THE PATHOLOGICAL AND HAEMATOLOGICAL EFFECTS OF *Aeromonas hydrophila* ON WALKING CATFISH (*Clarias gariepinus*)

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ABSTRACT

A comparative study was carried out to determine the effects of saline suspensions of either unwashed, washed or sonicated washed cells of a virulent strain of Aeromonas hydrophila on walking catfish, Clarias gariepinus. Three doses of 106, 107, and 108 cfu/ml were prepared from each of the above bacterial suspensions and injected intramuscularly into healthy catfish fingerlings. With the injection of 106 cfu/ml, both washed and sonicated washed cells caused a mild inflammation at the injected site which returned to normal after 48 hrs. Mortality was 60%, which occurred only from 3 to 12 hrs. In contrast, injection of the unwashed cells induced a stronger reaction than that of the washed and sonicated washed cells. Mortality was also 60% but occurred from 12 to 24 hrs. With an injected dose of 10^8 cfu/ml, both washed and sonicated cells induced more marked inflammation with haemorrhage at the injected site. Mortality (60% for W-injected and 90% for So- injected) occurred only from 3 to 12 hrs. The injured tissues of the survival took 120 hrs for recovery. On the other hand, an injection of 10^8 cfu/ml unwashed cells would kill all the fish at 48 hrs and their blood was completely hemolysed. The variations in haemotocrit value and blood differential counts after the injection of the different types and dilutions of bacterial preparations were discussed.

Keywords : *Aeromonas hydrophila*, inflammation, haemorrhage, ulcer, blood cells

The outbreak of *Aeromonas hydrophila* was first found in West Java, Indonesia, in 1980. It caused a loss of 82.288 tons of fish within a month (Angka *et al.*, 1982). By 1981, it had spread to Malaysia and Thailand and then to the whole South East Asia from Myanmar to the Philippines in 1985. By 1987, it had reached Sri Lanka and later Northeast India and Nepal in 1988 (Roberts *et al.*, 1992).

Thune *et al.* (1982) found out that this bacterium produces exotoxins (extracellular products) and endotoxin. The extracellular products (ECPs) are comprised of α and β haemolysin, cytotoxin, enterotoxin, protease and leucocidins (Allan & Stevenson, 1981; Lallier & Daigneault, 1984; Morgan *et al.*, 1985; Kanai & Takagi, 1986; Thune *et al.*, 1986; Santos *et al.*, 1987). Among these, haemolysin is the major active lethal factor to fish (Allan & Stevenson, 1981 ; Thune *et al.*, 1986).

Few in vivo studies with the effect of *A. hydrophila* ECPs on fish have been done (Allan & Stevenson 1981; Kanai & Takagi 1986). Since haematology is an important criteron for diagnosis and in assessing the health of fish (McCarthy *et al.*,1973; Allan & Stevenson, 1981 ; Boon *et al.*, 1990) the objective of this study was, therefore, to compare the pathological and haematological effects of live and sonicated *A. hydrophila* on walking catfish after intramuscular injection.

METHODS

Fish and *A. hydrophila*

Experimental fish were *Clarias gariepinus* fingerlings about 10 - 12 cm long, weighing 8 - 10 gm and were obtained from the same parental stock, obtained from catfish culture farm in Bogor. They were kept in 14l plastic containers. For the virulent test of LD_{50}, 10 fish were used for each treatment.

For the pathological and haematological experiment, triplicate lots of 25 fish each were intramuscularly injected with unwashed cell suspension (Su), washed cell suspension (W) or sonicated washed cell suspension (So) in a dose of 0.1 ml bacteria prepared per 10 gr fish. For control fish, they were similarly injected with 0.85% NaCl.

A. hydrophila strains were obtained from diseased fish in West Java. The virulence of the biochemical distinct strains were determined through LD_{50} (Reed & Müench, 1938) and haemolytic activity. The haemolytic activity was determined by culturing the bacterial strains on tryptic soy agar (TSA) which was mixed with 5% v/v defibrinated blood of either sheep, horse, cow, rabbit or man. LD_{50} $<10^6$ were considered as virulent strain; those 10^8 or greater, avirulent, while those between were referred to as weakly virulent. A virulent strain was chosen and diluted to three doses of 10^6, 10^7 and 10^8 cfu/ml cells. They were used to prepare live unwashed-cell suspension (Su), washed-cell suspension (W) and sonicated washed-cell suspension (So). Live unwashed-cell suspension contained bacteria harvested directly from the culture media and suspended in sterile saline (0,85% NaCl). Washed-cell suspension was prepared by washing the harvested bacteria in sterile saline 3 times by centrifugation. Sonicated washed-cell suspension was obtained by sonificating the washed-cell suspension for 5 minutes, and 0.1 ml from each dilution of each preparation was intramuscularly injected into each of 25 fingerlings. Controls were similarly injected but with 0.85% saline. Mortality, morbility, feeding appetite and behavior were monitored and recorded for 14 days.

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Blood Picture Analyses

The blood pictures of the above experimental fish were also analyzed for every 3 hours. For every treatment, 3 fish were removed for blood samples. Blood was obtained from the caudal artery by cutting the caudal peduncle and was spread on paraffin-coated watch glass for blood picture determination. The blood haemoglobin content was determined using Sahli method. Haematocrit was determined in heparinised capillary tubes (75 mm long and internal diameter 1 mm) by sealing one end with critocel and centrifuged at 3000 rpm for 5 mins. Blood smears were prepared and stained by the May Grünwald Giemsa method and differential counts of at least 2000 cells were routinely carried out.

RESULTS AND DISCUSSION

A. hydrophila is an aquatic bacterium which had many heterogeneous characteristics (Nieto & Ellis, 1991). In this study eighteen strains of *A. hydrophila* were isolated from diseased and healthy fish from West Java (Table 1). Their LD_{50} and haemolytic activity were studied. As shown in Table 1, all the isolated strains showed a heterogeneous degree of virulence. The two virulent strains (L_{31},L_{38}) with LD_{50} of $10^{5.4\ -5.5}$ cfu/ml were able to haemolyse all the different types of test red blood cells. However, similar haemolytic activity was also found in two weakly-virulent strains ($L_{35,38}$). The results also showed that most of the avirulent strains could haemolyse some of the test red blood cells. This, however, depends on their LD_{50} value.

From its haemolytic activity and the virulence LD_{50} test it was shown that all the isolated bacterial strains from the diseased and healthy catfish did not have the same characteristics. A virulent strain L_{38} was chosen to study its pathological and haematological effects on healthy catfish fingerlings.

No clinical signs were shown in all the experimental fish at 3 hrs after the injection of either unwashed cells (Su), washed cells (W) or sonicated cells (So) of L_{38} with a dose of 10^6 cfu/ml. During this time interval, they had good appetite and no mortality was encountered. However, from 6 to 48 hrs post-injection (PI), fish injected with Su showed inflammation (Fig.1). Their mortality was 60% and all the death was only occurred from 12 to 24 hrs. The inflamed tissues at the injected site of the survivals healed after 72 hrs. Fish injected with W and so exhibited inflammation from 12 to 48 hrses. The mortality of both groups was 60% and occurred between 6 and 12 hrs. The injected site of the survivals healed after 72 hrs. As the injected tissues became inflamed fish of the 3 injected groups showed a decrease of appetite and mortality began.

With a dose of 10^7 cfu/ml, inflammation appeared from 3 to 6 hrs in fish injected with Su and from 3 to 120 hrs in fish injected with W and So. The inflammation was followed by haemorrhage (Fig.1) at the injected site from 12 to 24 hrs and ulcer (Fig.2) appeared at 48 hrs with Su injection. The ulcerative tissues healed after 120 hrs, but mortality occurred between 12 and 120 hrs PI. For fish

Table 1. Source of strains and their virulence and haemolytic activity.

No Isolate	Source	Fish Condition	LD_{50}	Virulence	Hemolytic activity				
					Sheep	Man	Horse	Cow	Rabbit
L_1	Depok	Healthy	$10^{6.4}$	WV	+	-	-	-	-
L_7	Depok	Diseased	$10^{6.6}$	WV	+	+	-	-	-
L_{11}	Sukabumi	Diseased	$>10^8$	AV	+	+	+	-	+
L_{14}	Sukabumi	Diseased	$>10^8$	AV	+	+	-	+	-
L_{15}	Cibalagung	Diseased	$10^{6.6}$	WV	+	-	-	-	+
L_{18}	Cicurug	Diseased	$>10^8$	AV	+	-	-	-	-
L_{19}	Cicurug	Diseased	$>10^8$	AV	+	+	-	-	-
L_{21}	Sukabumi	Diseased	10^8	AV	-	-	-	-	-
L_{28}	Bekasi	Diseased	$>10^8$	AV	+	+	-	-	-
L_{31}	Cibalagung	Diseased	$10^{5.4}$	V	+	+	+	+	+
L_{33}	Sukabumi	Diseased	$>10^8$	AV	-	-	-	+	-
L_{34}	Sukabumi	Diseased	$>10^8$	AV	+	-	-	-	-
L^{35}	Sukabumi	Diseased	10^6	WV	+	+	+	+	+
L_{36}	Sukabumi	Diseased	10^6	WV	+	+	+	+	+
L_{37}	Sukabumi	Diseased	10^8	AV	-	-	-	-	-
L_{38}	Sukabumi	Diseased	$10^{5.5}$	V	+	+	+	+	+
L_{39}	Sukabumi	Diseased	10^8	AV	+	-	+	+	+
L_{40}	Sukabumi	Diseased	10^6	WV	+	+	-	-	-

Notes : WV = Weak Virulent
AV = Avirulent
V = Virulent

injected with W and So, the injected site became haemorrhage at 24 hrs. This was healed after 120 hrs but mortality occurred between 3 and 12 hrs PI. With the occurrence of inflammation at the injected site all fish showed anorexia. However, these fish returned to normal feed consumption as soon as the damaged tissues became healed. No changes have been encountered at the saline injected site of the controls (Fig. 2).

When fish injected with a dose 10^8 cfu/ml inflammation occurred from 3 to 6 hrs with Su, followed by haemorrhage from 6 to 18 hrs and ulcer started from 12 to 48 hrs. Fish lost appetite and showed lethargy during the experiment. Mortality began at 6 hrs and all the Su - injected fish died at 48 hrs showing haemolysis in their blood (Fig. 3). When fish injected with a dose of 10^8 cfu/ml of W and So inflammation appeared from 3 to 18 hrs. With W-injection, the inflammation became haemorrhage from 24 to 96 hrs and developed into ulcer from 120 hrs until the end of the experiment. But for fish injected with So, haemorrhage occurred at 24 hrs and ulcer developed from 48 to 120 hrs. When the injected site of all fish exhibited inflammation and haemorrhage, they showed anorexia and stopped eating completely when they had ulcer. At this stage, the diseased fish exhibited lethargy and remained at the bottom of the containers. The mortality of W and So-injected groups was 60 and 90% respectively and all occurred from 3 to 12 hours but without showing any haemolysis in their blood.

Their variations were also demonstrated in the pathological changes induced by intramuscuular injection of the bacteria into the catfish fingerlings. This pathogen heterogeneity could be attributed to the variations in production of extracellular products (exotoxins) and endotoxins by the different strains of the bacterium.

The in vitro studies by other workers (Ljungh & Wädstrom, 1982; Morgan *et al.*, 1985, Kanai & Takagi, 1986; Santos *et al.*, 1987; Lallier & Higgins, 1988) had shown that the virulent strains produced different kinds of extracellular products (ECPs) such as α and β haemolysin, proteolytic enzyme, leucocytic, enterotoxic, cytotoxic and dermonecrotic factors. This was further supported by the findings of the present, in vitro, haemolytic tests using various types of red blood cells from sheep, man, horse, cow and rabbit. Our results showed those virulent strains with LD_{50} of $10^{4.5-5.5}$ cfu/ml (Lallier & Daigneault, 1984) could haemolyse all the homiothermic blood being used in the present study. It is of interest to point out that Roberts (1993) recently reported that virulent strains of *A. hydrophila* produced β haemolysin, elastase and has a S-layer surface. He pointed out that the soluble haemolysins caused haemorrhage and also induced ulcerative skin lesions in fish.

So far, little has been done, in vivo, to study the effect of ECPs on fish (Allan & Stevenson, 1981; Kanai Takagi, 1986). The results of this study showed that the unwashed bacteria when injected into the fish was more pathogenic as compared to the washed (W) or sonicated cells (So). As shown in Fig.3, the red blood cells were completely haemolysed at 18 hrs in fish after the injection of 10^8 cfu/ml unwashed bacteria. This result suggests that exogenous factors present in the bacterial culture are involved in haemolysis. This observation is supported by the findings of Ljungh & Wädstrom (1982), Kanai & Takagi (1986) and Bloch & Monteil (1989) who reported that a β haemolysin was produced at the stationary phase while a β haemolysin at the end of log phase which is more proteolytic and is an indicator of enterotoxin (Rahim *et al.*, 1984).

It is interesting to point out that fish injected with a dose of 10^8 cfu/ml of sonicated-cell suspension (So) exhibited ulcer at 48 hrs which was occurred much earlier than that at 120 hrs in the washed-cell (W) injected fish. Both suspensions caused mortality (60% in the W-injected and 90% in the So-injected group) only from 3 to 12 hrs. However, no haemolysis was found in the fishes of both groups. This indicates that early death occurred in the fishes of the Sonicated-cell injected (So) group was most likely due to the action of endotoxins. On the other hand, haemolysis occurred in fish injected with unwashed-cells (Su); 60% of the injected fish died from 3 to 12 hrs and the rest died within 24 hrs. From the results of this study, it is obvious that haemolysin released by the bacteria in the culture medium is also lethal to the fish. The haematocrit values of these Su-injected fish were very low because their blood was completely haemolysed (Fig.6). This observation is in well agreement with the findings of the, in vitro, studies by various workers (Allan & Stevenson, 1981; Olivier *et al.*, 1981; Thune *et al.* 1986; Kanai & Takagi 1986; Ishimura *et al.*, 1988; Bloch & Monteil, 1989, Kanai & Wakabayashi, 1984).

Haemoglobin content of fish injected with the 3 different doses of W and So was not different from that of the control fish (Fig.4). The haematocrit value of fish injected with 10^6 cfu/ml of either Su, W or So showed no difference from that of the control fish (Fig.5). However, when bacteria-injected doses increased to 10^7 and 10^8 cfu/ml the haematocrit values were lower than the control fish, in particular to those showing haemorrhage and ulcer at the injected site. This was especially distinct for fish injected with Su in which their red blood cells were haemolysed at 18 hrs PI and a drop of haematocrit level appeared after 12 hrs.Thrombocyte value in fish injected with the 3 doses of W and Su was higher than that of the control fish (Fig.6) in particular when the inflammation, haemorrhage and ulcer were developed. On the other hand, the thrombocyte value of the So-injected fish was relatively lower as compared to the W- and Su-injected groups.

The number of lymphocytes in fish injected with the 3 doses of So was higher than the control fish (Fig.7). Higher value was also encountered in fish injected with 10^8 cfu/ml of Su. The monocyte value in all the So-injected fish was higher than the control while no difference was seen between the latter and the Su- and W-injected groups (Fig.9).

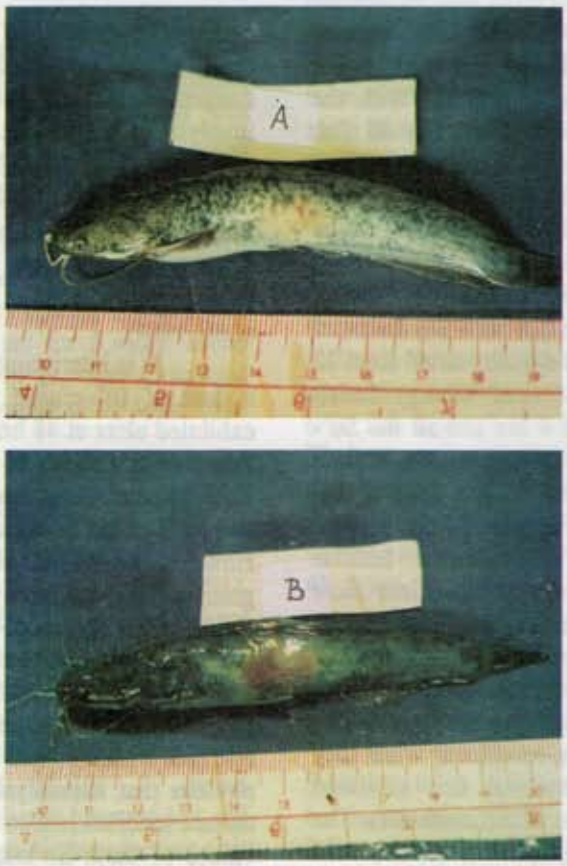

Figure 1. Inflammation (A) and haemorrhage (B) at the injected site.

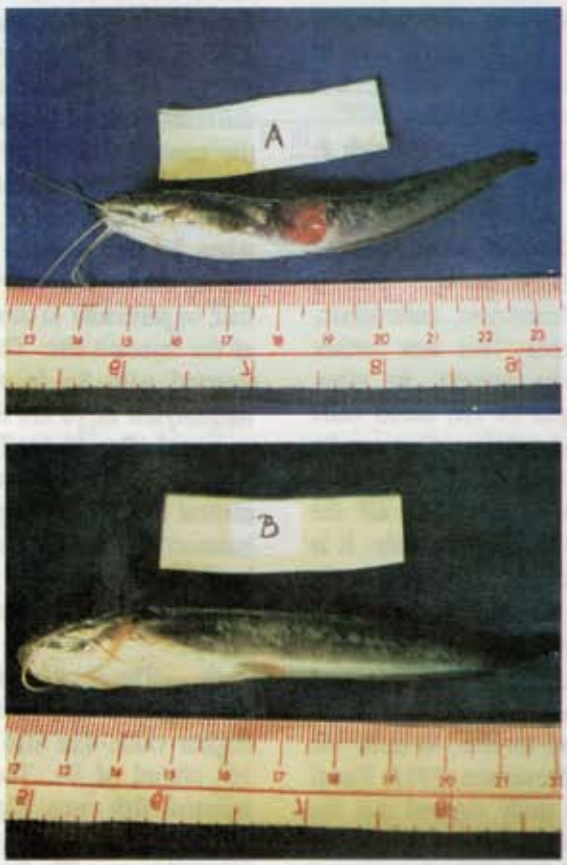

Figure 2. Ulcer at the injected site (A) and control fish injected with saline (B).

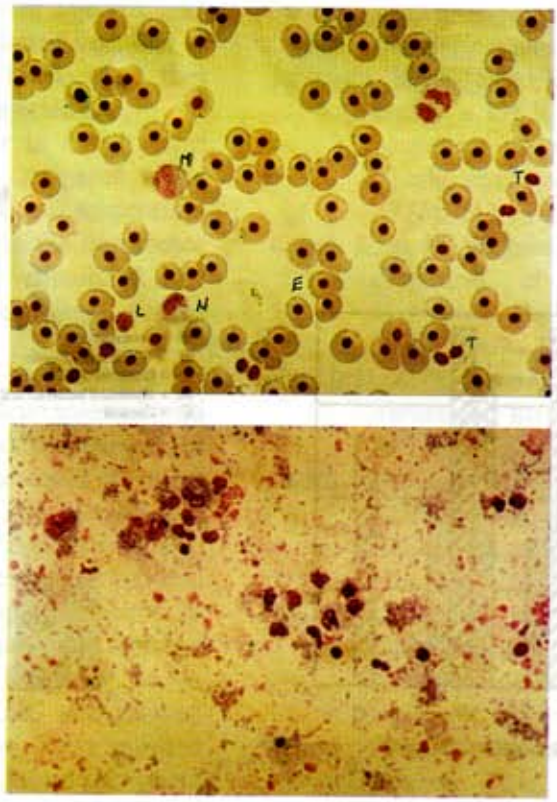

Figure 3. Normal blood smear and haemolysed blood 18 hrs after injected with bacterial cells + ECP (x 400 – Giemsa)
M = monocyte; L = lymphocyte; E = erythrocyte; N = neutrophil; T = thrombocyte.

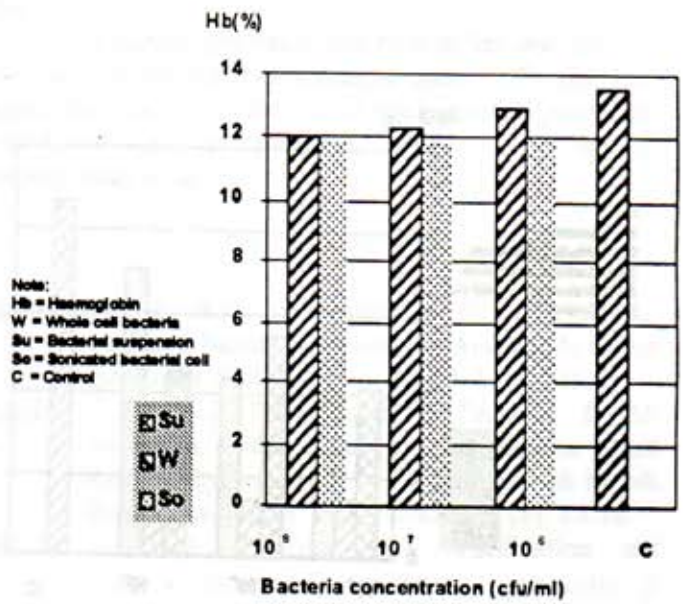

Figure 4. Haemoglobin value of fish injected with *A. hydrophilla*.

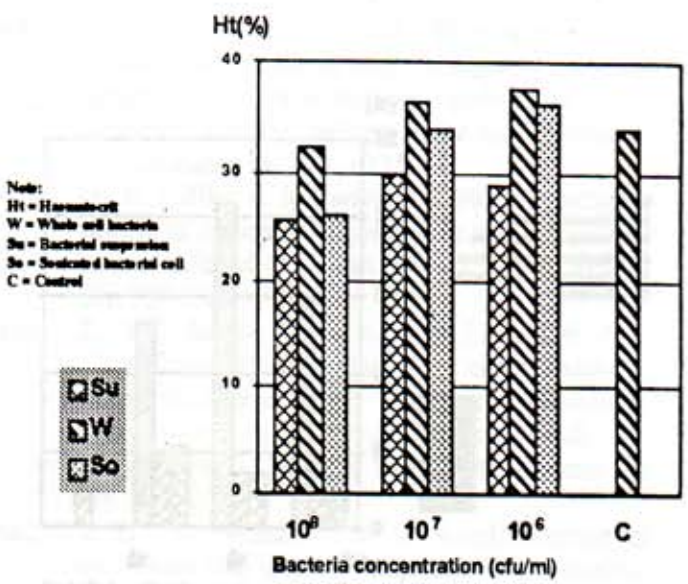

Figure 5. Haemotocrit value of fish injected with *A. hydrophilla*.

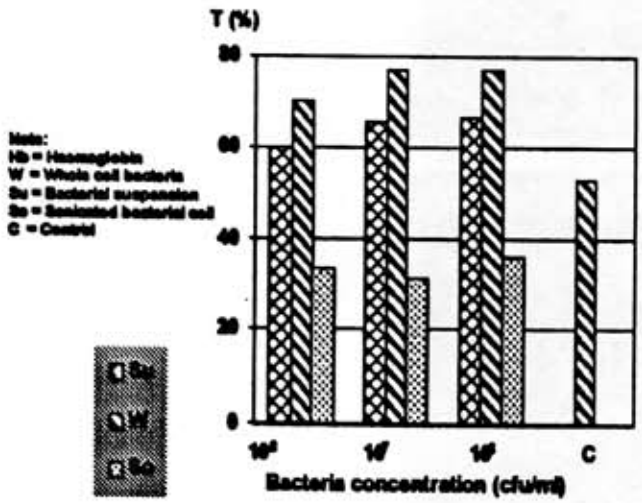

Figure 6. Thrombocyte value of fish injected with *A. hydrophilla*.

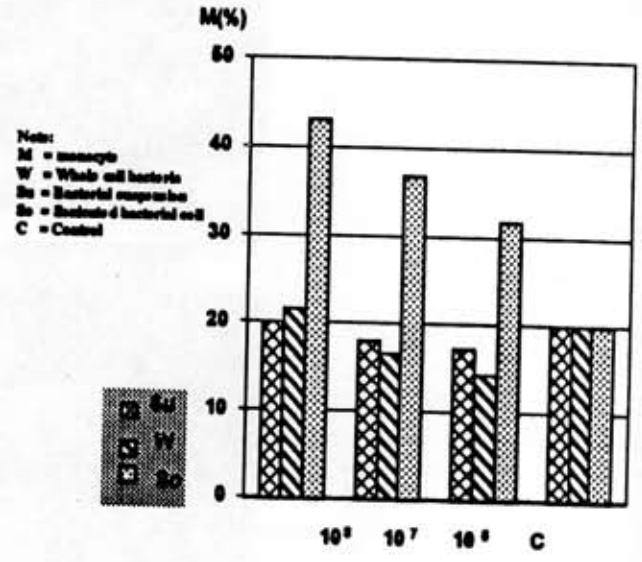

Figure 8. Monocyte value of fish injected with *A. hydrophilla*.

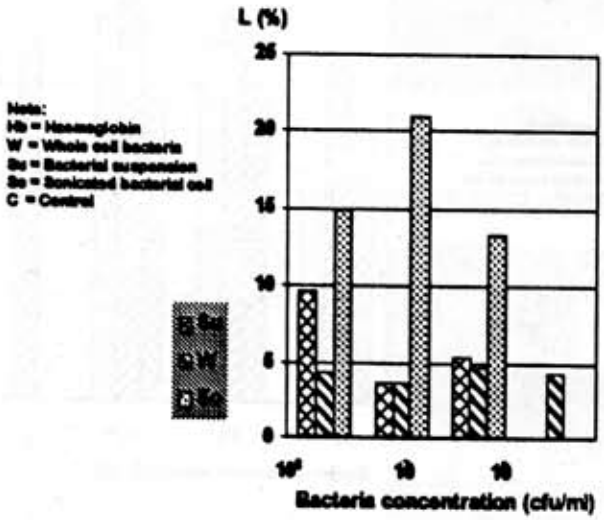

Figure 7. Lymphocyte value of fish injected with *A. hydrophilla*.

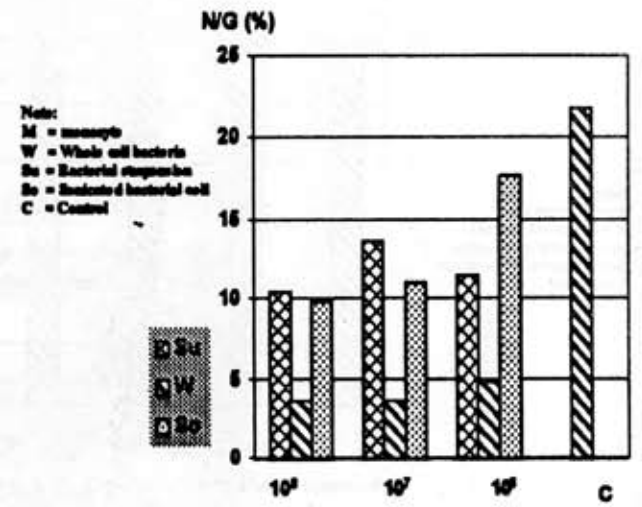

Figure 9. Granulocyte value of fish injected with *A. hydrophilla*.

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