Comparison of the Hemagglutination of Formalinized American Channel Catfish (*Ictalurus punctatus*) Erythrocytes Between Formalinized and Live *Aeromonas hydrophila* Isolated from the Catfish Rearing in Kasumigaura Lake in Japan

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ABSTRACT. Formalinized *Aeromonas* (*A.*) *hydrophila* agglutinated loosely with the formalinized American channel catfish erythrocytes (FACCE), while live *A. hydrophila* agglutinated tightly with the FACCE. There was a significant difference on the number of attaching bacterial cells to the FACCE ($p < 0.01$) ($n = 40$ erythrocytes) between formalinized and live *A. hydrophila*. The other bacteria such as *Salmonella* (*S.*) Typhimurium ST-5, *Escherichia* (*E.*) *coli* V-517 and *Staphylococcus* (*S.*) *hyicus* ATCC1249 used in this experiment did not attach the FACCE.

KEY WORDS: *Aeromonas hydrophila*, formalinized American channel catfish erythrocytes, hemagglutination.

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A. hydrophila is a causal agent of epizootic ulcerative syndrome disease of catfish [5–8, 11–13]. Kozaki *et al.* [8] succeeded in purifying a hemolysin from *A. hydrophila* [9], and American channel catfish erythrocytes can be hydrolyzed by the hemolysin as shown in our previous report [2]. Both fish erythrocytes with nucleus and sheep erythrocytes without nucleus were affected by the hemolysin [3–6, 10, 13]. However, hemagglutination of catfish erythrocytes with *A. hydrophila* has never been reported until now.

For this experiment, 8 out of 48 isolates (Ah₂– Ah₇₀) of *A. hydrophila* from American channel catfishes rearing in Kasumigaura Lake in Japan have been used. The isolates were identified by Api 20E (biomérieux, France). American channel catfishes have been kindly supplied by The Ibaraki Prefectural Institute of Fresh Water Fish. The bloods were taken from vein of the lateral line by heparinized syringes and kept in the Alsever's solution before use.

For hemagglutination test, American channel catfish erythrocytes (ACCE) were prepared as previously described [2]. *A. hydrophila* were grown in 15 ml of Trypticase soy broth (BBL, U.S.A.) for 18 hr at 37°C. One ml of the culture was taken and washed three times with saline by the centrifugation at $5,000 \times g$ for 5 min at 5°C. The precipitate was resuspended in 0.5 ml saline, mixed with equal volume of saline containing 1% formalin and then, left for 3 hr at room temperature. Formalinized American channel catfish erythrocytes (FACCE) were prepared by the centrifugation of ACCE at $450 \times g$ for 20 min at room temperature and the precipitate were mixed with 9 ml of saline containing 10% formalin and left for 4 hr at room temperature. The FACCE were washed 3 times with saline by the centrifugation at $450 \times g$ for 20 min at room temperature. Before the hemagglutination test, the bacterial cell number in the suspension was adjusted at McFarland standard No. 1. Two hundred and

fifty μ l of the suspension was taken and mixed with equal volume of FACCE in the test. *Salmonella* (*S.*) Typhimurium ST-5, *Escherichia* (*E.*) *coli* V-517 and *Staphylococcus* (*S.*) *hyicus* ATCC1249 were also used for the comparison of the hemagglutination with *A. hydrophila*. The mixtures were left at each of 5, 10, 20, 30 and 37°C for 12 hr and then, the reaction was observed under a phase contrast microscope (Olympus, Japan). The number of the attaching bacteria per 40 FACCEs was counted and the number of the attaching bacteria per one FACCE was shown as the mean $\pm$ standard deviation (SD).

The hemagglutination (adherent bacterial cells/one FACCE) between live and formalinized *A. hydrophila* was statistically treated with *t*-test (Microsoft Excel software).

Eight *Aeromonas* isolates which were selected on the basis of the preliminary experiment results concerning the hemagglutination, agglutinated well with FACCE (Table 1). Among the five different temperatures, best hemagglutination condition was at 10°C for 6 hr (Fig. 1). The isolates Ah₂₅, Ah₂₇, Ah₂₈ and Ah₇₀ attached well with FACCE and the selected number of attaching bacterial cells per one FACCE were 2.99 ± 0.21 (mean $\pm$ SD) ($n = 40$ erythrocytes), 2.97 ± 0.14 , 3.19 ± 0.18 and 3.11 ± 0.11 respectively, while the number of bacterial cells in the isolates Ah₂, Ah₃, Ah₄ and Ah₈ were 2.65 ± 0.19 , 2.53 ± 0.13 , 2.69 ± 0.16 and 2.74 ± 0.22 , respectively (Table 1, Fig. 1). However, there was no significant difference ($p < 0.01$) among the isolates on the hemagglutination with FACCE (Table 1). Microscopic observation results demonstrated that live *Aeromonas* cells agglutinated well with FACCE (Fig. 3L) while formalinized *Aeromonas* cells agglutinate loosely with the FACCE (Fig. 3F). Amongst the *Aeromonas* isolates, both live and formalinized Ah₂₈ attached well to FACCE and the numbers of the attaching bacterial cells per FACCE were 3.19 ± 0.18 in case of live bacteria and 2.02 ± 0.18 in case of formalinized bacteria, respectively (Table 1). There was a significant difference ($p < 0.01$) between live and formalinized bacterial

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Table 1. Comparison of the hemagglutination between formalinized and live bacteria with FACCE

Isolates	Adherent of formalinized bacteria/one FACCE ^{a)}	Adherent of live bacteria/one FACCE ^{b)}
<i>Aeromonas. hydrophila</i>		
Ah ₂	1.64 ± 0.12 ^{c)}	2.65 ± 0.19 ^{d)}
Ah ₃	1.66 ± 0.17	2.53 ± 0.13
Ah ₄	1.72 ± 0.22	2.69 ± 0.16
Ah ₈	1.69 ± 0.19	2.74 ± 0.22
Ah ₂₅	1.78 ± 0.16	2.99 ± 0.21
Ah ₂₇	1.86 ± 0.21	2.97 ± 0.14
Ah ₂₈	2.02 ± 0.18	3.19 ± 0.18
Ah ₇₀	2.07 ± 0.15	3.11 ± 0.11
<i>Salmonella. Typhimurium</i>		
ST-5	0.00 ± 0.00	0.00 ± 0.00
<i>Escherichia. coli</i>		
V-517	0.00 ± 0.00	0.00 ± 0.00
<i>Staphylococcus. hyicus</i>		
ATCC1249	0.00 ± 0.00	0.00 ± 0.00

a) Not significantly different among the isolates; b) Not significantly different among the isolates; c) Adherent bacterial cells/one FACCE; d) The value was expressed as mean ± SD (n=40 erythrocytes). There was significant difference between a and b by *t*-test ($p<0.01$).

Fig. 1. Comparison the hemagglutination (adherent *A. hydrophila* bacterial cells/ one FACCE) of live *A. hydrophila* isolates in FACCE in different temperatures and different time intervals. The number of the attached *A. hydrophila* per FACCE was shown as mean ± SD (n=40 erythrocytes). The vertical bar indicates standard deviation value mentioned above.

Fig. 2. Comparison of the hemagglutination (adherent *A. hydrophila* bacterial cells/ one FACCE) of live and formalinized *A. hydrophila* isolate in FACCE. The number of the attached *A. hydrophila* per FACCE was shown as mean ± SD (n=40 erythrocytes). The vertical bar indicates standard deviation value mentioned above. There was a significant difference in point of the attaching bacteria per one FACCE, between formalinized and live *A. hydrophila* by *t*-test ($p<0.01$).

cells as shown in Fig. 2 in point of the attaching bacteria number to FACCE. In contrast, *S. Typhimurium* ST-5, *E. coli* V-517, and *S. hyicus* ATCC1249 were used for contradiction to non-specific hemagglutination with *Aeromonas* isolates and they never attached the FACCE (Fig. 3L & 3F). American channel catfish erythrocytes have been used in this experiment because the catfishes were real host to the isolates. In addition, the formalinized erythrocytes have been used for the observation of the real hemagglutination because non-formalinized erythrocytes were broken quickly by the hemolysin. On the other hand, in case of chick erythrocytes, the hemagglutination with *Aeromonas* isolates have been observed but it was weak, compared with those of the

American channel catfish erythrocytes (unpublished data).

In the hemagglutination contributing the human isolates of *A. hydrophila*, human erythrocytes has been used [1]. In our experiment, *A. hydrophila* from American channel catfish has been used for the analysis of the agglutination with American channel catfish erythrocytes. This is the first experiment using American channel catfish erythrocytes. Hemagglutination between *Aeromonas* cells and FACCE is

Fig. 3. Hemagglutination with (L) live, (F) formalinized bacteria in FACCE. Ah, *A. hydrophila*; EC, *E. coli* V-517; ST, *S. Typhimurium* ST-5; SH, *S. hycus* ATCC1249; N, negative control without bacteria; E, erythrocyte. Arrows show the bacteria. Bar=20 μ m.

very important for the investigation of the adhesions concerning the virulence factors. Furthermore, FACCE still kept receptor activity. Therefore, we thought it may consist of a kind of glycoprotein. Hemolysin is also one of the virulence factors of *A. hydrophila*. In our previous experiment on *Aeromonas* hemolysin, we found Ah₂₅, Ah₂₇, Ah₂₈ and Ah₇₀ showed strong hemolysis on agar containing ACCE, while the isolates Ah₂, Ah₃, Ah₄ and Ah₈ showed weak hemolysis [2]. This result may indicate the good correlation between hemagglutination and hemolysis. This means strong hemolytic and hemagglutination isolates may be high virulent *A. hydrophila*. We will attempt to investigate electronmicroscopically the adhesion of *Aeromonas* to the receptor of catfish erythrocyte and will clarify the adhesin and the receptor biochemically concerning the hemagglutination.

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