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# Assays optimized for detection and quantification of antibacterial activity in shark cell lysates under high salt conditions

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ABSTRACT

To assess non-cellular innate immune mechanisms that play a role in the antimicrobial defense of an organism several assay systems have been devised to screen for such factors. Most assays, however, have been developed to measure activity against clinical isolates of medical importance. There is scant information on methods optimal for assaying material from sharks and other marine fish for antimicrobial activity particularly against salt tolerant organisms that are likely to be encountered in the marine environment. We have modified and optimized agar diffusion and broth dilution assays for detection and quantification of antibacterial activity of shark leukocyte lysates. By replacing marine agar, typically used for marine organisms, with artificial sea water complete medium (SCM) enriched with tryptone and yeast extract has resulted in an improved inhibition zone assay that uses *Planococcus citreus*, a salt-tolerant organism as the target organism. Antibacterial activity is correlated to the size of zone of no bacterial growth around wells containing bioactive test sample. An alternative broth based microdilution growth assay uses the 96 well format and the antibacterial effect of the sample on growth of *P. citreus*, the target organism, is measured spectrophotometrically as percent inhibition of bacterial growth when compared to the growth of *P. citreus* grown in medium alone that represents 100% growth. The assay can also be used to titrate antibacterial activity and express the level of growth inhibition as a titer.
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## 1. Introduction

Innate immunity is the body's immediate response to assault by microorganisms and antimicrobial proteins and peptides are an integral part of the innate immune defense and can be found in various body locations, in all kinds of body fluids and secretions, and intracellularly in a variety of cells [1–6]. The last decade has seen a rapidly growing interest in the identification and characterization of antibacterial factors of a diverse group of invertebrates and vertebrates [7–19]. Antimicrobial factors are found and have been isolated from a variety of biological sources, and since their spectrum of activity and biochemical structure and mode of action can vary significantly, investigators have developed, adapted, modified and optimized assay methods for their particular model system [20,21]. Although assays have been described for assaying shark material for cytotoxic activity against cancer cells [22] little has been published on methods optimal for assaying shark samples for antimicrobials.

Here we describe two simple assays to detect antibacterial activity in shark cell lysates using a salt tolerant bacterium as the target organism. Since most bioassays commonly employed are not optimal for shark samples and typically do not take into account the high levels of urea (0.35 M) and salt (0.25 M) present in most shark body fluids, we have developed a protocol that provides an isotonic environment for cell isolation and have modified an agar diffusion and a microdilution growth assay for detecting and estimating antibacterial activity in shark samples. Since many antimicrobial molecules are often present in low concentrations in biological samples, particularly in crude cell lysates, assays should be sufficiently sensitive to detect antibacterial activity present at relatively low levels while at the same time providing conditions conducive for the growth of the target organism preferably one likely to be found in the marine environment. Furthermore, crude biological samples often also contain inhibitory and synergistic molecules which have to be taken into consideration. The assays described herein have been used successfully in our laboratory to screen and identify antibacterial proteins and peptides in shark samples (unpublished data).

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Two popular assay formats used to screen for antimicrobials are the radial diffusion assay and the microdilution growth assay. The two tier agar diffusion assay described by Lehrer et al. [20] has wide application particularly when screening for antibacterial activity against target organisms of clinical significance. It has since been modified by several investigators to assay antibacterial activity in biological samples from marine organisms and uses marine agar medium (Difco Labs, Detroit, MI) with *Psychrobacter immobilis* or *Planococcus citreus*, as the target organism [14,23–26]. Briefly, the assay plate consists of two agar layers: the bottom non-nutritious agar underlay is seeded with sufficient target organism to form a lawn when provided nutrients by an agar overlay containing essential nutrients for the growth of the organism. Before application of the overlay the test sample is added to wells in the bottom agar underlay and the plate is incubated for a short period for test material to diffuse into the agar before applying the overlay. The plate is further incubated for growth of the target organism. Antibacterial activity is indicated by clear zones of no growth around test wells in a background lawn of the target organism. When the assay was employed in our laboratory to initially screen shark cell lysates for antibacterial activity against the salt tolerant organism, *P. citreus*, the cloudiness of the marine agar medium (resulting from precipitated material on reconstitution of dehydrated sea water) interfered with recording of zone size. To obtain a more definitive sharpness of zone margins we changed the composition of the agar medium and by doing so have significantly improved clarity of the medium resulting in sharply defined inhibition zones and at the same time the medium supports good growth of the target organism. As an alternative to agar diffusion assays here we also provide details of a broth based antibacterial assay optimized for shark samples. Both assays are simple to set up and can be used to screen for and quantify antibacterial activity in samples from sharks and other marine fish.

## 2. Materials and methods

### 2.1. Preparation of leukocyte lysates

Sodium citrate (3.5% in shark medium: RPMI-1640 containing 0.35 M urea and 0.2 M NaCl, pH 7.4) was added to whole blood withdrawn from the caudal sinus of an anesthetized (1 ppm 3-aminobenzoic acid ethyl ester in sea water) nurse shark (*Ginglymostoma cirratum*). Citrated blood was diluted with an equal volume of shark medium (SM) and layered onto a gradient mixture consisting of two parts Lymphocyte Separation Media, LSM (*Sigma*, *MO*), and one part saline (0.15 M NaCl). Following centrifugation at 435× *g* for 30 m, the interface layer along with the thin buffy coat layer present on the surface of sedimented erythrocytes was removed and centrifuged at 435× *g* for 20 m. The few remaining erythrocytes present in the leukocyte pellet were lysed by addition of 5 times its volume of hypotonic lysing solution (0.2% NaCl) and the tube inverted several times to resuspend cells completely. Immediately following, to neutralize the effect of the lysing solution, an equal volume of 2.5% NaCl was added. The disrupted cell suspension was centrifuged at 435× *g* for 15 m and the pellet suspended in 1–2 ml of 0.25 M NaCl (isotonic for shark leukocytes). Cell viability employing the Trypan blue exclusion test [27,28] was performed to confirm that at least 85–90% of cells were intact and not 'leaking' intracellular contents. Frozen (−20 °C) leukocyte pellets were subjected to several freeze (−80 °C) and thaw (RT) cycles which facilitated subsequent cell disruption by mechanical shearing using a glass homogenizer and/or ultrasonic disruption in a sonicator (Sonicator 3000, *Misonix*). For sonication up to 1 ml 0.25 M NaCl was added to the cell pellet and sonicated at 18–27 W for 10 m with 1 m pulses and 2 m rest periods between pulses. The cell lysate (CL) was centrifuged for 90 m at 21,000× *g* at 4 °C (Micromaxx FF, *IEC*) and the debris pellet discarded. Protein concentration of lysate was estimated using the BCA colorimetric protein assay protocol (*Pierce*, *IL*). Before screening for antibacterial activity each thawed lysate sample was clarified by centrifugation at 1000× *g* for 5 m. Lysates for fractionation by column chromatography were concentrated by lyophilization and samples were reconstituted in double distilled water before use.

### 2.2. Target bacterium

*P. citreus*, a salt tolerant Gram positive coccus, was the target bacterium for both assays. Stock cultures of *P. citreus* (NCIMB 1493) were maintained at 30 °C on SCM agar plates and subcultured every other day. Colonies were used to inoculate 50 ml of SCM broth and a 5 ml aliquot of the exponentially growing SCM broth culture (6–8 h at 30 °C) was inoculated into 50 ml of fresh medium and further incubated for 8 h. The broth culture was centrifuged (1085× *g* for 5–7 m) and the bacterial pellet washed twice with 3.2% NaCl solution and suspended in 0.005 M PBS, pH 7.4. The bacterial suspension was standardized to $10^6$ CFU/ml and used to set up the inhibition zone assay. For the microdilution growth inhibition assay the bacterial suspension was prepared in a similar manner, except the final bacterial suspension was in SCM and standardized to $2.5 \times 10^4$ CFU/ml.

### 2.3. Antibacterial assays

#### 2.3.1. Inhibition zone assay

The two tier assay used by Findlay and Smith (1995) was modified by replacing the marine agar medium with artificial sea water complete medium (SCM) enriched with peptone and yeast extract [29]. SCM is a clear translucent agar medium (minimal precipitation) which permits sharper visualization of the zone margins of areas of no bacterial growth around sample wells and facilitates accurate measurements. SCM (0.5 M NaCl, 25 mM $MgSO_4$, 25 mM $MgCl_2$, 10 mM KCl, 0.02% glycerol phosphate, 0.3% peptone, 0.1% yeast extract, 3 ml glycerol/L $ddH_2O$) was prepared, autoclaved and stored as a 5× stock solution. It supports good growth of *P. citreus*. The assay was set up in duplicate square grid plates and consisted of an underlay layer of 10 ml of 1% agarose in 0.005 M PBS, pH 7.4, seeded with 100 μl of the standardized bacterial suspension ($10^6$ CFU/ml). Test samples (5 μl) were added to 3 mm diameter wells cut in the underlay agar. Each sample, set up in duplicate, was allowed to diffuse into the agarose at 30 °C for 3 h. Following diffusion, a nutritious overlay consisting of 10 ml 1% agarose in SCM was layered on top and plates further incubated overnight at 30 °C. Parallel samples consisting of saline, SCM and HNP-1 (25 μg/ml) were run as controls. The zone of growth inhibition around sample wells, measured as the average of two readings taken at right angles across the circular zone area, was estimated by measuring for each sample the average diameter of the zone around duplicate sample wells minus the diameter of the well (3 mm). Antibacterial activity was expressed in arbitrary activity units (AU) calculated according to the formula

$$\text{Activity Units} = \text{Average diameter of inhibition zone (mm)} - \text{diameter of sample well (mm)} \times 10$$

#### 2.3.2. Microdilution growth inhibition assay

Optimal conditions for a microdilution growth assay to detect and measure antibacterial activity in shark cell lysates were established with *P. citreus* as the target organism. Briefly, a reaction mixture of 50 μl of test sample (cell lysate), 75 μl of 2× SCM and

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