Crocodylus siamensis Serum and Macrophage Phagocytic Activity

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Antimicrobial activity of sera from many crocodilian species has been recognized. This activity was proposed to be mediated, at least in part, by complement. Due to the fact that complement proteins have different functions in the immune system, they may be involved in phagocytic process of phagocytes. In the present study, the effects of Siamese crocodile serum on phagocytic activity of macrophages as well as the possible involvement of complement in this process were examined. The results showed increases in the phagocytosis of both Escherichia coli and to a lesser extent, Staphylococcus aureus upon incubation of murine macrophage cell line with fresh crocodile serum (FS). Similar to FS, other crocodile blood products, including freeze dried serum (DS) and freeze dried whole blood (DWB) exhibited phagocytosis-enhancing property. However, the ability of DWB to enhance phagocytosis was less efficient than that of FS and DS, suggesting that serum factors were involved in this process. Treatment of FS with heat at 56°C for 30 min deteriorated the effect of FS on bacterial uptake of macrophages, suggesting that complement proteins play a role in the modulation of the phagocytic process. Collectively, the results of the present study suggested that crocodile serum enhances the macrophage phagocytic activity through complement activity and, therefore, may be taken as an alternative medicine for supporting the human immune responses.

Keywords: *Crocodylus siamensis, Crocodile serum, Phagocytosis, Complement*

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Macrophages play an important role in the first line immune defense. Their primary function is phagocytosis, which depends largely on receptors on the cell membrane. These receptors include Toll-like receptors (TLR), mannose receptor (MR) and scavenger receptor (SR), which bind directly to pathogen surface structures, so-called pathogen-associated molecular pattern (PAMP). In addition, phagocytosis is facilitated through opsonization, a process by which coated pathogens are recognized mostly through Fc- (FcR) and complement receptors (CR). The multiple ligand-receptor interactions initiate pathogen internalization, and ultimately killing of pathogens[1,2].

Sera from several crocodilian species have been reported to have antimicrobial activity[3]. American alligator (*Alligator mississippiensis*) serum was shown to exhibit potent antimicrobial activity against a wide variety of bacteria[4], viruses[5] and amoeba[6]. From these studies, complement proteins were suggested to have significant antimicrobial functions in alligators. This activity was proposed to be related to the complement protein C3 since antimicrobial activity was inhibited by polyclonal anti-human C3 antibodies[7]. In Siamese crocodiles *(Crocodylus siamensis)*, antibacterial and antifungal activities were also detected[8,9]. In addition, the antibacterial activity of crocodile complement was observed and appeared to be more effective than that of human[10]. Although the antimicrobial peptide, named crocosin, was recently identified from Siamese crocodile plasma[11], complement proteins are expected to take part in antimicrobial activity in *C. Siamensis*. It is well documented that activation of C3 results in the formation of the anaphylatoxin, C3a and the opsonin, C3b. C3a recruits inflammatory cells whereas C3b can either bind to microbial membrane or initiate the formation of membrane attack complex (MAC)[12,13].

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Previous studies have shown hemolytic activity of serum from many crocodilian species[7,14,15], suggesting the role of MAC in crocodilians. However, it is not known whether crocodile serum is able to mediate phagocytosis. Thus the present study was designed to evaluate the effect of crocodile serum on phagocytic activity of macrophages and to determine the involvement of complement proteins in this process. The results from the present study will provide some insights into the benefit of crocodile blood products for human health.

Material and Method

Crocodile blood

Twenty milliliter of blood was drawn from anterior dorsal sinus of 2-4 years old farm raised Siamese crocodiles using 18 guage needles. Blood samples were transfer immediately to sterile heparinized or serum clot activated VACUETTE tubes (greiner bio-one, Germany). Freeze dried whole blood (FWB) and freeze dried serum (DS) were prepared using freeze dryer (Lyophilization System, Inc. USA) and kept at -20°C.

Bacteria preparations and FITC labeling

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 were grown on blood agar plates for 16-20 h. Up to three colonies of bacteria were transferred to Muller-Hinton broth and cultured at 37°C for 2 h to obtain exponential growing cultures. The bacteria were labeled with FITC as previously described[16]. Briefly, bacteria were washed 3 times in PBS, suspended in 0.1 mg/ml FITC/ 0.1 M $NaHCO_3$, pH 9.0 (Sigma-Aldrich, USA), incubated at 25°C for 60 min in the dark and washed four times in PBS. The FITC-labeled bacteria were diluted to a density of 1 x 10^8 CFU/ml in PBS prior to use.

Cell culture

RAW 264.7 murine macrophages were maintained in RPMI 1640 media, supplemented with 10% (v/v) fetal calf serum (FCS) (Hyclone, Logan, UT), at 37°C in a 5% CO_2 atmosphere. For phagocytosis experiments, cells were seeded in 6-well culture plates (Corning, USA) at 1.0 x 10^6 cells per well. Cells were allowed to attach to the bottom of the plate at least 14h prior to the experiments. For microscopic experiments, cells were grown on glass coverslips placed in 6-well plates.

Phagocytosis assay

Measurement of phagocytosis of FITC-labeled *S. aureus* and *E. coli* was performed as described previously[16]. In brief, 1.0 x 10^6 RAW 264.7 cells were incubated with fluorescently labeled bacteria at a ratio of 10:1 in the presence of 10% (v/v) fresh serum (FS), 10 mg/ml freeze dried serum (DS), or 10 mg/ml freeze dried whole blood (DWB) that were either left untreated or heat-inactivated at 56°C for 30 min. After 2-hour incubation, cells were placed on ice to stop internalization and were maintained on ice for the remainder of the assay. Non-phagocytosed bacteria were removed by extensively washing cells with ice-cold PBS. Cells were harvested and stained with 2 µg/ml propidium iodide (PI) to exclude dead cells. Total cell-associated fluorescence was measured by flow cytometry (20,000 events) using FACS calibur and the CellQuest program (BD bioscience, USA). Data were analyzed by Win MDI 2.9 software (Trotter@Scripps.edu, USA). The amount of phagocytosis (phagocytic level) was calculated as: percentage of fluorescence-positive cells X mean fluorescence intensity (MFI) of fluorescence-positive cells.

Percent increase of phagocytic level was calculated by the following formula:

Percent inncrease of phagocytic level = 100 X [phagocytic $level_{test}$ - phagocytic $level_{control}$]/phagocytic $level_{control}$

Microscopic enumeration of ingested bacteria

RAW 264.7 cells were allowed to adhere to glass coverslips for 14 h before the addition of FS, DS, or DWB as previously described. Bacteria were added at a ratio of 10:1 into each well. Phagocytosis was stopped by vigorous washing, and cells were stained with gram stain. The amount of phagocytosis was determined by counting the number of cells, which had internalized at least one bacterium and the number of bacteria they phagocytosed. Over 500 cells were enumerated for typical experiments. The phagocytic index was calculated as: percentage of macrophages that phagocytose bacteria X average numbers of phagocytosed bacteria per cell. Percent increase of phagocytic index was calculated by the following formula:

Percent increase of phagocytic index = 100 X [Phagocytic $index_{test}$ - phagocytic $index_{control}$]/phagocytic $index_{control}$

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows. Quantitative data are expressed as the mean $\pm$ SD of triplicate samples and are

representative of more than three separate experiments. The difference between two groups was statistically analyzed by t-test. Analysis of variance was performed by the ANOVA procedure. Significant differences between means were determined by LSD. p values of ≤ 0.05 were considered significant.

Results

Effect of Siamese crocodile serum on phagocytic capacity of macrophages

In order to examine the effect of Siamese crocodile serum on phagocytosis, murine macrophages **(**RAW 264.7) were treated with 10% (v/v) fresh serum (FS) from *C. siamensis* and subsequently incubated with FITC-labeled bacteria at 37°C for 2 h. To evaluate the effect of crocodile blood on macrophage phagocytosis of both gram-positive and gram-negative bacteria, *S. aureus* and *E. coli* were used in the experiments. The amount of phagocytosis was measured using flow cytometry and presented as the phagocytic level. As shown in Fig. 1A and Table 1, macrophages treated with FS had a significantly higher level of phagocytosis than that of untreated cells ($p < 0.05$). Treatment of cells with crocodile serum conferred a two-fold increase in phagocytosis of *S. aureus* and a four-fold increase with *E. coli* over untreated control cells. Furthermore, in the absence of serum, the phagocytic level of cells incubated with *S.aureus* [244.9 ± 73.8 ($x10^2$)] was much higher than that of *E.*coli [3.5 ± 1.7 ($x10^2$)]. However, when incubated with FS, macrophages had a greater increase in *E.coli* phagocytic level [14.6 ± 4.8 ($x10^2$)] than that of *S.aureus* [460.4 ± 130 ($x10^2$)]. These data reflected increases of both the percentage of cells that engulfed bacteria as well as the quantities of engulfed bacteria (Fig. 3), suggesting that differences in surface molecules of gram positive and gram negative bacteria were involved in the phagocytosis-enhancing effect of FS. Alternatively, the efficiency of the phagocytic process was quantified by measuring numbers of phagocytosed bacteria and expressed as the phagocytic index. Consistent with previous results, marked increases in phagocytic index were observed in serum treated cells, incubated with either *S. aureus* or *E.coli* (Fig. 1B and Table 1). Phagocytic indices obtained from serum treated cells incubated with *S.aureus* or *E.coli* were 531.9 ± 94 and 259.5 ± 35.7, respectively. These values were approximately two and five folds higher than those of their control counterparts. Similar levels of phagocytosis enhancement were obtained when cells were treated with fresh plasma (data not shown). Together, these results indicated that crocodile serum facilitated macrophage uptake of both gram-negative and, to a lesser extent, gram-positive bacteria.

Comparison of phagocytic ability induced by variety of crocodile blood products

For practical usage purposes, crocodile blood has to be freeze dried and placed into capsules for taken as a dietary supplement. In order to test whether

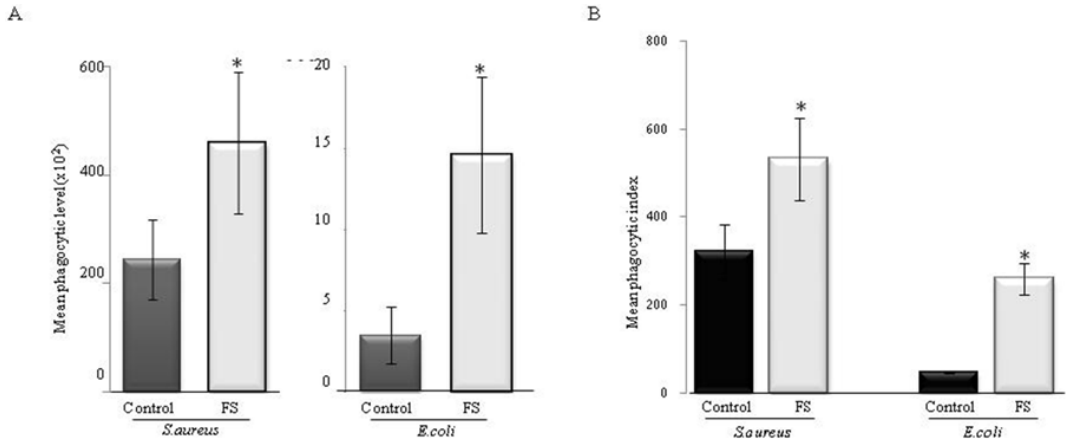

Fig. 1 The phagocytic-enhancing effect of Siamese crocodile serum. RAW 264.7 murine macrophages were incubated with FITC labeled *S.aureus* or *E.coli* at a ratio of 10:1 in the presence of 10% (v/v) fresh serum (FS) or PBS (control) for 2h. Cell-associated fluorescence was measured by flow cytometry. Results are expressed as the phagocytic level (A). Alternatively, cells were incubated with bacteria together with FS or PBS as described above and stained with gram stain. The numbers of engulfed bacteria were enumerated and calculated. Results are expressed as the phagocytic index (B). Statistical analysis was performed by t-test. *, $p \leq 0.05$

Table 1. Effects of Crocodile serum and heat inactivated crocodile serum on phagocytosis of macrophages (≥ 30)

Treatment	*S.aureus*		*E.coli*	
	Phagocytic Level ($x10^2$)	Phagocytic index	Phagocytic Level ($X10^2$)	Phagocytic index
Control	244.9 ± 73.8	322 ± 61.3	3.5 ± 1.7	48.8 ± 1.9
Crocodile serum	460.4 ± 130*	531.9 ± 94*	14.6 ± 4.8*	259.0 ± 35.7*
Heat inactivated crocodile serum	298.8 ± 69.2	410.1 ± 105	2.2 ± 0.7	51.6 ± 7.7

*$p \leq 0.05$, significantly different from control

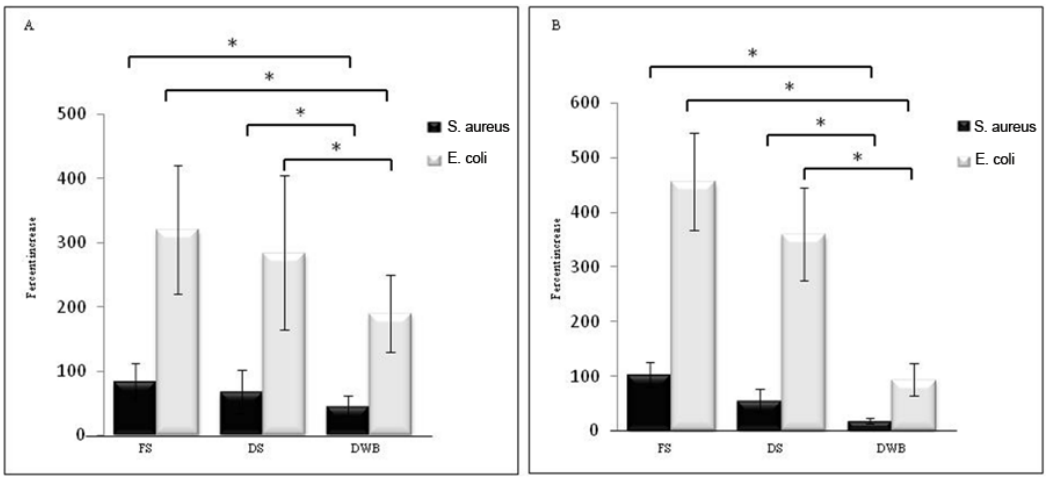

Fig. 2 Effect of crocodile blood products on phagocytic activity of macrophages. RAW 264.7 were treated with 10% FS, 10 mg/ml freeze dried serum (DS), or 10 mg/ml freeze dried whole blood (DWB) and subsequently incubated with bacteria as prviously described. Cell-associated fluorescence was measured by flow cytometry. Percent increase in phagocytic level of each blood product was calculated, as described in materials and methods, and compared (A). Similarly, percent increase in phagocytic index of each blood product was compared (B). Statistical analysis was performed by ANOVA. Significant differences between means were determined by LSD*, $p \leq 0.05$

the enhancement ability of serum was reduced by freeze dried process, various crocodile blood products, include freeze dried serum (DS) and freeze dried whole blood (DWB) were further tested. Cells were treated with 10 mg/ml DS or 10 mg/ml DWB prior to incubation with bacteria. All blood products tested exhibited significant increases in phagocytic ability of macrophages ($p < 0.05$). Therefore, the data were calculated and expressed as percent increases in phagocytotic level as well as phagocytic index. Then, data were compared between groups of blood products (Fig. 2A and B). Both phagocytic level and phagocytic index were not significantly different between cells incubated with FS and DS ($p > 0.05$), suggesting that freeze dried process did not deteriorate the phagocytosis-enhancing property of serum. Although DWB was found to augment phagocytosis, its ability was less effective than that of FS and DS, indicating that serum factors, most likely complement, may involved in the process.

Effect of complement-deficient serum on phagocytic activity

To define an involvement of crocodile complement on phagocytosis-enhancing activity, serum was subjected to heat treatment at 56°C for 30 min. This condition is well known to abrogate complement activity. Compared to FS treatment, ingestion of bacteria was diminished when macrophages were incubated with heat inactivated crocodile serum (Fig. 3 and Table 1). The phagocytic levels and phagocytic indices of cells treated with heat

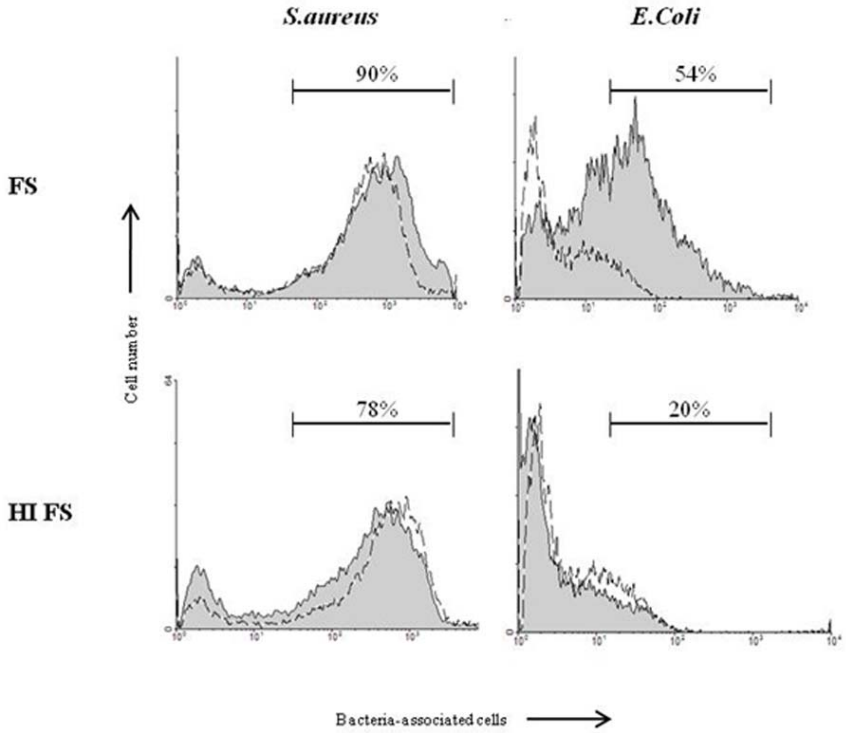

Fig. 3 Effects of crocodile serum and heat inactivated crocodile serum on phagocytosis of macrophages. RAW 264.7 murine macrophages were incubated with FITC labeled *S.aureus* or *E.coli* at a ratio of 10:1 in the presence of 10% (v/v) fresh serum (FS) 10% (v/v) heat inactivated serum (HIFS) or PBS (control) for 2h. Cell-associated fluorescence was measured by flow cytometry. Histogram showed the percentages of cells that engulfed bacteria (solid filled) in the presence of FS (top) or heat inactivated serum (HI FS, bottom). The percentages of cells that engulfed *S.aureus* and *E.coli* in the absence of serum (dash line) were 82% and 25%, respectively. The typical experiments were shown

inactivated serum were reduced to almost the same level as those of untreated controls ($p > 0.05$), suggesting that the phagocytosis-enhancing activity was mediated by complement.

Discussion

The immune system is a crucial defense mechanism of the body that fights infection. It is made up of a wide variety of effector cells and molecules. As part of the immune system, macrophages have central roles in uptake and killing pathogens. This process is mediated through various receptors, expressed on the cell surface. While recognition through pattern recognition receptors (PRRs) is a primary feature of phagocytosis, opsonization promotes phagocytic internalization of diverse types of pathogens. The process of phagocyte recognition through opsonin involves multiple proteins including C3b, which is generated from complement activation cascade[17].

Crocodilians were recognized for their resistance to many pathogens[3]. Several lines of evidence showed anti-microbial activity in both serum[4,5,8] and leukocyte extracts[18] from crocodiles and alligators. Recently, novel anti-microbial peptides, crocosin[11] and leucrosin[19], were isolated from *C. siamensis* and were shown to have anti-bacterial activity against several gram-positive and gram-negative bacteria. Apart from anti-microbial peptides, complement activity in serum from many crocodilian species has been demonstrated to inhibit growth of a wide variety of pathogens[4,6,7,14,15]. Due to the fact that complement has many functions, raising the possibility that serum complement from crocodiles may involve in opsonization of pathogens. Therefore, it is of interest to demonstrate the influence of crocodile serum on phagocytic ability of macrophages, which will provide some insights into the antimicrobial properties of crocodile that can be beneficial to human health.

The results from the present study showed that serum from *C. siamensis* was able to increase

phagocytic activity of RAW264.7 macrophages. The phagocytosis levels as well as phagocytic indices of both *S.aureus* and *E.coli* were enhanced by crocodile serum. In addition, crocodile serum caused greater increase in phagocytosis of *E. coli* than that of *S. aureus*, suggesting that surface molecules of gram negative bacteria were involved in phagocytosis-enhancing property of crocodile serum. Many non-opsonized membrane receptors on macrophages have been shown to recognize bacteria through their surface molecules such as lipoteichoic acid and lipopolysaccharide[1,2,20,21]. The authors' results suggested that these PRRs were not sufficient for effective recognition and phagocytosis of live gram negative bacteria. On the other hand, engulfment of live gram positive bacteria through non-opsonic recognition is relatively more efficient than that of gram negative bacteria. Hence, FS showed a lesser effect on *S. aureus* phagocytosis.

Phagocytosis-enhancing activity of whole blood-treated macrophages was immensely lower than that of serum-treated cells. Therefore, the enhancement of bacterial phagocytosis was confirmed to be due to the serum components. Many serum factors have been shown to influence macrophage phagocytosis including the classic opsonins, complement activation fragments and antibody[17]. Heat treatment of serum, known for complement inactivation, abrogated the enhancement of phagocytic activity, indicating that complement was involved in this activity. Although complement activity has been demonstrated in earlier studies[7,10,14,15], information on complement fragments of crocodilians is limited. Complement C3, a central component of opsonization, was detected in alligator serum[7]. Therefore, the results observed in this study suggest the roles of crocodile C3 activation in phagocytosis. In human, C3b, the cleavage product of C3, was demonstrated to initially bind covalently to the microbial surface. Subsequent proteolysis of the bound C3b generates iC3b, C3c and C3dg fragments that are recognized by different receptors on phagocytes. Interactions of C3b as well as its degradation fragments with their specific receptors on macrophages mediate the clearance of pathogens[12,13]. Therefore, it is postulated that a comparable scenario may apply for crocodile complement system. Previous studies have shown that C3-mediated opsonization plays an important role in the control of *S. aureus* infection in animal[22] as well as *E. coli* phagocytosis and oxidative burst in human granulocyts and monocytes[23]. Taken together, the results from the present study suggested that crocodile serum might be beneficial in limiting the severity of infection.

In conclusion, the findings of the present study indicate that serum from *C. siamensis* contains complement that mediates phagocytosis of pathogens. Hence, crocodile serum might be the source of complement for potential therapeutic use in patients suffered from infection.

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Potential conflicts of interest

None.

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กระบวนการกลืนกินของมาโครฟาจ และซีรัมจาก Crocodylus siamensis

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การศึกษาซีรัมจากจระเข้หลายสายพันธุ์พบฤทธิ์ต้านจุลชีพหลายชนิด ซึ่งมีผู้สันนิษฐานว่าเป็นผลมาจากฤทธิ์ของคอมพลีเมนท์ในซีรัม เนื่องจากคอมพลีเมนท์มีหลายบทบาทในระบบภูมิคุ้มกัน จึงมีความเป็นไปได้ที่ซีรัมของจระเข้จะสามารถกระตุ้นกระบวนการกลืนกินของมาโครฟาจ การศึกษานี้จึงได้ทำการทดสอบฤทธิ์ของซีรัมจากจระเข้พันธุ์ไทยในการเพิ่มประสิทธิภาพกระบวนการกลืนกินของมาโครฟาจ ตลอดจนศึกษาความเกี่ยวข้องของคอมพลีเมนท์ในกระบวนการนี้ ผลจากการศึกษาพบว่าซีรัมจากจระเข้ (fresh serum, FS) สามารถทำให้มาโครฟาจเพิ่มประสิทธิภาพการกลืนกินแบคทีเรียทั้ง Escherichia coli และ Staphylococcus aureus โดยสามารถเพิ่มประสิทธิภาพการกลืนกิน E. coli ได้ดีกว่า S. aureus นอกจากนี้ตัวอย่างเลือดจระเข้ประเภทอื่น ๆ ได้แก่ freeze dried serum (DS) และ freeze dried whole blood (DWB) สามารถเพิ่มประสิทธิภาพการกลืนกินของมาโครฟาจได้เช่นกัน หากแต่ฤทธิ์ของ DWB ในการเพิ่มประสิทธิภาพการกลืนกินของมาโครฟาจน้อยกว่าฤทธิ์ของ FS และ DS แสดงให้เห็นว่าฤทธิ์ดังกล่าวเป็นผลจากสารที่มีอยู่ในซีรัม โดยเมื่อนำซีรัมไปให้ความร้อนที่ 56 องศาเซลเซียส เป็นเวลา 30 นาที พบว่าฤทธิ์ในการเพิ่มประสิทธิภาพการกลืนกินลดลงอย่างมาก ชี้ให้เห็นว่าคอมพลีเมนท์มีบทบาทในกระบวนการนี้ ดังนั้นการศึกษานี้แสดงให้เห็นฤทธิ์ของเลือดจระเข้ในการเพิ่มประสิทธิภาพของกระบวนการกลืนกิน ซึ่งสามารถนำไปประยุกต์ใช้ในการบำบัดโรค ในกรณีที่ต้องการเพิ่มประสิทธิภาพการทำหน้าที่ของระบบภูมิคุ้มกัน