Application of Cell-free Hemolymph of Horseshoe Crab in Antimicrobial Drug Screening

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Abstract: Horseshoe crabs are an ancient invertebrate which possesses powerful innate immune defense against microbes. The simplicity, specificity and rapidity of its antimicrobial response have accorded the horseshoe crab as an excellent animal model from which immune responsive tissues may be procured for biomedical research. Such usefulness is exemplified by the extensive application for nearly four decades, of the limulus amebocyte lysate (LAL) for sensitive detection of endotoxin in the medical industry. Apart from the amebocytes, the cell-free hemolymph (CFH) of this arthropod offers a large repertoire of evolutionarily conserved proteins, which are highly sensitive to pathogens. This makes the hemolymph an ideal physiological microenvironment for simulating an in vitro infection. We therefore propose to employ the CFH as a quick and convenient tool for antimicrobial drug screening in vitro. This specific drug screening system also provides further optimization of drug design, and selection of drugs with antioxidant properties. Being an easily accessible natural resource, and allowing high-throughput screening with uniform and reliable data output, the horseshoe crab CFH provides a desirable physiological milieu for drug screening and development.

Keywords: Horseshoe crab, innate immunity, cell-free hemolymph, antimicrobial drug screening.

INTRODUCTION

Emerging bacterial resistance to existing drugs over the last decade has increased the demand on the search for new and more potent antimicrobial agents to treat infectious diseases [1]. This has necessitated the medical industry to explore new strategies and technologies to hasten the process of drug discovery. Although genome-based drug discovery is capable of providing large numbers of drug candidates, further screening and validation of such lead candidates are required to confirm any potent antimicrobial drugs. Conventional "whole-cell" testing assay is regarded as a golden method employed for such screening [2]. However, there are disadvantages such as: (a) the lack of linkage of the mode-of-action, being likely attributable to the absence of physiological conditions in relation to environmental interactions in the extracellular milieu; (b) overlooking of valuable drug candidates which are bioactive against the target but not non-target microorganisms; (c) difficulty in high-throughput running mode [2]. Thus a system, which could provide a physiologically relevant environment for targeting specific pathogens, could possibly overcome the abovementioned limitations. Here, we propose a novel in vitro model system using the cell-free hemolymph (CFH) of horseshoe crabs as a simple, convenient and economical system for rapid screening of antimicrobials. Being readily available, the horseshoe crab, which thrives in a habitat teeming with disease-causing pathogens, harbor a repertoire of well-known bioactive molecules. Its CFH offers an ideal physiological milieu for examining the efficacy of antimicrobial drugs in a simulated microenvironment of infection.

WHY IS THE HORSESHOE CRAB CFH BIOLOGICAL RELEVANT FOR ANTIMICROBIAL DRUG SCREENING?

Having survived over hundreds of millions of years in a microbiologically challenging environment, the horseshoe crabs have evolved a fascinating defense system to effectively fight against microbial invasion. Hence, this invertebrate can be used as an excellent model in biomedical research for studying innate immune strategy to improve human healthcare. Its most remarkable contribution to mankind is the extensive application of the limulus amebocyte lysate (LAL) in the pharmaceutical industry for detecting endotoxin, a ubiquitous pathogenic molecule from the Gram-negative bacteria. Endotoxin, also known as lipopolysaccharide (LPS), can induce septic shock in humans if intravenously administered [3]. The predominant hemocyte, also known as amebocyte elicits coagulation defense, which is the basis of the LAL assay [4]. Altogether, the hemolymph forms a formidable defense fortress for the horseshoe crabs.

The amebocytes, which are highly sensitive to LPS, would undergo immediate degradation at the site of infection, to exocytose a cascade of clotting enzymes and coagulogen where upon activation, forms a coagulin gel clot for immobilizing Gram-negative bacteria [5-9]. LPS induces sequential activation and limited proteolysis of three serine proteasezymogens (Factor C → Factor B → proclotting enzyme) and a clottable protein (coagulogen), all of which are stored in the large granules of the amebocytes [10-12]. Furthermore, a cell wall component of the fungi – 1,3-β-D-glucan, can activate the serine proteasezymogen. Factor G, which constitutes an alternate pathway in this cascade to activate the proclotting enzyme and converts coagulogen to a coagulin gel matrix [13]. Besides these coagulation components, a pool of natural antimicrobial peptides e.g. tachyplesins [14-16], polyphemusins [14], big defensin [17], tachycin [18], tachystatins [19], are also found in the small granules of the amebocytes, which will be simultaneously released in the proximity of infection to kill the invading microbes. The accumulated wealth of evidence indicates the immediate responsiveness and specificity of such amebocyte-driven innate immune defense against Gram-negative bacterial infection. Thus, for antimicrobial drug screening, it will be necessary to remove the amebocytes from the hemolymph, hence the cell-free hemolymph (CFH). Furthermore, the absence of amebocytes would eliminate any potential interference on the antimicrobial activities of the drugs for testing in this in vitro CFH-based system.

The CFH (also known as plasma) is the blue-colored fluid component of the horseshoe crab hemolymph. This is where a collection of innate immune effectors constituting pathways are present and involved in humoral antimicrobial defense and/or regulating the innate immune response when confronting microbes. Versatile evolutionarily entrenched innate immune molecules - complement components [20], three different isoforms of C-reactive protein...
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(CRPs) [21-23], and various lectins [24-31] have been identified and characterized. These essential innate immune factors not only act on their own in modulating host defense, but also crosstalk with each other or with other CFH proteins to form multifunctional pathogen-recognition complexes, which target pathogens specifically. For instance, the lectins; the pentraxin family members like CRP and CrOoctin; and galactose-binding protein were observed to form such complexes [32-34], which are tightly regulated by plasma serine proteases and protease inhibitors. [35-40]. Thus, the CFH contains a rich resource of fast-acting innate immune molecules, which are immunomodulated in a timely and collaborative manner to exert host self defense-cum-self protection.

As the most abundant (90-95%) protein in the CFH [41], hemocyanin (HMC) is an extracellular oxygen carrier in the horseshoe crab [42]. The copper ion present in the HMC makes the blood blue. The HMC also represents the only identifiable prophenoloxidase (PO) defending system which can be activated into phenoloxidase (PO) to produce microbial reactive oxygen intermediates [43]. Besides antimicrobial peptide and clotting enzymes [44, 45], extracellular microbial proteases were recently found to directly activate the HMC-proPO to PO during the host-pathogen interaction [46].

Being capable of binding to invading microbes further substantiates the function of defending molecules in the CFH as primary pattern-recognition-receptors, which recognize the pathogen-associated-molecular-patterns, like LPS or peptidoglycan displayed by the pathogens. This implies that the CFH factors are frontline inducible antimicrobial factors. As such, the CFH is much simpler than its equivalent counterparts – the plasma, in mammals. Taken together, such simple and unique microenvironment present in the CFH corroborates its physiological relevance for the antimicrobial drug screening in vitro.

AN OUTLINE OF CFH-BASED ANTIMICROBIAL DRUG SCREENING - AN IN VITRO SYSTEM

A proposed protocol for using the CFH in antimicrobial drug screening is illustrated in Fig. 1A. Here, the CFH is separated from the amebocytes of the horseshoe crab hemolymph by centrifugation [47]. The CFH is then heated-inactivated at 56˚C for 30 min, a widely used procedure for immunological assays [48], to eliminate potential pathogen-specific activity from the known complement system present in the CFH [20]. The inactivated CFH may be diluted with buffers such as phosphate buffered saline (PBS) or HEPES. As a proof of concept, we show that this dilution step is favorable since firstly, microbes have been shown to grow well in the presence of inactivated CFH at 10-20% (v/v) (Fig. 1B); secondly, the diluted CFH provides an ideal physiological milieu to simulate an in vitro infection for high throughput antimicrobial drug screening in 96-, 192- or 384-well microtitre plates (Fig. 1C). In view of the abundance, effectiveness and sensitivity of innate immune factors in the CFH of the horseshoe crabs [34, 49], the proposed set-up allows for: (a) a flexible application of the CFH to be utilized under different buffered conditions to suit different needs or requirements for optimal function of the drug under study and (b) a micro-scaled single-step testing, which enables simultaneous high-throughput screening of the drug candidates.

The in vitro simulated infection is reconstituted by incubating the target microbes with the CFH contained in the microtitre-plate wells. Generally, a culture density of 10^5 colony-forming unit (cfu)/ml of microbes can be used according to the guideline of minimum inhibitory concentration (MIC) tests [50]. Following the addition of drug compounds into the wells, the antimicrobial activities of the drug candidates can be determined based on the inhibition of microbial growth over a time course of incubation. In this screening method, negative controls comprise wells containing either CFH alone or CFH and microbes without test drug. Additional controls to test the dose-responsiveness of drugs to different concentrations of CFH should be conducted to help ascertain whether any other heat-insensitive components of the CFH might contribute to the antimicrobial activity against the target microbes. A drop in microbial growth in the test well would indicate the presence of antimicrobial activity of the drug, which can be monitored by turbidometry or indirect conductimetric assay. The turbidometry measures the residual microbial density based on the change in optical density, whilst the indirect conductimetric assay is based on the change in electrical conductivity in the liquid, which is a more sensitive approach and can be applied when determination of turbidity is impossible [51]. Standard procedures for the selection of drug hits based on the inhibited growth, validation of dose response of potent drug candidates and determination of drug leads can be followed according to the current practice employed in the pharmaceutical industry.

Liquid dispensation involved in this proposed protocol can be operated by automated robots. This will not only significantly increase the speed and accuracy of the assay, but also reduces systematic errors when generating the data. Thus, it ensures statistically uniform and reliable data for comparison and analysis. Meanwhile, the high throughput testing afforded by this proposed system makes it possible for examining dose responses of the drug candidates during the screening process. Hence, this CFH-derived in vitro screening system is a rapid and cost-effective approach, which can be easily implemented in the pharmaceutical industry to obtain biologically meaningful antimicrobial drug leads.

THE HMC-RICH CFH FOR SCREENING OF ANTIMICROBIAL DRUGS IS APPLICABLE AGAINST PROTEASE (+)/(-) BACTERIA

The effectiveness of the horseshoe crab in defense against bacterial invasion, in particular against the Gram-negative bacteria, has prompted the potential application of the CFH system in screening antibacterial drugs. The removal of LPS-sensitive amebocytes from the proposed system extends the scope of the screening for drugs targeting both Gram-positive and -negative bacteria. However, the major protein, HMC-proPO, in the CFH can be specifically activated to PO by microbes which produce extracellular proteases, resulting in the generation of microbial reactive oxygen intermediates [46, 49], which indicates the specificity of HMC-derivates against the protease-producing bacteria. Thus, based on the unique characteristic of such inducible HMC-based defense, this HMC-rich CFH system is useful for testing the antimicrobial drugs which target either known protease non-producing [protease (-)] or protease producing [protease (+)] bacteria. This is an added advantage to the pharmaceutical industry since the virulent bacteria employ extracellular proteases as a powerful invasive factor to attack the host.

Figures 2A-C illustrate how the CFH is employed to screen the drugs which target protease (-) or (+) bacteria. We do not anticipate microbial reactive oxygen intermediates to be generated from the HMC (Fig. 2A). Therefore, by deduction any antimicrobial activity should be attributable to the antimicrobial drug being tested as demonstrated in a sample test (Fig. 2D). This certainly increases the efficiency of pathogen-directed specific drug screening, which will be rapid and cost-effective. On the other hand, this system will also provide valuable information on further development of the drugs which target known protease (+) bacteria (Fig. 2B). The limited antimicrobial activity (~ 60 – 80%) resulting from the activated HMC by the microbial proteases [46, 49] is an advantage that can be exploited in this assay. By comparison with the negative controls, which are the incubations without drugs, it is possible to infer whether the potential antimicrobial drug under test cooperates with
Horseshoe crab “cell-free hemolymph”-based antimicrobial drug screening

A. cell-free hemolymph from horseshoe crab
   ↓
   heat-inactivation of cell-free hemolymph
   ↓
   dilution in buffers & dispensation in micro-plate
   + antimicrobial drug
   + targeting microbes
   ↓
   incubation over a time course
   ↓
   determination of microbial growth by turbidometry or indirect conductimetric assay
   ↓
   selection of hits based on the inhibition of microbial growth
   ↓
   validation: dose responses
   ↓
   selection of drug leads

B. bactericidal activity of antimicrobial drugs
   ↓
   bacterial survival (%)
   ↓
   selection of drug leads

Fig. (1). Horseshoe crab heat-inactivated “Cell-free hemolymph”-based antimicrobial drug screening system.
(A) Schematic illustration of the procedure for horseshoe crab “cell-free hemolymph” (CFH)-based antimicrobial drug screening in vitro. (B) Sample test for the effect of differently diluted heat-inactivated CFH on the growth of bacteria. Two representative Gram-positive or -negative bacteria, *Staphylococcus aureus* (*S. aureus*) or *Pseudomonas aeruginosa* (*P. aeruginosa*) were used in the test. The heat-inactivated CFH was diluted with PBS. Growth of the bacteria after overnight incubation was monitored spectrophotometrically at wavelength of 600nm. (C) Antimicrobial assays with or without 10% CFH. Four representative antibiotics (A, B, C and D) with different concentrations were tested on one Gram-positive or -negative bacterial species (*S. aureus* or *P. aeruginosa*). In (B) & (C), the bacterial survival rate (%) was calculated based on the negative controls where only bacteria were incubated.

microbial protease(s) when accomplishing antimicrobial activity. For example, as presented in a sample test (Fig. 2E), a potential scenario of three resulting inhibited rates on microbial growth could be deciphered as follows: higher, lower or similar levels as compared to the negative controls, indicating that the drugs tested in this system could be synergistic, or antagonistic, or has no correlation to the presence of the protease(s). An understanding on how the synergy or antagonism is generated between the drugs under test and the bacterial protease(s) will assist in further modification of antimicrobial drug design or the development of a drug cocktail via modulating the protease activities of a virulent microbe.

THE HMC-RICH CFH ENABLES THE ASSESSMENT OF THE ANTIOXIDANT ACTIVITY OF ANTIMICROBIAL DRUGS

The enhanced production of reactive oxygen intermediates by the activated HMC has prompted the application of the HMC-rich CFH for testing the antioxidant activity of the antimicrobial drugs...
**Fig. (2).** Potential applications of the "cell-free hemolymph"-based antimicrobial drug screening system.

Illustration of the application of heat-inactivated "cell-free hemolymph"-based system for screening antimicrobial drugs targeting known bacteria which are (A) non-protease producing (-), (B) protease producing (+), and (C) determining the antioxidant activity of the drugs. In this system, the "HMC" (hemocyanin) represents 90-95% and the "others" represents the remaining 5-10% of the total proteins contained in the cell-free hemolymph (CFH). The corresponding bar charts depict arbitrary plots of the anticipated results for the drugs harboring either antimicrobial activity against the protease (-): P(-), protease (+): P(+), or antioxidant activity. These potential applications are derived based on the protease-mediated activation of the HMC-proPO to PO, which results in the generation of microbicidal reactive oxygen intermediates and the subsequent antimicrobial activity. 

(D) & (E) Sample data of antimicrobial assays using 10% CFH-based screening system. The antimicrobial activities of four representative antibiotics (A, B, C and D) were tested against protease non-producing [P(-)] or protease producing [P(+)] Gram-positive or -negative bacteria, respectively. The bacterial survival rate (%) was calculated based on the negative control where only bacteria were incubated.

*P(-): protease non-producing, P(+): protease producing, drug<sup>antimicrobial</sup>: drug with antimicrobial activity, drug<sup>antioxidant</sup>: drug with antioxidant activity*
(Fig. 2C). For this application, non-targeting bacteria which will not be killed by the drugs under test, and which produce extracellular protease(s), can be employed to reconstitute an infection in the CFH screening system where the protease will activate the HMC to generate microbicidal reactive oxygen intermediates. If the antimicrobial drug harbors a potent antioxidant activity, it will scavenge such cytotoxic derivatives, abolishing the HMC-associated antimicrobial activity as compared to the negative controls (incubations without drugs). Positive controls may be set up by incubations with known antioxidant e.g. ascorbic acid [52] to compare and verify the antioxidant activity of the drug under test. Therefore, this additional feature of the CFH screening system can be expedient for testing the antioxidant activity of antimicrobial drugs.

In summary, the proposed CFH-based in vitro antimicrobial drug screening system is advantageous, being an easily accessible resource, which is time- and cost-effective and it provides an evolutionarily conserved host-pathogen interaction environment. Statistically uniform and reliable data can be anticipated as compared to other invertebrate models or traditional screening methodology. Hence, it can be used as an ideal bioassay complementing other in vitro screening assays in formulating optimal parameters for further in vivo testing in higher order animal models.

ABBREVIATIONS
CFH = Cell-free hemolymph
CRP = C-reactive protein
HMC = Hemocyanin
LAL = Limulus amebocyte lysate
LPS = Lipopolysaccharide
PO = Phenoloxidase
proPO = Prophenoloxidase

ACKNOWLEDGEMENTS
This work was supported by the Ministry of Education, Singapore (MoE Tier 2 grant).

REFERENCES


