Antibacterial and Antifungal Activities from Siamese Crocodile Blood

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***Objective**: To evaluate the in vitro antimicrobial activity of the Siamese crocodile blood against bacteria and fungi.*
***Material and Method**: Thirty Siamese crocodile blood samples including freeze dried whole blood (FDWB), fresh serum (FS), and freeze dried serum (FDS) were evaluated for antimicrobial susceptibility and MIC values against ATCC-registered strains of nine bacterial species and two fungal species and one fungus isolated from a clinical specimen, by using the standard broth microdilution method and a modified resazurin microtiter plate assay.*
***Results**: The result showed that FS (80 mg/ml) and FDS (100 mg/ml) inhibited Gram negative bacteria including Enterobacter aerogenes ATCC 13048, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 27736, Salmonella typhimurium ATCC 13311 and Pseudomonas aeruginosa ATCC 27853 with the susceptibility rate at 23.30%, 10.00%, 40.00%, 70.00%, and 86.67%, respectively for FS, and 30.00%, 10.00%, 43.33%, 76.67% and 90.00%, respectively for FDS. The MIC and MBC were in the range of 12.50-100.00 mg/ml and 25.00-100.00 mg/ml, respectively. FS and FDS also inhibited Cryptococcus neoformans 250309 and Aspergillus niger with the susceptibility rate at 90.00% and 80.00%, respectively for FS and 100.00% and 83.33%, respectively for FDS. The MIC was in the range of 25.00-100.00 mg/ml. However, FS and FDS did not inhibit Gram positive bacteria and did not kill fungi. FDWB (100 mg/ml) could neither inhibit bacteria nor fungi.*
***Conclusion**: FS and FDS from Siamese crocodile exhibited potential antibacterial and antifungal activities.*

***Keywords** : Antimicrobial susceptibility test, Siamese crocodile blood, MIC, MBC*

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The powerful antibiotic was first discovered in the 1940s[1], and many of which originate from natural sources found among all classes of life. Meanwhile, resistance microbes to those antibiotics continuously emerge over time and spread all over the world. This situation motivated scientists to search for new naturally occurred bacterial agents that may potential antibiotics. Reptiles including alligators and crocodiles were subjected for an exploration of the antibiotic properties from their body. Naturally, the crocodilians, which are aggressive and often fight between its members, do not suffer from infection despite having serious injuries such as open wounds. Even though they live in a marsh environment which harbors myriad of microbes, they survive with relatively few infectious occurrences. Merchant and colleagues have studied the innate immune systems of American alligators and found that the complement systems from their serum and cationic peptides (1-10 kDa range) from their WBCs exhibited potent and broad-spectrum anti-microorganism effects against bacteria, protozoa, fungi, and viruses[2-6]. Our preliminary report showed that saltwater crocodile serum sample exhibited strong antibiotic activity (both peptide and non-peptide based antimicrobial activity) in several fractions serum[7]. Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum antimicrobial activities[8]. In this regard, we speculate that the Siamese crocodile, which is a relative to the American alligator and saltwater crocodile, would possess the antimicrobial properties within its serum. The aim of this study is to further investigate the antibacterial and

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antifungal activities of Siamese crocodile's blood products including FDWB, FS and FDS from *in vitro*.

Material and Method

Bacterial and Fungal strains

The following ATCC-registered bacterial and fungal strains were used: *K. pneumoniae* 27736, *E. coli* 25922, *S. typhimurium* 13311, *P. aeruginosa* 27853, *E. aerogenes* 13048, *S. aureus* 25923, *S. pyogenes* 19615, *B. subtilis* 6633, *S. epidermidis* 14990, *C. albicans* 10231, and *C. neoformans* 250309. Bacterial ATCC strains were purchased from the NIH, Department of Medical Science, Nonthaburi, Thailand, and fungal ATCC fungal strains were given from Preclinical Science Institute, Faculty of Medicine, Thammasat University, Pathumtani, Thailand. *A. niger* isolated from a clinical specimen was so included in the test.

Siamese crocodile blood

Thirty 2-4-year-old male and female Siamese crocodiles from Dontoom Nakhon Pathom Farm (Nakhon Pathom, Thailand) were made unconscious by an electrical shock and then their mouth was tied. Blood samples were collected from anterior dorsal sinus by using 3.8 cm 18 gauge needles and lithium heparinized vacutainer tubes. The blood tubes were kept in ice box and were centrifuged to separate fresh serum. Fresh serum was kept at -20°C. The FDS and FDWB were prepared by the freeze dryer (Lyophilization System, Inc.,USA) and kept at -20°C.

Ethical Consideration

Permission and approval for animal studies were obtained from the Animal Ethics Committee of National Research Council of Thailand (ID#DACKU 00151, 1 July 2008).

Inhibition of complement activity

Crocodile serum were incubated at 56°C 30 min to inhibit the complement activities.

Preparation of bacterial culture

Bacteria were cultured on nutrient agar plate or blood agar plate (for *S. pyrogenes*) at 37°C, overnight and were subcultured into 3-ml nutrient broth and further incubated at 37°C for 3-4 hours to obtain the log-phase culture. Cultures were then adjusted to a 0.5 McFarland standard prior to the susceptibility testing.

Preparation of fungal culture

C. albicans 10231 and *C. neoformans* 250309 were subcultured onto Sabouraud dextrose agar (SDA) and incubated for 24-48 hours at room temperature to obtain a freshly grown pure culture. 2-3 yeast colonies were then transferred into 3-ml RPMI-1640 medium (with glutamine and phenol red, without bicarbonate, HyClone, USA) and incubated at 35°C for 3 hours to reach the log phase. The cultures were then diluted to a 0.5 McFarland standard with RPMI-1640 medium. *A. niger* was subcultured onto patato dextrose agar (PDA) and incubated for 7 days at room temperature to ensure maximum sporulation. Mold colonies were transferred into 3-ml RPMI-1640 medium and incubated at 35°C for 3 hours. Dilutions of this conidial suspension to a 0.5 McFarland standard were then prepared in 5 ml of RPMI 1640, adjusted to a final concentration of 0.4-5.0 x 10^4 CFU/ml.

MICs determination

The antimicrobial susceptibility testing was performed by using the NCCLS and CLSI methodology. The NCCLS, the CLSI M27 A2, the CLSI M38-A were intended for bacterial testing, yeast testing, and mold testing, respectively[9-11]. The resazurin microtiter plate assay as described by Sarker[12] was used for the detection of crocodile blood MIC. The sterile cefazolin sodium or ceftazidime, amphotericin B 1 mg/ml (The Government Pharmaceutical Organization, Thailand) were used as positive control for bacterial and fungal MICs determination. Serial twofold dilutions of samples and antibiotics were prepared directly in each plate. The plate containing 50 ml samples in each well were inoculated with 50 ml of 0.5 McFarland standard bacteria or yeast or mold suspension. To each well 10 ml of the sterile resazurin indicator solution (1 mg/ml) was added. All tests were carried out in triplicate. Microtiter plates were incubated at 37°C, 16-18 hours for bacteria; at room temperature, 1 day for *C. albicans*, 2 days for *C. neoformans*. The color change was then assessed visually. The blue or purple color (oxidized form) was recorded as positive. Any color changes from blue or purple to pink or colorless (reduced form) were recorded as negative. The lowest concentration at which the blue or purple color occurred was taken as the MIC value.

MBC and MFC determination

The MBC/MFC were determined by inoculating 1 loop of sample in wells that showed no apparent growth from the MIC assays onto MHA or SDA or PDA plates. The plates were incubated at 37°C, 24 hours or at room temperature for 48 hours, and were

examined for colony growth or lack of growth for each dilution subculturing. No growth indicated that the crocodile blood sample was bactericidal or fungicidal at that dilution. Growth indicated that the sample was bacteriostatic or fungistatic at that dilution. The least concentration showing no visible growth on agar subculture was taken as MBC/MFC value.

Statistical analysis

All experiments were performed in triplicate to obtain valid statistical evaluation of the results.

Results

The susceptibility testing of 30 Siamese crocodile blood samples showed the FS and 100 mg/ml FDS inhibited five ATCC-registered strains of Gram-negative bacteria and two fungi (*C. neoformans 250309* and *A. niger*) (Table 1). However, both FS and FDS did not inhibit any of four Gram-positive bacteria nor *C. albicans ATCC 10231*. FDWB (100 mg/ml) did not inhibit any bacteria or fungi (Table 1).

The results of the MIC study showed that FS inhibited four bacteria (including *E. aerogenes*, *E. coli*, *K. pneumoniae*, *S. typhimurium*) and two fungi (*C. neoformans* and *A. niger*) at undiluted condition -1:2 dilution and inhibited *P. aeruginosa* at 1:2-1:4 dilution. As for FDS, MIC value at 100 mg/ml was found to inhibit *E. aerogenes*, *E. coli*, *K. pneumoniae* and MIC values at 50.00-100.00 mg/ml were found to inhibit *S. typhimurium*, yet, no bactericidal effect. The MIC values at 12.50-25.00 mg/ml were found to inhibit *P. aeruginosa*, and the MBC values were 25.00-100.00 mg/ml. Cefazolin and ceftaxidime were used as positive control and the MIC and MBC against the five of these bacterial strains were 0.97-31.25 mg/ml and 1.95-62.50 mg/ml, respectively. The MIC values of FDS for *C. neoformans* and *A. niger* were 25.00-50.00 mg/ml and 50.00-100.00 mg/ml, respectively. However, both FS and FDS had no fungicidal activity. Amphotericin B was used as positive control and the MIC against these isolates were 3.90 – 7.81 mg/ml (Table 2).

Discussion

The present study showed that the Siamese crocodile serum (both 80 mg/ml FS and 100 mg/ml FDS) exhibited a potential antibacterial effect. They were highly effective as an antibacterial agent against *S. typhimurium* and *P. aeruginosa* with the percentage of susceptibility up to 90%. In addition, the results showed the antibacterial property against *P. aeruginosa*, a notoriously resistant pathogen. Furthermore, FS and FDS also have moderate effects against *E. aerogenes*, *E. coli*, and *K. pneumoniae*. Interestingly, MIC/MBC study revealed that *P. aeruginosa* showed the highest susceptibility to the FDS with the MIC value of 12.50-25.00 mg/ml and the MBC value of 25.00-100.00 mg/ml. We also confirmed the reproducibility of this finding by using the modified Kirby-Bauer method and found that 14 of 30 (47%) samples showed an inhibition with 7-10 mm inhibitory zone (positive control = 35 mm) (result not showed). The controversial results between resazurin microtiter plate assay and modified Kirby-Bauer method might be explained by the antimicrobial proteins or peptides in the serum possibly trapping on

Table 1. Percentage of susceptibility testing of bacteria and fungi from crocodile blood using broth micro-dilution method.

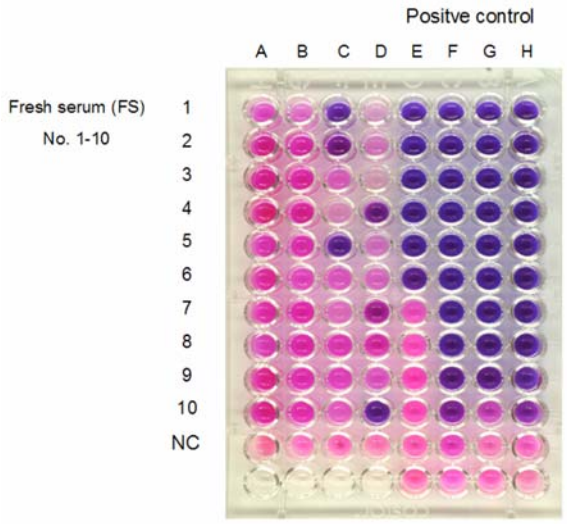
Species	FDWB (100 mg/ml)	Susceptibility (%) FS (80 mg/ml)	FDS (100 mg/ml)	Positive controlMIC(mg/ml)
E. aerogene ATCC 13048	0	23.33	30.00	31.25
E. coli ATCC 25922	0	10.00	10.00	1.95
K. pneumoniae ATCC 27736	0	40.00	43.33	3.90
S. typhimurium ATCC 13311	0	70.00	76.67	1.95
P. aeruginosa ATCC 27853	0	86.67	90.00	0.975
S. aureus ATCC 25923	0	0	0	31.25
S. pyogenes ATCC 19615	0	0	0	1.95
S. epidermidis ATCC 14990	0	0	0	15.62
B. subtilis ATCC 6633	0	0	0	1.95
C. neoformans ATCC 250309	0	90.00	100.00	3.90
A. niger	0	80.00	83.33	7.80
C. albicans ATCC 10231	0	0	0	3.90

Table 2. Antimicrobial activity of FS and FDS

Species	FS		FDS		Positive control	
	MIC value	MBC/MFC value	MIC value (mg/ml)	MBC/MFC value (mg/ml)	MIC value (mg/ml)	MBC/MFC value (mg/ml)
E. aerogenes ATCC 13048	Undiluted serum (n = 7)	- -	100.00 (n = 9)	-	31.25	62.50
E. coli ATCC 25922	Undiluted serum (n = 3)	-	100.00 (n = 3)	-	1.95	3.90
K. pneumoniae ATCC 27736	Undiluted serum (n = 12)	-	100.0 (n = 13)	-	3.90	15.60
S. typhimurium ATCC 13311	Undiluted serum-Dilute1:2 (n = 21)	-	50-100.00 (n = 23)	-	1.95	3.90
P. aeruginosa ATCC 27853	Dilute 1:2-1:4 (n=26)	Undiluted serum-Dilute 1:2 (n=10)	12.50-25.00 (n=27)	25.00-100.00 (n=15)	0.975	1.95
S. aureus ATCC 25923	-	-	-	-	31.25	ND
S. pyogenes ATCC 19615	-	-	-	-	1.95	ND
S. epidermidis ATCC 14990	-	-	-	-	15.62	ND
B. subtilis ATCC 6633	-	-	-	-	1.95	ND
C. neoformans ATCC 250309	Undiluted serum-dilute1:2	-	25-50.00	-	3.90	ND
A. niger	Undiluted serum-dilute 1:2	-	50.00-100.00	-	7.80	ND
C. albicans ATCC 10231	-	-	-	-	3.90	ND

Note: - = No MIC/MBC/MFC, ND = not done

the filter paper disc. We confirmed whether the antibacterial property was depended on the effect of complement or not. The subsequent analysis of heat inactivation serum at 56°C for 30 min revealed that the antibacterial activities of the serum could be completely depletion. The results demonstrated that the antibacterial activities were the effect of the serum complement, consistent with the study in American alligator serum[2]. Conversely, the result showed FS and FDS were not effective against all four gram positive strains tested presumably due to the expression of some proteins that may inhibit complement function or the concentration of the complement in crude serum was insufficient for Gram positive cell wall destruction. This result was consistent with human serum that could not form membrane attack complex (MAC) on Gram positive cell wall because of the thick layer of peptidoglycan[13]. However, Merchant and colleagues found the controversial results that the American alligator serum was highly effective as an antibacterial agent against both Gram-positive and Gram-negative bacteria[2]. They demonstrated that in



A = antibacterial activity of FS to inhibit *E. aerogenes*
B = antibacterial activity of FS to inhibit *E. coli*
C = antibacterial activity of FS to inhibit *K. pneumoniae*
D = antibacterial activity of FS to inhibit *S. typhimurium*
E = antibacterial activity of cefazolin (1mg/ml, 2 fold dilution) FS to inhibit *E. aerogenes* (MIC=31.25 mg/ml)
F = antibacterial activity of cefazolin (1mg/ml, 2 fold dilution) FS to inhibit *E.coli* (MIC=1.95 g/ml)
G = antibacterial activity of cefazolin (1mg/ml, 2 fold dilution) FS to inhibit *K. pneumoniae* (MIC=3.90 mg/ml)
H = antibacterial activity of cefazolin (1mg/ml, 2 fold dilution) FS to inhibit *S. typhimurium* (MIC=1.95 mg/ml)
NC = Negative control (MHB+bacteria+resazurin)

Fig. 1 Antibacterial activity of FS by modified resazurin microtiter-plate assay

addition to the complement systems, antimicrobial peptides from alligator's WBCs can destroy bacteria both Gram positive and Gram negative. For the modes of action by which antimicrobial peptides kill bacteria is varied and includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components[14]. The initial contact between the peptide and the target organism would be electrostatic, as most bacterial surface are anionic so bacterial membrane will be prone to be attacked by the positively charged antimicrobial peptides. Gram negative bacteria differ from gram positive bacteria in having a smaller cell wall peptidoglycan layer, but possessing an outer membrane (LPS, protein) in addition to the common cytoplasmic membrane. The antimicrobial cationic peptides, utilize the uptake pathway across the outer membrane, termed self-promoted uptake, often work as well or better against gram negative than gram positive bacteria[15].

The results of antifungal activity showed both 80 mg/ml FS and 100 mg/ml FDS had the potential antifungal activities. The serum was highly effective against *C. neoformans* and *A. niger*, but not *C. albicans*. This result may be due to *C. albicans* expresses several virulence factors such as adhesins, phenotypic switching, aspartyl proteases and phospholipases that contribute to pathogenesis or antifungal resistant properties[16]. Among the three strains of fungi, the results revealed that *C. neoformans* showed the highest susceptibility (100%) to the FDS with the MIC value at 25.00-50.00 mg/ml, yet no MFC (Table 2). Additional results showed the antifungal properties of the serum could not be depletion by serum incubation at 56°C for 30 min indicating that these activities were not dependent on the complement activity. This result was in agreement with the report of Merchant et al (2006) that studied in leukocyte extracts from the American alligator[5]. They found that the antimycotic activities against *C. parapsilosis* were heat-stable. Merchant and colleagues confirmed whether the antifungal properties were depended on the effect of antimicrobial peptides or not. The subsequent analysis with 10 units of trypsin treated leukocyte extracts for 15 min revealed that the antifungal activities could be completely depletion. The result providing strong evidence that these activities were due to the presence of antimicrobial peptides; however, the exact mechanism was not known. In addition, this finding demonstrated that the antimicrobial peptides in the crocodile serum were fungiostatic against *C. neoformans* and *A. niger* rather than fungicidal.

The study of antibacterial and antifungal activity of FDWB showed that FDWB could not inhibit any tested pathogen. The FDWB was reconstituted in sterile distilled water. The hemolysis RBC content such as hemoglobin, Fe^{2+}, and other divalent cations might be effect the result of the susceptibility testing. In addition, the concentration of the antimicrobial substance in crude FDWB might be insufficient for bacterial or fungal destruction.

Overall, this finding suggests a new insight into the feasibility of utilizing the Siamese crocodile serum as an alternative option for combating infections caused by bacteria and fungi.

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ฤทธิ์การต้านเชื้อแบคทีเรียและเชื้อราจากเลือดจระเข้พันธุ์ไทย

ราตรี ลีละวงค์เทวัญ, จินดาวรรณ สิรันทวิเนติ, วิน เซยชมศรี, ชิษณุชา สัตพนพันธ์

***วัตถุประสงค์**: เพื่อประเมินประสิทธิภาพทางห้องปฏิบัติการของเลือดจระเข้พันธุ์ไทยต่อเชื้อแบคทีเรีย และเชื้อรา สายพันธุ์มาตรฐาน 11 สายพันธุ์ และเชื้อราที่แยกได้จากผู้ป่วย 1 สายพันธุ์*

***วัสดุและวิธีการ**: ทดสอบความไวและหาค่า MIC ของเลือดแห้ง ซีรัมสด และซีรัมแห้งจากจระเข้พันธุ์ไทย ชนิดละ 30 ตัวอย่าง กับแบคทีเรียและเชื้อราสายพันธุ์มาตรฐานจำนวน 9 สายพันธุ์ และ 2 สายพันธุ์ ตามลำดับ รวมทั้งเชื้อราที่แยกได้จากผู้ป่วย 1 สายพันธุ์ ด้วยวิธี broth microdilution method และ resazurin microtiter plate assay*

***ผลการศึกษา**: พบฤทธิ์ยับยั้งการเจริญของแบคทีเรียแกรมลบ ได้แก่ E. aerogenes ATCC 13048, E. coli ATCC 25922, K. pneumoniae ATCC 27736, S. typhimurium ATCC 13311 และ P. aeruginosa ATCC 27853 ในซีรัมสด 80 mg/ml (Fresh serum; FS) คิดเป็นร้อยละ 23.30, 10.00, 40.00, 70.00 และ 86.67 ตามลำดับ และเซรัมแห้ง 100 mg/ml (Freeze dried serum; FDS) คิดเป็นร้อยละ 30.00, 10.00, 43.33, 76.67 และ 90.00 ตามลำดับ โดย MIC/MBC อยู่ระหว่าง 12.50-100.00 mg/ml และ 25.00-100.00 mg/ml ตามลำดับ โดยมี cefazolin หรือ ceftaxidime เป็นสารควบคุมซึ่งให้ค่า MIC/MBC ต่อแบคทีเรียทั้ง 5 สายพันธุ์ระหว่าง 0.97-31.25 mg/ml/1.95-62.50 mg/ml และฤทธิ์ยับยั้งการเจริญของเชื้อรา เช่น C. neoformans ATCC 250309 และ A. niger ของ FS คิดเป็นร้อยละ 90.00 และ 80.00 ตามลำดับ และ FDS คิดเป็นร้อยละ 100.00 และ 83.33 ตามลำดับ โดย MIC อยู่ระหว่าง 25.00-100.00 mg/ml โดยมี amphotericin B เป็นสารควบคุมซึ่งให้ค่า MIC 3.90 -7.81 mg/ml แต่อย่างไรก็ตามไม่พบฤทธิ์ยับยั้งการเจริญของเชื้อแบคทีเรียและเชื้อราในเลือดครบแห้ง 100 mg/ml (freeze dried whole blood; FDWB) และไม่พบฤทธิ์ยับยั้งการเจริญของเชื้อแบคทีเรียแกรมบวกและฤทธิ์ฆ่าเชื้อราทั้งใน FS และ FDS*

***สรุป**: FS และ FDS มีศักยภาพในการเป็นสารยับยั้งเชื้อแบคทีเรียและเชื้อราบางชนิดได้*