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Review Article

Biomolecules of the Horseshoe Crab's Hemolymph: Components of an Ancient Defensive Mechanism and Its Impact on the Pharmaceutical and Biomedical Industry

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Without adaptive immunity, invertebrates have evolved innate immune systems that react to antigens on the surfaces of pathogens. These defense mechanisms are included in horseshoe crab hemocytes' cellular responses to pathogens. Secretory granules, large (L) and small (S), are found on hemocytes. Once the invasion of pathogens is present, these granules release their contents through exocytosis. Recent data in biochemistry and immunology on the granular constituents of granule-specific proteins are stored in large and small granules which are involved in the cell-mediated immune response. L-granules contain most clotting proteins, which are necessary for hemolymph coagulation. They also include tachylectins; protease inhibitors, such as cystatin and serpins; and anti-lipopolysaccharide (LPS) factors, which bind to LPS and agglutinate bacteria. Big defensin, tachycitin, tachystatin, and tachyplesins are some of the essential cysteine-rich proteins in S-granules. These granules also contain tachycitin and tachystatins, which can agglutinate bacteria. These proteins in granules and hemolymph act synergistically to fight infections. These biomolecules are antimicrobial and antibacterial, enabling them to be drug resistant. This review is aimed at explaining the biomolecules identified in the horseshoe crab's hemolymph and their application scopes in the pharmaceutical and biotechnology sectors.

1. Introduction

Horseshoe crabs are aquatic arthropods from the Animalia kingdom, Arthropoda phylum, Xiphosura or Xiphosurida order, and Limulidae family. Around 400 million years ago, a horseshoe crab species roamed on the Earth, at least 200 million years older than the dinosaurs [1–3]. Currently, four horseshoe crabs exist on the Earth though they are restricted to specific regions. *Limulus polyphemus* is called Atlantic horseshoe crab. The North American continent's Atlantic coast is home to the *Limulus polyphemus*. The *Tachypleus tridentatus*, which is called trispine horseshoe crab, can be found along the Western islands of the Philippines as well as the Northern beaches of Japan and South Vietnam. *Carcinoscorpius rotundicauda* known as the mangrove horseshoe crab is a crab species found in the northern part of the Bay of Bengal. The Bay of Bengal is where *Tachypleus gigas* is predominantly found [4–7]. Figure 1 depicts the global distribution of living and extinct horseshoe crab species.

Mostly, horseshoe crabs are captured for biomedical and pharmacological applications due to their extraordinary blue hemolymph properties. Prosoma, opisthosoma, and telson are 3 parts of horseshoe crab body [2]. There are seven pairs

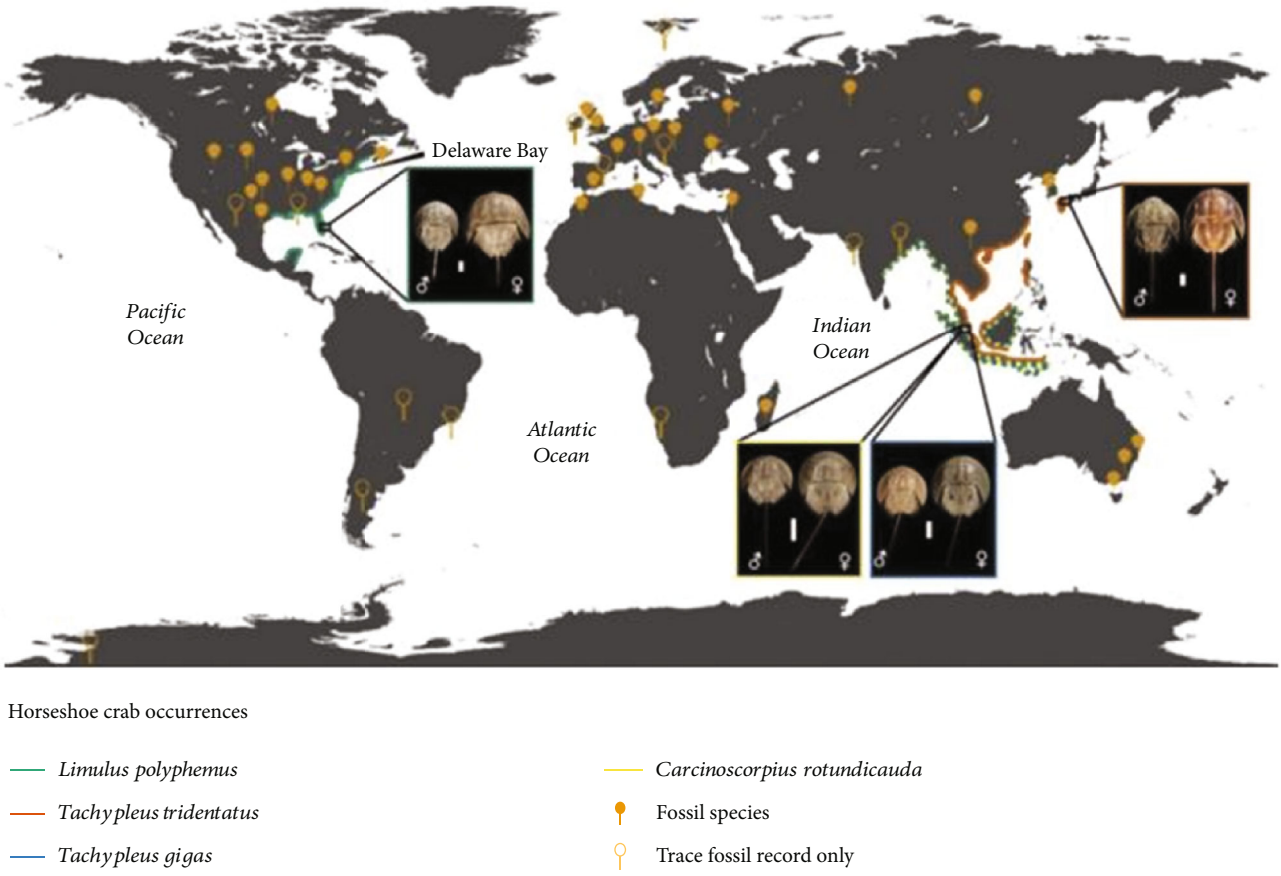

Figure 1: Horseshoe crab species distribution worldwide [1].

of appendages on the concave ventral side of the prosoma. The first four pairs of walking legs help with the breakdown of food. It has four leaf-like legs stretching out when the appendages are forced against the ground, either pushing sand back or propelling the animal ahead. The ventral side of the abdomen is lined with 6 pairs of membrane appendages. Five pairs of book gills provide the natural surface for gas exchange. As a weapon, the tail spike is used for swimming, correcting, and digging rather than articulating with the posterior abdomen [3]. The horseshoe crab's distinct body sections are shown in Figure 2.

Amebocytes or granulocytes are special type of cell that are susceptible to bacterial endotoxins found in the horseshoe crab hemolymph [4–8]. Large (L) granules which are less dense and smaller (S) granules which are dense are two types of granules which fill the cytoplasm of the hemocyte which is shown in the electron micrograph [9, 10]. LPS-induced exocytosis in hemocyte releases granular components into hemolymph plasma. Immunocytochemical examination of these granules revealed three clotting factor including factor C [11, 12] which is a proclotting enzyme [11, 13–17]. L- and S-granules store biologically active substances and function as a host defense mechanism against pathogens [10, 18].

Limulus amebocyte lysate and tachypleus amebocyte lysate from horseshoe crab blood are blessings widely used in the pharmaceutical business to determine endotoxin [20]. Bacterial endotoxin test follows the sensitivity of 0.005 EU/ml, which amounts to 0.0005 to 0.001 ng/ml [21]. US Pharmacopeia and FDA have approved the test as an acceptable endotoxin testing strategy for liquid suspensions, biomedical devices [22], drug delivery strategies, and orthodontic procedures [23, 24]. LAL is a widely preferred assay for the testing the endotoxin clinically and environmentally [25]. Furthermore, the rise of pathogens immune to numerous antibiotics has put the health of populations in danger, and horseshoe crabs may be a potential source of antibacterial peptides [26]. To the safe fabrication of immunizations and injectable medications, the horseshoe crabs have become vital for last 40 years [5].

The article is aimed at exploring the remarkable properties of horseshoe crab biomolecules, which are linked to their hemolymph, and their potential applications as biologics and therapeutics in the biomedical and pharmaceutical fields.

2. Source of Information

Information from recent literature (no time restriction) was acquired from the Google Scholar, PubMed, and Scopus databases to choose the material on horseshoe crab and biomolecules of its hemolymph for this review. The

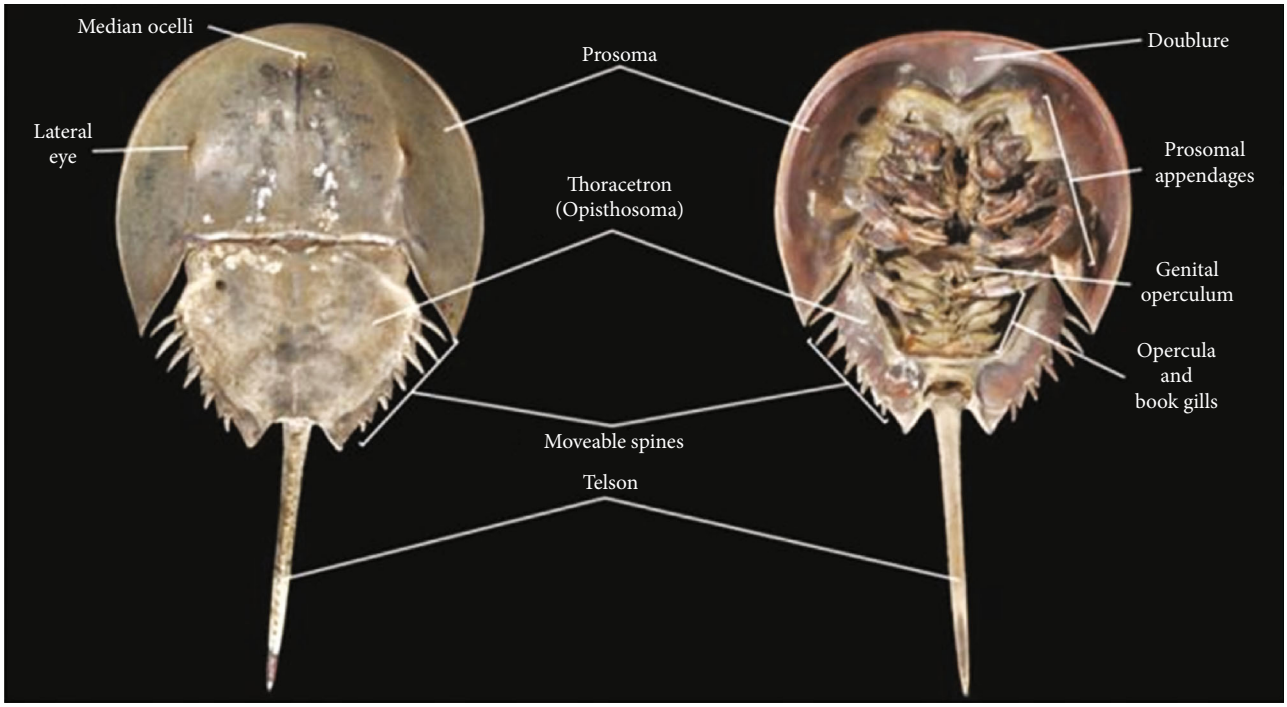

Figure 2: Basic morphological features of horseshoe crab [19].

horseshoe crab, roaming of horseshoe crab, horseshoe crab's blood components, immune system of the horseshoe crab, biomedical use of horseshoe crab, LAL kit, TAL kit, COVID-19, and horseshoe crab hemolymph and endotoxin were considered as keywords for the literature search. Protein diagrams have been obtained from Protein Data Bank (PDB), and BioRender and MS PowerPoint have drawn other illustrations.

3. Features of Horseshoe Crab's Hemolymph

To counter microbial invasion successfully, horseshoe crabs have evolved a new defense system over hundreds of millions of years [27]. The horseshoe crab's innate immune system is concentrated mainly in the hemolymph, which contains hemocytes or amebocytes [15]. Hemolymph includes two types of hemocytes: granular and nongranular cells [28]. The predominant hemocyte is the amebocyte (Figure 3) [27]. Large and small granules are distinguished by electron microscopy due to their distinctive electron concentration [29]. Table 1 describes the different features of large and small granules.

The hemolymph contains mainly three types of proteins; they are hemocyanin, C-reactive protein, and α2-macroglobulin [30, 31]. Table 2 represents the hemolymph of different species of horseshoe crab.

Additionally, horseshoe crab blood contains plasma and cell-free hemolymph (CFH). The blue hemolymph fluid is called the cell-free hemolymph (CFH). CFH contains hemocyanin (HMC), a predominant (90-95%) protein enriched with blue copper ion which is the reason behind the horseshoe crab's blue blood. Only the HMC can generate microbicidal reactive oxygen intermediates from prophenoloxidase (proPO). HMC-proPO is converted to PO by extracellular microbial proteases during host-pathogen interactions. The innate immune system effectors are located in the CFH that control humoral antimicrobial defense and bacteria response. Complement elements, CRP isoforms, and lectins are examples of innate immune components with evolutionary roots [27].

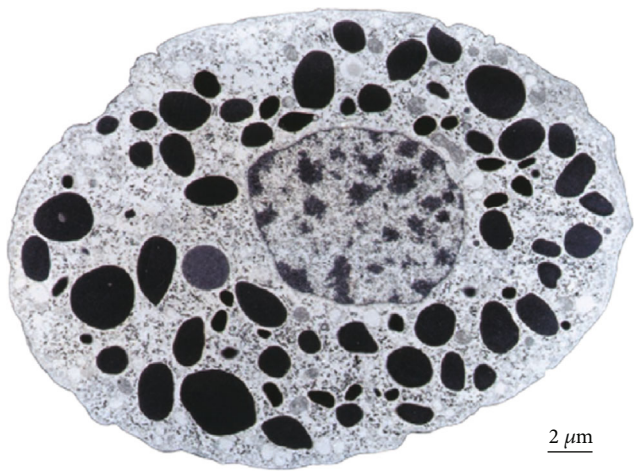

Figure 3: A cross-section of a granular hemocyte under electron microscopy [32].

4. Coagulation Reaction of Horseshoe Crab's Hemolymph

Coagulation occurs because of the presence of LPS secreted by the Gram-negative bacteria. It starts with three serine protease zymogens: proclotting enzyme, factor C, and factor

Table 1: Features of large and small granules.

Types of hemocyte	Features of hemocyte
Large (L) granules	(1) The L-granules consist of approximately 20 proteins with molecular weights ranging from 8 to 123 kDa [19]. (2) After isolating the L-granules, the extract showed that it caused the LPS to gel [29].
Small (S) granules	(1) The S-granules include at least six proteins with molecular weights less than 30 kDa, as well as the antimicrobial peptide tachyplesin and its derivatives. Recent studies on S-granules have revealed that they also accumulate tachycitin, tachystatins, and large defensin in addition to tachyplesins (all of which are also found in L-granules), all of which have antibacterial properties against Gram-positive and Gram-negative bacteria as well as fungus [30–33].

Table 2: Under the transmission and scanning electron microscopy (TEM and SEM) features of hemolymph of different species of horseshoe crabs.

Species	Features of hemolymph
Limulus polyphemus	(1) Blood contains only the large granulocyte [29]. (2) Blood cell count of *L. polyphemus* (20,000-60,000 cell mm^{-3} as reported by Wu et al. [34].
Tachypleus tridentatus	(1) Hemocytes come in two different types: granular and nongranular (Figure 3). Three clotting factors and one antimicrobial component are present in the large granules, while the other antimicrobial compounds are only available in the small granules [11].
Tachypleus gigas	(1) Under light microscope, blood cells of *T. gigas* were ovoid with light pink cytoplasm packed with red granules. TEM of normal granular cells of *T. gigas* revealed that the cytoplasm consisted of large (1 μm) and small (0.5 μm) granules [35]. (2) The average blood cells count of *T. gigas* was 33,000 cell mm^{-3} (29,000 for females and 37,000 for males) as reported by Wu et al. [34].

B, resulting coagulogen, a clottable protein. Factor C (123 kDa) is an LPS-responsive biosensor [11, 12, 33, 36, 37]. In the presence of LPS or synthetic lipid A analogs, it is autocatalytically converted to an active form, factor C. The activation of factor B (64 kDa) by factor C results in its active form (factor B), turning proclotting enzyme into a clotting enzyme (54 kDa) [35, 38–40]. The active clotting enzyme converts coagulogen to an insoluble gel known as coagulin. Although the actual process by which this coagulin gel form is unexplained, a recent compositional analysis of the Tachypleus tridentatus's coagulogen provided a foundation for understanding the crosslinking mechanism [41]. Sometimes due to fungal infections, bacterial endotoxin test (BET) shows pseudopositive result caused by glucans. A potential beta-D-glucan-sensitive protease zymogen in a hemocyte lysate was discovered in 1981 [42]. Since then, the instability of this protein is known as factor G. This beta-D-glucan-mediated coagulation mechanism may be activated on the surface of fungi [43].

The *Limulus* intracellular coagulation inhibitors, LICI-1, LICI-2, and LICI-3, are three kinds of serpins identified from the hemocytes [44–46]. Each and every LICI belongs to the serpin family and creates complex compounds with the target serine proteases. LICI-1 exclusively inhibits factor C*, whereas LICI-2 and LICI-3 suppress factor C*, factor G*, and activity of the clotting enzyme. LICI-2 has a more substantial inhibitory effect on the clotting enzyme, but LICI-3 favors factor G* over other enzymes. One of the antibacterial compounds, big defensin (Figure 4), is copurified with LICI-1 and interacts only with LICI-1, not with LICI-2 or LICI-3 [44].

5. Biomolecules of Horseshoe Crab Hemolymph

Granular hemocytes, which make up 99% of all hemocytes in horseshoe crabs, are responsible for the storage and release of several defensive chemicals, such as the anti-LPS factor, clotting protein coagulogen, protease inhibitors, serine protease zymogens, lectins, and antimicrobial peptides. Table 3 and Figure 5 summarize the defense or biomolecules found in horseshoe crab hemocyte and hemolymph plasma.

5.1. Anti-LPS Factor. Anti-LPS factor (102 amino acid residues) and tachyplesins (17 residues for tachyplesin I) or polyphemusins (18 residues for polyphemusin I) were first found in hemocytes as defensive molecules that negate a range of LPS activities [13, 15–17, 48–57]. The anti-LPS factor is a molecule in the shape of a disc that has a great charge distribution and is amphipathic. It has a single domain with three α-helices packed against a four-stranded sheet. Gram-negative bacteria cannot grow because they can attach to lipids, which is most likely why this works [49]. Tachyplesin, a peptide with an amphiphilic structure, increases bacteria's permeability to potassium, including *S. aureus* and *E. coli* [50, 51].

5.2. Big Defensin. Big defensin (Figure 6) is a peptide present in both large and small molecules [59]. Gram-negative and Gram-positive bacteria and fungus like *Candida albicans* are all inhibited by this chemical (Table 4). The isolated molecule, coined "big defensin," is constructed with 79 amino acid residues [60]. However, big defensin differs in size from

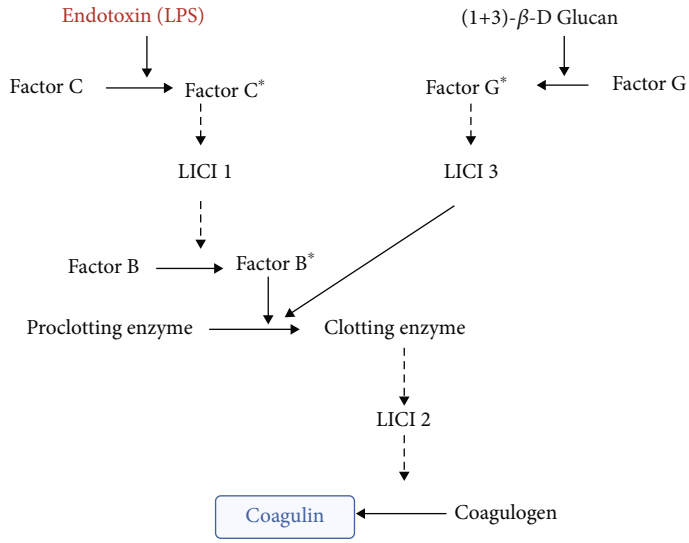

Figure 4: Cascade of horseshoe crab coagulation by coagulation inhibitors LICI-1, LICI-2, and LICI-3.

mammalian defensins, which typically comprise just 29–34 residues [61].

5.3. Tachycitin. Tachycitin (Figure 7) is a 73-amino acid protein that has five disulfide bridges but no N-linked sugars [63]. Although tachycitin has only mild antibacterial properties on its own, it dramatically enhances the antimicrobial action of large defensin. In the presence of a little amount of tachycitin, the concentration of big defensin required to inhibit the development of Gram-negative bacteria by 50% (i.e., the half-maximal inhibitory concentration [IC50]) is decreased to one-fiftieth of its normal value (Table 4) [64]. Chitin is a crucial protein which is a basic structural unit of fungi as well as the cell wall of bacteria. They may stimulate and accelerate wound healing [58].

5.4. Tachystatins. A novel tachystatin family with extensive antibacterial effectiveness against Gram-negative and Gram-positive bacteria and fungi was discovered by Osaki et al. in 1999. The most potent of these tachystatins is tachystatin C (Table 4). Tachystatin A is homologous to tachystatin B (Figure 8), although the two proteins' sequences do not share a lot of similarities [65].

5.5. Factor D. Copurified with the separation of horseshoe crab serpins from hemocyte lysates was a novel antimicrobial glycol protein of 43 kDa designated as factor D. Factor D is composed of 394 amino acids and a signal sequence. L-granules of hemocytes contain factor D [67].

5.6. α2-Macroglobulin. In the plasma of the American horseshoe crab, *L. polyphemus*, they are the third most common protein. Horseshoe crabs have α2M in their plasma and red blood cells [68, 69]. *Limulus* α2M has a lot in common with mammalian α2Ms [70]. A cDNA that codes for *Limulus* α2M has a 25 amino acids signal sequence at the NH2-terminus, and the protein it makes is 1,482 amino acid long [71]. It has a similar structure to the human complement factor C8 chain, which is consistent with *Limulus* α2M playing a role in host defense [58].

5.7. Transglutaminase. Therefore, it is anticipated that a TGase may participate in crosslinking the coagulin gel and in the immobilization of invasive microbes in the horseshoe crab clotting system [72]. The 8.6 kDa and proline-rich proteins are essential in hemocytes' L-granules, although horseshoe crab TGase is cytosolic. The crosslinking of coagulin or microbial cell walls with other proteins may be facilitated by TGase [58].

5.8. Factor C. Factor C is linked to the amebocyte membrane and activated by LPS in vivo. This causes a signal transduction cascade involving G-protein-coupled receptors (GPCRs), which causes the amebocyte to degranulate and release defensive chemicals, such as the zymogens of the coagulation cascade [73]. It is roughly 38 kDa in size [74]. Higher concentrations of the proclotting enzyme protein and factor C are linked to increased LAL reactivity [75].

5.9. Coagulogen. In horseshoe crabs, the coagulation cascade is made up of a clottable protein coagulogen (Figure 9) and four serine protease zymogens, which include factor C, factor B, factor G, and the proclotting enzyme. LPS and β-1,3-glucans of fungal cell wall components act as biosensors for factor C and factor G, which cause the coagulation factors to be sequentially activated, resulting in the conversion of coagulogen to coagulin [76].

Pathogen-associated compounds in horseshoe crab encourage the rapid formation of a gel generated by the cleavage of coagulogen into coagulin, which then interacts with proxins to build a matrix that immobilizes the pathogen in a network of hemocytes and coagulin polymers [41, 77]. The three-dimensional composition of coagulogen shows a major polymerization mechanism in which the release of the helical peptide C exposes a hydrophobic cove on the "head," which interacts with the water-insoluble edge

Table 3: Biomolecules exist in horseshoe crab hemolymph and hemocytes [58].

Types of molecule	Proteins and peptides	Mass (kDa)	Function/specificity	Localization
Larger granules	*Coagulation factors*			
	Factor C	123	Serine protease	L-granule
	Factor B	64	Serine protease	L-granule
	Factor G	110	Serine protease	L-granule
	Proclotting enzyme	54	Serine protease	L-granule
	Coagulogen	20	Gelation	L-granule
	Protease inhibitors			
	LICI-1	48	Serpin/factor C	L-granule
	LICI-2	42	Serine clotting enzyme	L-granule
			Serine/factor G	
	LICI-3	53	Complement	L-granule
	α2-Macroglobulin	180	GNB	Plasma, L-granule
	Anti-LPS factor	12	GNB	L-granule
	Factor D	42	GNB	L-granule
	Lectins			
	Tachylectin-1	27	LPS (KDO), LTA	L-granule
	Tachylectin-2	27	GlcNAc, LTA	L-granule
	Tachylectin-3	15	LPS (O-antigen)	L-granule
	Tachylectin-4	470	LPS (O-antigen), LTA	L-granule
	Tachylectin-5A	41 (SP) 31.185 (Se) 210,/300 (GF)	Acetyl group	Plasma
	Tachylectin-5B	41 (SP) 33.069 (Se) 21	Acetyl group	Plasma
	Big defensin	8.6		L-granule
	LEBP-PI	12	GNB, GPB, and FN	L-granule
	Limulus cystatin	12.6	New type	L-granule
	Limunectin	54	Cystatin family	L-granule
	18 K-LAF	18	PC	L-granule
	8.6 kDA protein	8.6	Hemocyte aggregation	L-granule
	Proxins	80	TGase substrate	L-granule
	L1	11	TGase substrate	L-granule
	L4	11	Unknown Unknown	L-granule
Smaller granules	Tachyplesins	2.3	GNB,GPB,FN	S-granule
	Tachycitin	8.3	GNB,GPB,FN	S-granule
	Tachystatins	6.5	GNB,GPB,FN	S-granule
	Big Defensin	8.6	GNB,GPB,FN	S-granule
	Polyphemusin	2.3	GNB,GPB,FN	S-granule

Table 3: Continued.

Types of molecule	Proteins and peptides	Mass (kDa)	Function/specificity	Localization
Other molecules	Trypsin inhibitor	6.8	Kunitz type	ND
	LTI	16	New type	ND
	Chymotrypsin inhibitor	10	ND	Plasma
	Limulin	300	HLA/PC, PE, SA, and KDO	Plasma
	LCRP	300	PC, PE	Plasma
	TCRP-1	300	PE	Plasma
	TCRP-2	330	HLA/PE, SA	Plasma
	TCRP-3	340	HLA/SA, KDO	Plasma
	Polyphemusin	ND	LTA, GlcNAc	Plasma
	TTA	ND	SA, GlcNAc	Plasma
	Liphemin	400-500	SA	Hemolymph
	Carcinoscorpin	420	SA, KDO	Hemolymph
	GBP	40	Gal	Hemolymph
	PAP	40	Protein A	Hemolymph
	(1→3) β-D-glucan	168	Pachyman	Hemocyte
	Transglutaminase(TGase)	86	Crosslinking	Cytosol
	Limulus kexin	70	Precursor processing	ND
	Toll-like receptor (tToll)	110	Precursor processing ND	Hemocyte
	Hemocyanin	3600	Oxygen transporter	Plasma

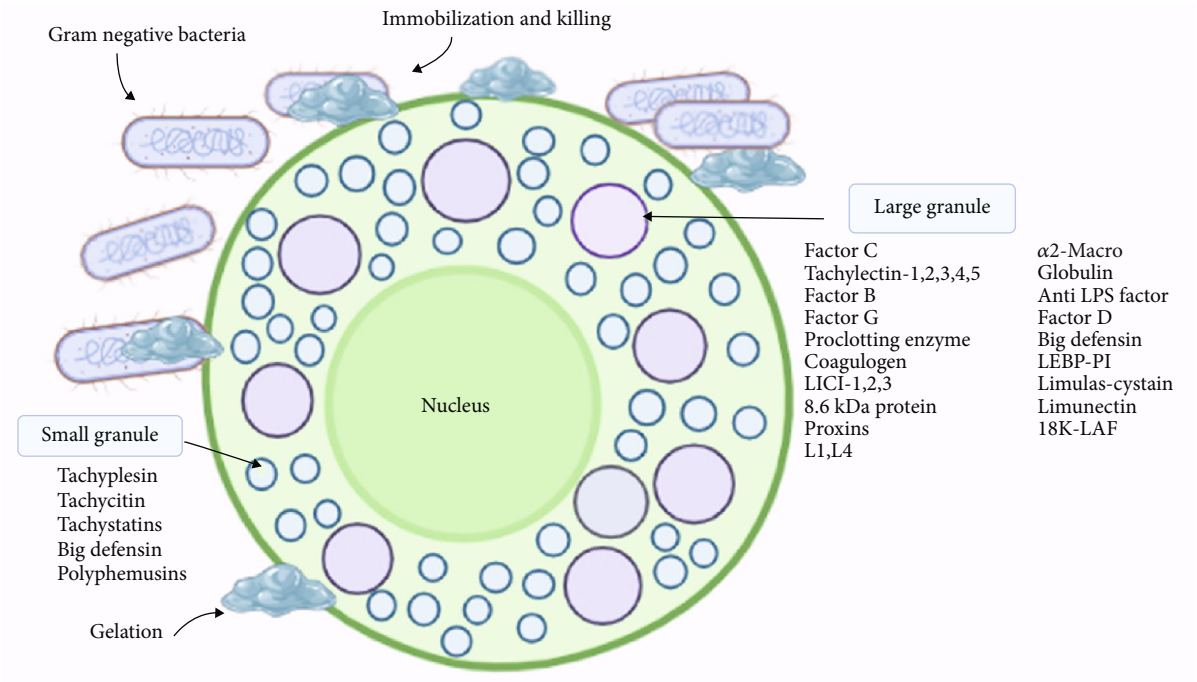

Figure 5: Defense systems associated with defense molecules of horseshoe crab hemolymph and their localization [47].

or "tail" of another molecule, leading to the formation of a coagulin homopolymer [78].

5.10. CRP. The horseshoe crab, *Carcinoscorpius rotundicauda*, has a great deal of CRP in its blood [79]. In the species *Tachypleus tridentatus* from Japan, the CRP families are called CRP-1, CRP-2, and CRP-3 [58, 80]. In the American species *Limulus polyphemus*, CRP-2 is known as limulin. The CRP functional criteria are met because CRP-1 and CRP-2 bind phosphorylcholine in a way that depends on calcium [79]. Pure horseshoe crabs CRP-1, CRP-2, and CRP-3 did not affect *E. coli* K12, *Enterococcus hirae*,

Figure 6: Big defensin solution structure of *Tachypleus tridentatus* (pdb id: 2RNG) [62].

Micrococcus luteus, or *Staphylococcus aureus* 209P in terms of clumping or stopping growth [80]. CRP is essential to the first line of defense against infections. It was found that when CRP is tested with plasma or hemolymph, it binds to a broader range of bacteria than when CRP is tested on its own [79].

5.11. Cystatin. Cystatin may be essential for biological defense mechanisms against invaders and shielding cells from unwanted proteolysis by intracellular and extracellular cysteine proteases. Limulus (L) cystatin, a single-chain protein with 114 amino acids and a molecular weight of 12.6 kDa, was isolated from the Japanese horseshoe crab. It was discovered by immunoblotting to be present in hemocyte's L-granules. Cystatin formed from L-granules cooperates with other defense compounds produced in response to external stimuli to effectively resist invasive pathogens [58].

6. Impact of Horseshoe Crab Blood on the Biomedical Industry

Horseshoe crab blood has unique qualities that benefit the biomedical sector. That is why it has broad range of use.

6.1. LAL Test. Limulus amebocyte lysate (LAL) is an integral feature of the horseshoe crab's innate immune system [81]. The activated components of LAL originate in the amebocytes of the horseshoe crab. Active ingredients are released when the amebocytes are broken or lysed. Possessing (1,3)-β-D-glucan lysate can identify Gram-negative bacteria and fungi [42]. There are two main methods for endotoxin detection: the *in vitro* pyrogen test (IPT) and the test Limulus amebocyte lysate (LAL) [82].

LAL is manufactured by "bleeding" adult horseshoe crabs of both sexes and isolating the amebocytes from the plasma or hemolymph. It is worth noting that commercial LAL can be different depending on the brand or manufacturer. Due to manufacturing variations, there are qualitative and quantitative discrepancies among LAL brands. The assessment can be evaluated in various ways, and the manufacturing technique will vary depending on the type of analysis selected. Levin and Bang introduced the approach [4]. Figure 10 shows the diversified uses of LAL test.

6.1.1. LAL Test in Radiopharmaceutical, Biopharmaceutical, and Pharmaceutical Industry. The LAL test has been shown to be reliable since its establishment. Particularly for radiopharmaceuticals, the LAL test has demonstrated its accuracy. Recombinant drugs are produced through genetic engineering approaches derived from living organisms. LAL is used as a tool to measure the purity of the recombinant drugs because mostly the sources of the drugs are bacteria, fungi, and different types of cell lines where the growth medium, fungi, or bacteria are the potential sources of endotoxin. Gram-negative bacteria and endotoxins easily contaminate water. That is why in the pharmaceutical industry, water is the substance that has the most LAL tests because it is used in all drugs and devices, either as an integral component or as a processing agent. The level of the FDA and the USP permits the level of endotoxin 0.25 endotoxin units (EU) ml^{-1}. Water testing for renal dialysis is a particular type of testing which is followed by the kidney dialysis industry. Concern about endotoxin toxicity is equal to the production of injection water as kidney dialysis water. The water used to make intravenous drugs must already have low levels of endotoxin. Individual components and finished products are tested with LAL to ensure that the intravenous solution and its container meet the endotoxin limit [25].

Pharmaceuticals classified as biologicals are those derived from compounds taken from people and animals, such as clotting factors and insulin. Additionally, biologicals include vaccinations that may contain bacterial or animal components, such as chicken eggs. It is well established that Gram-negative bacteria may easily infect biologicals, resulting in batches containing high levels of endotoxin. Fortunately, biologicals are often given in small amounts and are frequently injected intramuscularly. Nonetheless, in 1976, following severe reactions to a new batch of swine flu vaccination, it was established, in one of the early applications of the LAL test, that the batch of vaccine included an abnormally high quantity of endotoxin that was causing the bad effects [25].

6.1.2. LAL Test in the Biomedical Industry. Syringes, catheters, and other medical devices like needles are frequently made using extremely clean manufacturing processes. Implanted devices, such as porcine heart valves or orthopedic implants with intricate manufacturing processes, might, however, include endotoxins at levels that lead to localized inflammation and ultimately the rejection of the implant. In these circumstances, it is crucial that LAL evaluates the devices [25].

6.2. Utility of Horseshoe Crab Blood in COVID-19. Horseshoe crab blood is essential to the story of scientific progress that spans the 1960s and continues right up to the present day when the globe is coping with a pandemic that has

Table 4: Antimicrobial activities (IC50 μg/ml)* of the horseshoe crab hemocyte-derived proteins and peptides [32].

Protein/peptide	*E. coli* K12	*Staphylococcus aureus* 2O9P	*C. albicans* M9	*Pichia pastoris*
Tachyplesin	<2.5	.3	.2	.1
Polyphemusin	6.3	6.3	6.3	ND
Big defensin	2.5	<2.5	20	42
Tachystatin A	25	4.2	3.0	0.5
Tachystatin B	NI	7.4	3.0	0.1
Tachystatin C	1.2	0.8	0.9	0.3
Tachycitin	33	56	52	41
Anti LPS factor	>20	1.3	ND	ND
Factor D	36	NI	ND	ND
Limulus cystatin	>86	100	ND	ND

*Half-maximal inhibitory concentration. ND: not determined; NI: no inhibition at 100 μg/ml.

Figure 7: Solution structure of tachycitin, an antimicrobial protein with chitin-binding function of *Tachypleus tridentatus* (pdb id: 1DQC) [64].

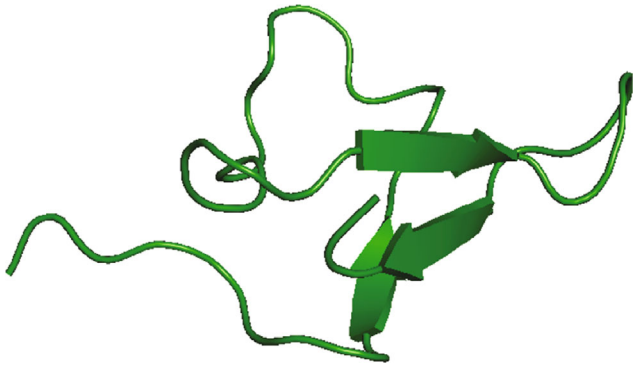

Figure 8: Tachystatin B isolated from the horseshoe crab, *Tachypleus tridentatus* (pdb id: 2DCW) [66].

claimed more than 3 million lives. COVID-19 vaccinations that are restoring hope for an end to the epidemic are scientific marvels of the twenty-first century [83]. LAL is used to test each antibody test, each batch of vaccine, and each syringe and vial used to administer the COVID-19 vaccinations [84].

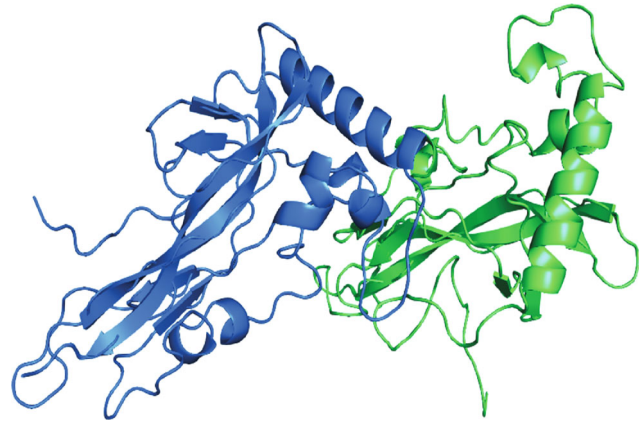

Figure 9: Coagulogen of *Tachypleus tridentatus* (pdb id: 1AOC) [41].

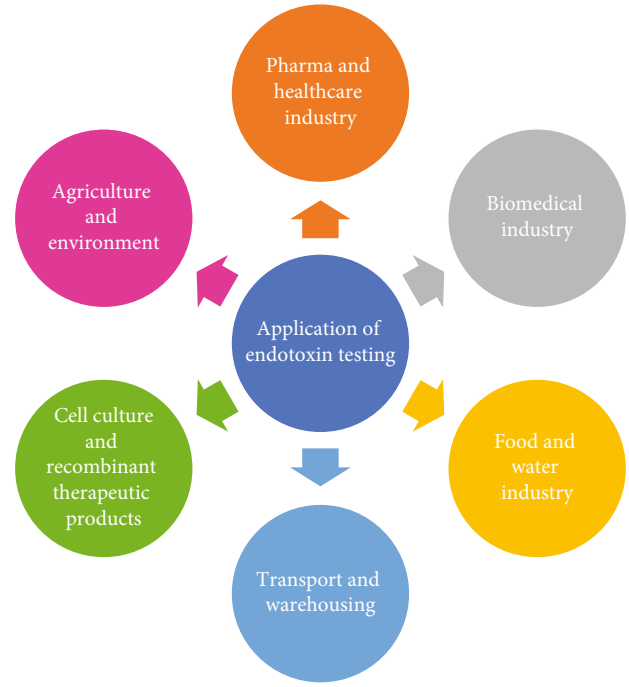

Figure 10: Application of LAL test.

Table 5: Potential drug, antibiotics, and therapeutic effect of hemolymph's molecules of horseshoe crab.

Biomolecules	Potential effect
Tachystatin	(i) Tachystatins A, B, and C are hemocyte-derived antimicrobial peptides in *Tachypleus tridentatus*. Tachystatin B contains B1 and B2 isopeptides. Tachystatins B1 and B2 are more potent than tachystatins A and C at countering fungi and Gram-positive bacteria [65, 66]. (ii) Stabilized tachystatin B can be a potent antifungal peptide with medicinal applications [66].
Tachyplesin I (TPI)	(i) Tachyplesin I (TPI) is a cationic β-hairpin antimicrobial peptide having broad antibacterial efficacy against Gram-positive and Gram-negative bacteria with very low MICs (0.8 to 12.5 g/ml) [17]. (ii) Compared to TPI, the all-d-amino acid derivative (TPAD) demonstrates significantly increased stability against enzymatic degradation and decreased hemolytic activity, indicating greater therapeutic potential. The combination of TPAD and LED209, a QseC/B inhibitor, greatly enhanced the bactericidal effect against three multi-drug-resistant bacterial strains [89]. (iii) Tac has been shown to stop the growth of some tumor cells, such as prostate cancer (TSU), melanoma (B16), hepatocarcinoma (SMMC-7721), human promyelocytic leukemia (HL-60), human gastric adenocarcinoma (BGC-823), and lung cancer (SPCA-1) cells [90]–[91]. Cyclic analogues of tachyplesin I are anticancer and cell penetrating [92]. (iv) Biomaterials surface coated with tachyplesin I and antimicrobial agents. In a bacterial-contaminated mouse skin wound model, wound healing potential was demonstrated [93]. (v) Tachyplesin worked as a nonviral macromolecule nanocarrier in both biological systems, transporting cargo molecules [94]. (vi) Tachyplesin I made Gram-negative bacteria more sensitive to the killing effects of subinhibitory concentrations of novobiocin and nalidixic acid, but tachyplesin I was still able to kill polymyxin B-resistant strains with changed lipopolysaccharides. In 40 minutes, tachyplesin I stopped the growth of bacteria forever. A *Salmonella typhimurium* phoP strain that was sensitive to defensin was also sensitive to tachyplesin I. The inverted inner membrane vesicles of Escherichia coli were quickly depolarized by tachyplesin I. These results show that tachyplesin-mediated killing of Gram-negative bacteria is linked to the cytoplasmic membrane's depolarization and the outer membrane's permeabilization [95].
Tachycitin	(i) A chitin-binding antimicrobial protein known as tachycitin demonstrated notable effectiveness against fungus [96].
Big defensin	(i) A wide range of uses are demonstrated by the purified big defensin's antibacterial effectiveness against Gram-negative and Gram-positive bacteria and its antifungal activity. As a result, it may complement the anti-LPS factor in protecting from invasive bacteria [97].
Lectin	(i) A *Tachypleus tridentatus* hemolymph-derived isolated lectin performed significantly against human colon cancer [98].

7. Future Aspects of the Biomolecules in the Biomedical and Pharmaceutical Industry

In the past 70 years, horseshoe crab blood has gone from curiosity to vital in pharmaceuticals. Not only bacterial endotoxin tests but also diversified pharmaceutical and biomedical products are dependent on horseshoe crab blood. With the advancement of biological research, more sectors are opening for the utilization of horseshoe crab biomolecules.

7.1. Potential Drug, Antibiotics, and Therapeutic Nature of Hemolymph Molecules. The recent surge in the number of drug and multi-drug-resistant microbial pathogens poses a significant worldwide concern. As a result, identifying novel techniques for developing novel anti-infectives and therapeutic targets is one of the top priorities in global health care. According to a recent study, Gram-positive *cocci* are the leading cause of nosocomial infections. About 16% infections with *Staphylococcus aureus* and 14% infections with *Enterococcus* species take the lead. Invasive fungal illnesses have also posed a life-threatening hazard to people with impaired immune systems because they are difficult to identify, treat, and prevent [85, 86]. However, some living species encounter illnesses that rely on the host's defensive system or immunity to survive. Vertebrates spontaneously produce antibodies, a kind of security and safety known as innate immunity. Immunity against infectious pathogenesis is mainly developed by ingesting antibodies, commonly known as acquired immunity. Antimicrobial peptides (AMPs) have potent antibacterial action against many bacteria that cause illnesses. As a result, AMPs have lately been regarded as a potential class of antibiotics [66, 87]. Among the invertebrate species, horseshoe crab's hemolymph can be an enriched source of antimicrobial peptides. Table 2 shows that the antimicrobial activity of this protein is stronger against Gram-negative bacteria than against Gram-positive bacteria [88].

Table 5 describes potential drugs, antibiotics, and therapeutic effects of hemolymph molecules of horseshoe crab. Figure 11 evolves some bases of potential antibiotic components obtained from horseshoe crab's blue blood.

7.2. Endotoxin Detection Biosensor. The LAL test may have drawbacks like poor stability, high cost, and inconvenience. To address these issues, a variety of novel methods—including electrophoresis, fluorescence, chemiluminescence, and electrochemical methods—have lately been used in the production of endotoxin sensors [99]. Table 6 describes different types of endotoxin detection biosensor.

7.3. Recombinant Factor C. To conserve the horseshoe crab, recombinant factor C (rFC), a synthetic replacement, has been introduced. Factor C molecules from numerous

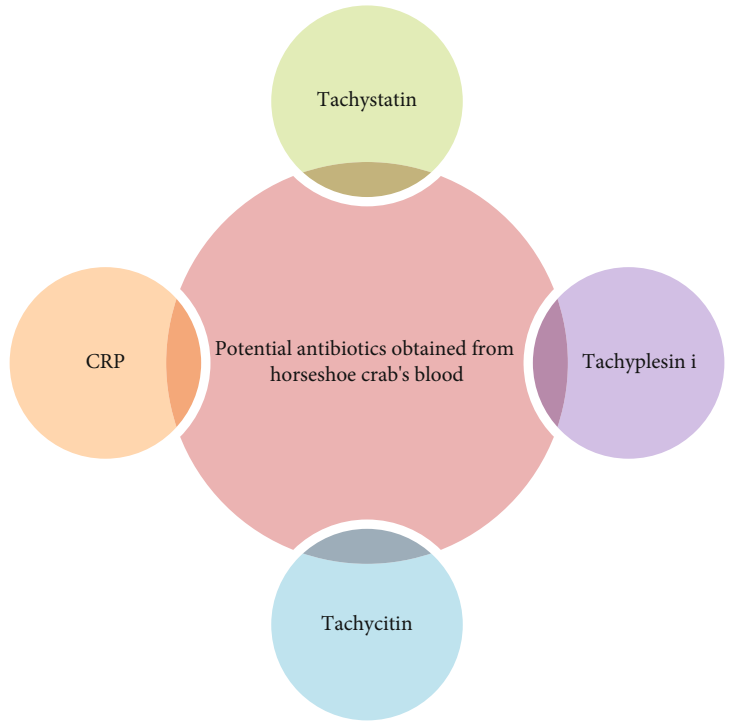

Figure 11: Potential antibiotics obtained from horseshoe crab's blood.

Table 6: Endotoxin detection biosensor and their features.

Endotoxin detection biosensor	Features of the biosensor
(1) Endotoxin-induced Limulus amebocyte lysate gel-clot process	(1) Electrochemical behavior of the sensor has already been studied. For the electrochemical examination, screen-printed electrodes with excellent stability, easy operation, and low cost are developed [100]. (2) The electrochemical signals of the gel-clot process are then applied to the endotoxin assay [101]. (3) The LAL contained zymogens that were activated by an endotoxin to produce p-nitroaniline (pNA), which was then electrochemically measured by differential pulse voltammetry (DPV). Within an hour, this chip device could detect endotoxins with as few as 10 units/l at ambient temperature. This method has been shown to improve the LAL assay sensitivity by 200 times compared to the commercial standard methods, reduce the assay time by more than half, and eliminate the need to incubate the test samples [102].
(2) A unique open-microcavity photonic-crystal biosensor	(1) To monitor the change in the refractive index due to the reaction between LAL regents and endotoxins.
(3) A nanoplasmonic biosensing method	(1) In the pharmaceutical industry, a nanoplasmonic biosensing method for endotoxin detection has previously been developed [103].
(4) Highly sensitive endotoxin assays using DNA-modified gold nanoparticles and peptide/graphene oxide	(1) This sensor can detect as little as 0.001 EU ml^{-1}. Additionally, this technique has been effectively used to assess endotoxins in complicated biological samples, which may have a lot of potential applications [104].

horseshoe crab species have been cloned and thoroughly studied. Using methods of genetic engineering, the recombinant factor C was produced by cloning the DNA of a factor C molecule and serving as a synthetic replacement for the LAL test (rFC). It has demonstrated a broad sensitivity and valid range [5]. The proenzyme activation of rFC is facilitated by minute amounts of endotoxin. The sensitivity of endotoxin detection increases from 0.005 EU ml^{-1} to 0.001 EU ml^{-1} when the amount of rFC is increased from 10 g to 80 g. When compared to commercial LAL under the same test conditions, rFC showed a lower background reading and a more sensitive reactivity to endotoxin [105].

Table 7 includes multiple researches on the usefulness of rFC as bacterial endotoxin testing on various samples.

Table 7: Different studies conducted on recombinant factor C (rFC).

Author and year	Study title	Study findings	Reference(s)
Bolden and Smith [106]	Application of recombinant factor C reagent for the detection of bacterial endotoxins in pharmaceutical products	In this study, the rFC reagent was used as part of an endpoint florescence-based endotoxin method to test a number of pharmaceutical products. The method is the same as or better than the bacterial endotoxin test (BET) method in the compendia.	[106]
Schwarz et al. [107]	Residual endotoxin contaminations in recombinant proteins are sufficient to activate human CD1c+ dendritic cells	Endotoxin units (EU) are commonly used to measure the amount of bacterial contamination in commercially produced recombinant proteins. Most suppliers guarantee less than 1 EU level, roughly equivalent to 100 pg of E. coli LPS per microgram of recombinant protein. 1-100 pg LPS may be present in protein preparations with 10-1000 ng/ml values. The current study examines the effects of extremely low endotoxin concentrations ranging from 0.002 to 2 ng/ml on human immune cells because the majority of in vitro research has concentrated on endotoxin effects produced by concentrations between 1 and 100 ng/ml.	[107]
Reich et al. [108]	Detection of naturally occurring bacterial endotoxins in water samples	In this study, three commercially available synthetic reagents showed a correlation of 94.4% when evaluating water from different sources.	[108]
Chen and Mozier [109]	Comparison of Limulus amebocyte lysate test method for endotoxin measurement in protein solutions	The kinetic turbidimetric approach, the kinetic chromogenic method, the endpoint chromogenic method, and the new recombinant factor C method (PyroGene) were all compared to the LAL test. The endotoxin test allows for a 2-fold error (50-200%) and a spike recovery of 50-200%. To rule out interfering molecules, such as LPS interaction with proteinaceous or lipophilic chemicals, this spike recovery research was done on all samples in the test plates. This practice is beneficial, but exact precision at low levels is difficult to determine. The majority of endotoxin values fall within a 2-fold range. Some samples, such as yeast samples, may require pretreatment before testing.	[109]
Grallert et al. [110]	EndoLISA®: a novel and reliable method for endotoxin detection	In this study, the primary receptor of the Limulus amebocyte coagulation cascade reacts with factor C to detect LPS. The detection range of EndoLISA (rFC) is 0.05 EU/ml to 500 EU/ml.	[110]
McKenzie et al. [111]	Evaluation of lot-to-lot repeatability and effect of assay media choice in the recombinant factor C assay	This study demonstrated that rFC assays of environmental materials are repeatable from lot to lot and that employing Tris buffer or water as the extraction and assay medium for endotoxin assessment in dust samples may be a viable choice for the development of a standardized method.	[111]
Thorne et al. [112]	Evaluation of the Limulus amebocyte lysate and recombinant factor C assays for assessment of airborne endotoxin	The results of this study show that the LAL and rFC assays deliver the same amount of information about how much endotoxin is in the air in livestock facilities.	[112]
Loverock et al. [113]	A recombinant factor C procedure for the detection of Gram-negative bacterial endotoxin	A photometric approach for detecting Gram-negative bacterial endotoxins is described in this article (lipopolysaccharide). The traditional Limulus amebocyte lysate (LAL) cascade is initiated by the activation of recombinant factor C (rFC). The rFC technique described here measures the cleavage of a fluorogenic substrate by an enzyme.	[113]

Table 7: Continued.

Author and year	Study title	Study findings	Reference(s)
Bolden et al. [114]	Results of a harmonized endotoxin recovery study protocol evaluation by 14 BioPhorum Operations Group (BPOG) member companies	A quantitative fluorometer is used in the endpoint fluorescence assay. Recombinant factor C, an endotoxin-sensitive protein, a fluorogenic substrate, and a buffer are used in this experiment. Fluorescence is analyzed at the beginning of the investigation and after 60 or 90 minutes of incubation. The variation in fluorescence is therefore proportional to the endotoxin concentration. The samples were diluted and then examined in accordance with site protocols. Diluted samples were mixed with recombinant factor C, a fluorogenic substrate, and buffer in a 96-well microplate. The endotoxin concentration was ascertained by contrasting the fluorescence variation after an hour of incubation with the standard curve. Linear regression was used to calculate the results.	[114]

The rFC test is quantitative, whereas the LAL test is qualitative or semiquantitative, and the rFC-based assay was equivalent to the LAL test for detecting Gram-negative bacterial endotoxin. LAL is triggered by peptidoglycan from Gram-positive bacteria, exotoxins from group A streptococci, and simple polysaccharides like dithiols, yeast mannans, and bacterial dextrans, which might result in false positive results. Surprisingly, the rFC-based assay is less prone to false positives than the LAL test because rFC lacks glucan-sensitive factor G. Regardless of the vendor, these nine investigations all showed that readily accessible rFC assays detected endotoxins as well as or better than LAL. A wide range of research has demonstrated the effectiveness of rFC. The breadth of these studies also revealed good properties for the pharmaceutical use of rFC, such as high precision, exceptional dependability, and excellent performance across various applications [5].

8. Conclusion

The horseshoe crab's blue blood has an advanced defense mechanism that is particularly sensitive to infections and other factors. Amebocytes detect a trace amount of LPS molecules by hemolymph coagulation reaction. Protease inhibitors, antimicrobial peptides, proteins, and agglutinins are abundant in the plasma and hemocytes. Recently, antimicrobial peptides have been considered potential candidates for therapeutic anti-infectives due to their fast bactericidal effect. Numerous antimicrobial peptides are now in clinical trials for the treatment of a variety of bacterial infections. Additionally, specific antimicrobial peptides have a broad spectrum of activity, which is beneficial in some therapeutic regions. Antimicrobial peptides obtained from horseshoe crab could be a promising sector for therapeutic and antimicrobial agents, which can play a significant role in the pharmaceutical and biomedical industries.

Abbreviations

LPS: Lipopolysaccharide
LAL: Limulus amebocyte lysate
FDA: Food and Drug Administration
CFH: Cell-free hemolymph
HMC: Hemocyanin
proPO: Prophenoloxidase
LICI: Limulus intracellular coagulation inhibitors
GPCR: G-protein-coupled receptor
TGase: Transglutaminase
DCA: Dansylcadaverine
CRP: C-reactive protein
AMP: Antimicrobial peptide
pNA: *p*-Nitroaniline
DPV: Differential pulse voltammetry
rFC: Recombinant factor C.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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