*

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septicum infections, such as myonecrosis (gas gangrene), underscoring the urgent need for vaccine development. A human vaccine could substantially reduce morbidity, mortality, and antibiotic use. This need has prompted the exploration of advanced antigen-discovery approaches, including:

Subtractive proteomics: Identifying *C. septicum*-specific proteins essential for virulence, resistance, or survival by excluding homologous host or commensal proteins. Reverse vaccinology: Using genomic and proteomic datasets to computationally predict suitable antigens without requiring pathogen cultivation [49]. While conventional vaccine development relies on toxoid-based or recombinant subunit platforms, alternative strategies such as passive immunization with recombinant antibodies have shown promise against clostridial toxins. For instance, phage display-derived scFvs targeting tetanus toxoid have demonstrated high specificity and neutralization potential, suggesting that similar approaches could be explored for ATX [50].

In a recent study, researchers utilized genetic engineering to develop a detoxified recombinant version of the *Clostridium septicum* alpha-toxin, designated rCSAm4/TMD. This variant was engineered by introducing four amino acid substitutions and an 11-amino acid fragment deletion, resulting in a protein devoid of the cytotoxicity and lethality associated with the wild-type toxin. Experimental data demonstrated that immunization with this recombinant protein conferred complete protection in murine models against ultra-lethal challenges with the native toxin. Furthermore, it elicited high titers of neutralizing antibodies in rabbits, providing robust protection against the disease. Consequently, rCSAm4/TMD has been proposed as a highly effective subunit vaccine candidate for the prevention of braxy in ruminants [51].Recent work also demonstrated that oral administration of *Lactococcus lactis* expressing the non-toxic region of alpha-toxin conferred 100% protection against mortality, offering a scalable, injection-free vaccine platform [52]. However, all such innovations require evaluation under field conditions to validate real-world efficacy [19].

In conclusion, *C. septicum* vaccines can be produced via multiple modalities. Traditional formulations rely on inactivated bacterial cultures and toxoids, whereas recombinant vaccines employ genetically engineered non-cytolytic forms of alpha-toxin. Recombinant platforms offer advantages such as defined antigen composition, stronger immunogenicity, and potentially lower production costs. Although no highly effective recombinant vaccine targeting ATX has yet been licensed, emerging approaches—particularly chimeric antigens combining ATX with other clostridial proteins—are promising future directions (Figure 5B) [11, 14].

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Table 1. Summary of key studies on *Clostridium septicum* alpha-toxin (ATX)-based vaccine candidates.

Vaccine Candidate	Study Type	Model System	Key Findings	Ref.
Purified ATX toxoid	In vivo	Murine	Prolonged survival post-challenge; rabbit polyclonal antibodies specific to ATX	[41]
Purified ATX	In vitro	Cell culture	Characterization of activation and pore-forming mechanism	[2]
NCAT (non-cytolytic ATX)	In vivo	Turkey	Partial protection against gangrenous dermatitis	[46]
Bivalent toxoid (C. perfringens / C. septicum)	In vivo	Turkey	Reduced cellulitis incidence in laboratory and commercial settings	[48]
Electroeluted ATX	In vitro	Protein purification	Rapid purification method; rabbit polyclonal antibodies produced	[20]
Purified ATX	In vitro	Protein purification	Cost-effective three-step purification protocol	[43]
Purified ATX (polyclonal IgG)	In vitro	Rabbit	High-titer IgG production; diagnostic potential	[44]
Polyvalent clostridial toxoid	In vivo	Sheep	Temporal antibody response characterized; effective disease reduction	[45]
rCSAm4/TMD	In vivo	Murine / Rabbit	Complete protection against ultra-lethal challenge; high neutralizing antibody titers	[51]
ntATX-D1 / ntATX-D2	In vivo	Turkey	ntATX-D2 showed superior protection; reduced inflammation	[19]
ntATX-D2	In vivo	Broiler chicken	Significant protection; increased body weight gain; reduced lesions	[47]
L. lactis-oral ATX	In vivo	Turkey	100% mortality protection; needle-free platform	[52]
Bacterin-toxoid	In vivo	Turkey	Evaluated hemolytic activity, administration route, and dosage for optimal response	[13]
Multi-epitope vaccine	In silico	Computational	Identified potential immunogenic epitopes via reverse vaccinology	[49]

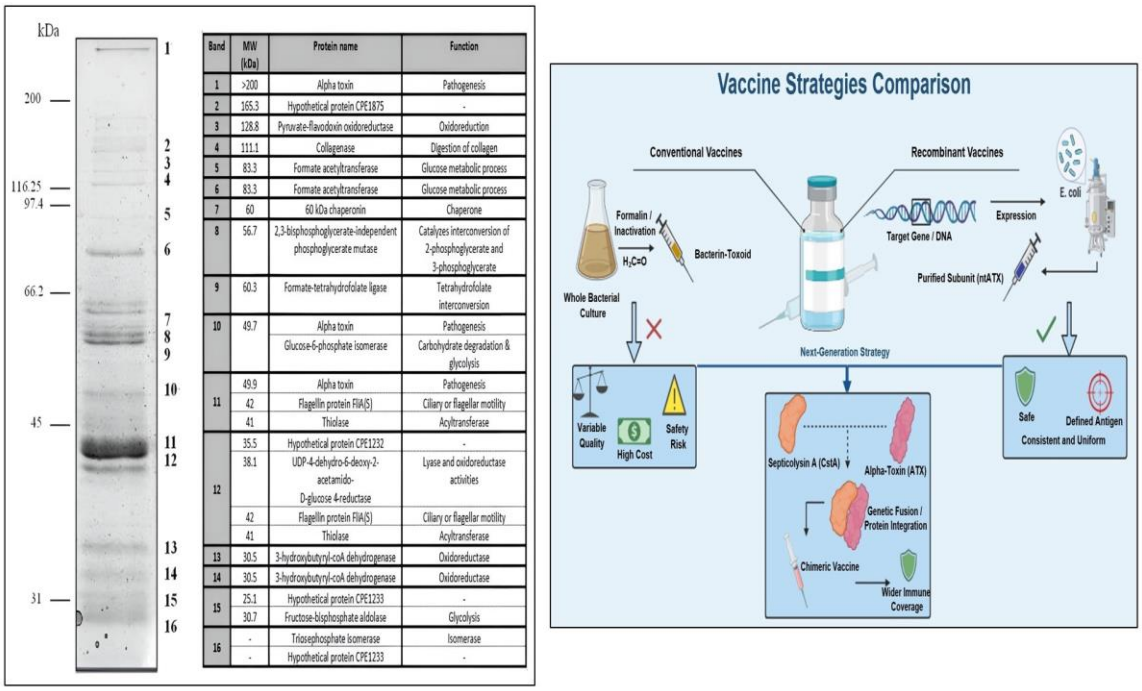


Figure 5. Vaccine development strategies and protein analysis of *C. septicum*. **(A)** SDS-PAGE profile and mass spectrometry identification of *C. septicum* extracellular proteins, including three distinct alpha-toxin species. (Reproduced from Vázquez-Iglesias et al. [20], 2017, an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0)). **(B)** Comparative overview of vaccine modalities: conventional bacterin-toxoid formulations (inactivated whole cultures), recombinant subunit vaccines (non-cytolytic ATX derivatives such as ntATX-D2 and rCSAm4/TMD), and next-generation chimeric strategies combining ATX with complementary antigens like septicolysin A (CstA). This diagram highlights the shift from conventional to modern recombinant approaches, offering advantages such as defined antigen composition, enhanced safety, and broader immune coverage. (Created by the authors using BioRender.com). Data synthesized from [11, 14].

4. Conclusion

Alpha-toxin (ATX) is the primary virulence factor of C. septicum, responsible for gas gangrene and other severe infections. Its pore-forming activity and immunomodulatory effects make it a key vaccine target. Other toxins (beta, gamma, delta, and CstA) also contribute to disease. ATX causes cell death through a multi-step mechanism involving receptor binding, pore formation, ion imbalance, and mitochondrial damage, while also suppressing inflammatory responses.

Despite significant progress in understanding ATX biology and promising advancements in recombinant subunit vaccines—including non-cytolytic derivatives such as ntATX-D2 and rCSAm4/TMD—no licensed vaccine currently exists for human C. septicum infections. Conventional toxoid-based vaccines, while

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effective in veterinary settings, suffer from limitations in safety, consistency, and cross-strain protection. The emergence of chimeric antigens, nanoparticle-based adjuvants, and oral delivery platforms offers new avenues for overcoming these challenges.

However, several critical knowledge gaps must be addressed to accelerate vaccine development. First, the majority of protective epitopes identified to date have been characterized in veterinary models, and their conservation and immunogenicity in humans remain largely unexplored. Future research should employ immunoinformatics and advanced immunological assays to systematically identify epitopes that are broadly recognized across diverse human populations. Second, a major bottleneck is the absence of a standardized animal model that faithfully recapitulates the pathophysiology of human *C. septicum* infection. Current models vary considerably in their susceptibility to ATX, immune responses, and disease progression, complicating cross-study comparisons. Third, while recombinant subunit vaccines offer defined antigen composition, their immunogenicity is highly dependent on formulation parameters, and the optimal choice of adjuvant remains to be systematically determined. Fourth, although several candidates have demonstrated robust protection under controlled laboratory conditions, their efficacy in real-world field settings remains unproven, necessitating large-scale field trials. Fifth, the identification of septicolysin A (CstA) as a complementary virulence factor underscores the limitations of single-antigen vaccines, highlighting the need for multi-antigen or chimeric vaccine strategies. Sixth, the path to clinical translation is fraught with challenges, including the need for comprehensive toxicological profiling and large-scale production processes. Finally, emerging technologies such as reverse vaccinology and needle-free delivery platforms—exemplified by the recently reported Lactococcus lactis oral vaccine—offer transformative opportunities that, combined with traditional vaccinology and novel adjuvant formulations, hold the promise of overcoming current limitations.

The integration of structural biology, immunology, and innovative vaccine technologies will be essential for translating experimental evidence into clinical practice. Achieving a safe and effective vaccine against C. septicum—a goal that is both scientifically achievable and clinically imperative—remains a priority for future research.

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Statements and Declarations

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Author contribution

Aisan Heyrani: creating figures and writing original draft, review & editing, Investigation, Data curation. Maedeh Samianifard: review & editing, Validation and revision of the scientific content. Mehdi Jafari Anari:

review & editing, Validation. Ali Nazari: review & editing, Validation, Supervision, Project administration, Conceptualization.

Ethical approval

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Conflict of interest Data

The authors have no competing interests to declare that are relevant to the content of this article.

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No datasets were generated or analyzed during the current study.

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