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by M. Nishibuchi, K. Muroga, and Y. Jo

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Pathogenic *Vibrio* Isolated from Cultured Eels—VI. Diagnostic Tests for the Disease Due to the Present Bacterium

Mitsuaki NISHIBUCHI*, Kiyokuni MUROGA*, and Yasuhiko JŌ**

**Fac. Applied Biological Science, Hiroshima University, Fukuyama*

***Fish. Exp. Station of Tokushima Pref., Hiwasa, Tokushima*

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A search was made for the diagnostic tests for the disease of cultured eels due to the present bacterium.

It is considered impossible to differentiate the disease of eels caused by the present bacterium, by means of external signs of diseased fish, from that caused by other eel pathogens such as *Aeromonas hydrophila, Edwardsiella tarda, Vibrio anguillarum*, and *Pseudomonas anguilliseptica*. This necessitates the isolation and identification of the infectious agent for diagnosis.

Positive proof was obtained that the diluted rabbit antisera prepared against representative strains of the present bacterium can be used for diagnostic purpose. In addition, a simplified identification method by the use of API 20E was shown.

It was proposed that the disease of cultured eels caused by the present vibrio be named as vibriosis of eels Type B; that due to *V. anguillarum* as Type A.

The authors have clarified since the summer of 1975 that 125
a bacterium isolated from cultured eels in Tokushima, Shizuoka, and Kōchi Prefectures is a new type of a bacterium which belongs to the Vibrio species. (Muroga, et al., 1976 a, b; Nishibuchi, Muroga, 1977, 1980; Nishibuchi, et al., 1979, in writing). The epidemic disease of cultured eels caused by this bacterium breaks out sporadically even at present. Our survey, which was performed in April, 1979, at eel hatcheries in Tokushima Prefecture, confirmed that the disease occurs in heated ponds. However, as we will mention later, the diagnosis of this disease based on symptoms and/or the cultivation of the isolated bacterium is relatively difficult to make. It is considered that the disease

is not understood correctly in terms of epidemiology. Therefore, we think that, in order to help the research of the present disease, it it necessary to establish an easy and accurate method of diagnosis about it.

As we have mentioned in the first report (Muroga, et. al., 1976a), in the case that the progress of the disease is relatively slow, we could observe a characteristic swelling (Fig. 1-A) or an ulcer (Fig. 1-B) on the trunk of diseased eels which are contaminated by the present bacterium, and could sometimes diagnose from the symptoms. However, when the progress of the disease is fast and many eels die in a short period, the external signs of diseased eels are often only red colouration of the skin on each fin, and the dilatation and congestion of the anus. (Fig. 1-C & D). These symptoms are similar to those of hemorrhagic septicemia, which occurs frequently in eel hatcheries in Japan, caused by Aeromonas hydrophila, Edwardsiella tarda, Vibrio anguillarum, or Pseudomonas anguilliseptica. It is hard to differenciate, from the external appearances, the disease caused by the present bacterium from hemorrhagic septicemia.

The above-mentioned well known four eel pathogens grow relatively well, like the present bacterium, on a common agar culture medium at 20-25°C. They are all short bacteria and have a gram-negative mobility. However, the present bacterium grows faster than the other bacteria on a common agar culture medium. Therefore, it could be possible to a certain degree to distinguish it from the other four pathogens according to the cultured state, by experience, but this is not always an accurate way of judgement. 128

The slide agglutination tests of the present bacterium,

with rabbit antisera, discussed in the previous report (Nishibuchi, Muroga, 1980), are examined in the present report to see whether they could be used for the diagnosis of the disease in cultured eels. In addition, an API 20E System (Analytab Products), which is sold commercially as a simple identification kit for intestinal bacteria, is also discussed to see whether it could be used as a simple method of identification.

Materials and methods

Rabbit antisera: Antisera against the representative three strains of the present bacterium, which were discussed in the previous report, are used.

Slide agglutination tests: A total of 49 strains, which includes the present bacterium, the other various types of Vibrio bacteria, fish pathogens, and Escherichia coli, is used for the tests. Two platinum loopfuls (about 60 mg of wet bacteria) of the cultured bacteria grown on horizontal agar culture media were taken out, and were made to float on the 1 ml. of phosphate salty buffer solution (pH 7.0). This was used as a reaction antigen. The antigen was made to react againt the same amount of the antisera on a concave glass and was judged in three minutes.

Simple identification of eel pathogens using API 20E: An API 20E is used for the simple identification of pathogens from diseased eels. Strains isolated by the authors from diseased cultured eels in Tokushima Prefecture and other prefectures during 1971 through 1979, A. hydrophila, E. tarda, V. anguillarum, P. anguilliseptica, which were among the eel pathogens donated by Prof. Riichi Kusuda, Kōchi Univ., and V. anguillicida (tentative name), which is our main subject of this study, totalling 88 strains, were examined.

As a first step of our test, we examined six characteristic items at the genus level in order to identify these bacteria easily. That is, gram-staining characteristics cytochrome oxidase test, motility, and sensitivity to O/129 were examined following the conventional methods. Also O-F test and gas production from glucose were examined following the simplified method of Walters & Plumb (1978). Then, using the API 20E System, following its test procedure, 22 items (if the above-mentioned cytochrome oxidase test is included, 129 23 items) of biochemical characteristics were examined. In this experiment, a floating solutinn of the bacteria, which were cultured at 25°C for 24 hours on a common agar culture medium (NaCl 0.5%), was inoculated and cultured at 25°C. The final judgement was made after 48 hours, (because most reactions were weak in 24-hour culture).

Results and discussion

The slide agglutination tests of the present pathogen and the other bacteria in the control group were performed and the presence of peculiar and similar agglutinative reactions of the antisera were examined. All rabbit antisera against three representative strains shown in Table 3 were used in undiluted, diluted (1:5) and (1:10) solutions. The results are shown in Table 1. All strains of the present pathogen Vibrio show positive reactions against the undiluted and 1:5 diluted antisera. However, some negative reactions are shown against the 1:10 diluted antisera. Among the control group, P. putida ATCC 12633 and E. coli WP2 strains show strong positive reactions only against the anti-ET-517 and anti-KV-1 sera. However, after the absorption tests as shown in Table 2, it became clear that these reactions were not the reactions of a peculiar antibody against the present bacterium. Excluding these cases, there were no discrepancies due to the different samples of the antisera. Except the V. fischeri ATCC 15382 and A. hydrophilia ET-2 strains, which

showed positive reactions against the undiluted antisera solution, there were no strains which showed similar agglutinative reactions. Therefore, we conclude that properly diluted antisera against any strains could be used for the diagnosis.

As a means of distinguishing the present pathogen from the above-mentioned four types of bacteria, which have been known as pathogens of hemorrhagic septicemia of cultured eels, a slide agglutination test of antisera against the present bacterium is useful. In order to confirm this, we have performed slide agglutination tests of bacteria isolated from cultured eels in the various prefectures for several years. Different kinds of isolates from collected diseased eels were examined in simplified identification tests using the API 20E system. Table 3 shows morphological and biochemical properties of these isolates. As we have mentioned before, we tested 88 strains at first, but after the Step 2 tests using the API 20E system, 8 strains were excluded because of troubles in the preliminary identification. However, we kept 24 strains of the present pathogen, 7 strains of V. anguillarum, 11 strains of A. hydrophila, 18 strains of E. tarda, and 20 strains of P. anguilliseptica, to a total of 80 strains, and used them in experiments afterward. Among these strains, 19 strains of the present pathogen, 3 strains of V. anguillarum, 4 strains of A. hydrophila, 4 strains of E. tarda, 20 strains of P. anguilliseptica were tested following the conventional tests for differentiation. The results were confirmed to be the same as the results from the API 20E system.

Table 4 shows the results of the slide agglutination tests of the above-mentioned 80 strains, using the undiluted and diluted (1:5) solutions of the anti-ET-7617 serum which is among the antisera against the present pathogen. As shown in the table, while

all the strains of the present pathogen show positive reactions against the undiluted and diluted (1:5) solutions of the anti-ET-7617 serum, other strains show all negative reactions except A. hydrophila, which shows a positive reaction against the undiluted solution.

The above results lead us to think that the antiserum which was used in this experiment can be used for a serological diagnosis of the present disease of cultured eels, if it is diluted in 1:5 solution. However, we think that it is necessary, in order to make the serological diagnosis more accurate, to do also the simple identification tests against isolates as in Table 3. As seen in this table, in the conventional tests at the genus level, it is impossible to differenciate the present pathogen from V. anguillarum. However, if the API 20E system is added to the tests, an identification with a relatively high reliability becomes possible, because the present bacterium can be easily distinguished from the other bacteria including V. anguillarum.

The authors reported in the first report (Muroga, et. al., 1976a) that the occurrences of the epidemic disease of cultured eels due to the present bacterium are seen in hatcheries with salty water, and only during the high water temperature period when the water temperature becomes more than 20°C or in heated ponds. In the second report (Muroga, et. al., 1976b), we clarified that this is based on a physiological characteristic of the present pathogen 130 regarding salt and temperature. The occurrences of the present disease observed afterward are limited only in such hatcheries as described above. These environmental factors should not be forgotten in the diagnoses of the present disease. Including these factors, all-round diagnoses should be made.

In finishing this report, we would like to propose a name of the epidemic disease of cultured eels caused by the present pathogen. The epidemic disease of cultured eels by the present Vibrio was called, at first, ulcer disease. However, as mentioned before, not all diseased eels get ulcer formation on the body. Also in order to avoid getting mixed with the ulcer disease of seawater fish caused by Vibrio sp. (Kusuda, 1965), we think that it is better to call it vibriosis of eels. Furthermore, in order to distinguish it from vibriosis of eels caused by V. anguillarum, we would like to propose to call it vibriosis of eels Type B, because V. anguillicida was once called V. anguillarum Type B. Therefore, the disease by V. anguillarum is named vibriosis Type A, and the disease by the present vibrio, Type B. 131

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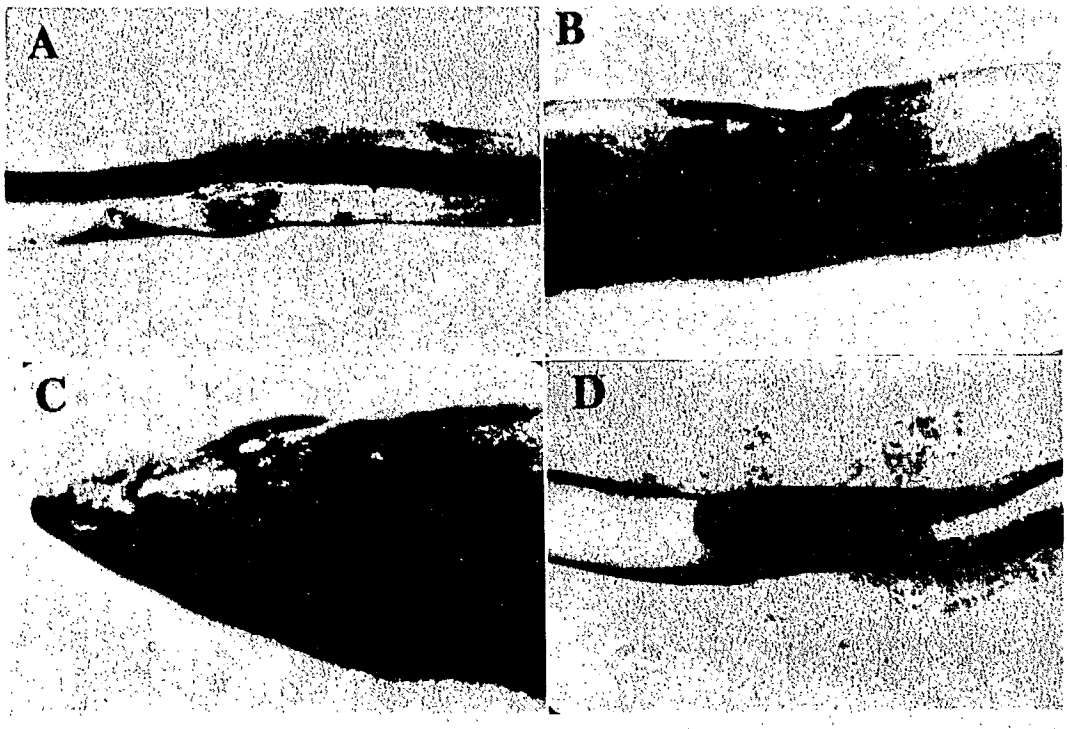

Fig. 1. External signs of diseased eels due to the present vibrio.
A. Swelling surrounded by zone of hemorrhage in the dorso-lateral area of the body.
B. Putrefactive ulcer lesion on the body surface.
C. Red coloration of the head part.
D. Congestion of the anal fin and dilatation of the anus.

Table 2. Relationship of the agglutinogens of eel isolate KV-1, *Escherichia coli* WP2, and *Pseudomonas putida* ATCC 12633

Antiserum against	Adsorbed with organism	Agglutinin titer with the formalin-killed cells of organism		
		Vibrio sp. KV-1	*Escherichia coli* WP2	*Pseudomonas putida* ATCC 12633
KV-1	Unadsorbed	1024	32	16
	Vibrio sp. KV-1	<2	32	16
	E. coli WP2	1024	<2	4

Table 3. Results of simplified identification tests of eel isolates

Item	Eel isolates identified as				
	Present vibrio 24 strains	*Vibrio anguillarum* 7 strains	*Aeromonas hydrophila* 11 strains	*Edwardsiella tarda* 18 strains	*Pseudomonas anguilliseptica* 20 strains
Step 1 (Conventional tests for differentiation)					
Gram-negative short rod	+	+	+	+	+
Motility (Wet mount)	+ (L)	+ (L)	+ (L)	+ (W)	+ (L)
O–F test (WALTERS & PLUMB's medium*1)	F	F	F	F	—*2
Gas from glucose	—	—	+	+	—
Cytochrome oxidase test	+	+	+	—	+
Sensitivity to O/129	+	+	—	—	
Step 2 (API 20E system*3)					
ONPG (= Beta-galactosidase)	d (.96)	+	d (91)	—	—
ADH (= Arginine dihydrolase)	—	+	+	—	d (.35)
LDC (= Lysine decarboxylase)	+	—	—	+	—
ODC (= Ornithine decarboxyl)	—	—	—	+	—
CIT (= Citrate utilization)	d (.92)	+	+	+	+
H_2S (= Hydrogen sulfide production)	—	—	—	d (.50)	—
URE (= Urease)	d (.04)	—	—	—	—
TDA (= Tryptophane deaminase)	—	—	—	—	—
IND (= Indole production)	—	+	+	+	—
VP (= Voges-Proskauer reaction)	—	+	+	—	+
GEL (= Gelatinase)	+	+	+	—	d (.20)
GLU (= Glucose utilization)	+	+	+	+	—
MAN (= Mannitol util.)	—	+	+	—	—
INO (= Inositol util.)	—	d (.14)	—	—	—
SOR (= Sorbitol util.)	—	d (.86)	d (.18)	—	—
RHA (= Rhamnose util.)	—	—	—	—	—
SAC (= Sucrose util.)	—	+	+	—	—
MEL (= Melibiose util.)	—	—	d (.09)	—	—
AMY (= Amygdalin util.)	+	d (.42)	d (.45)	—	—
ARA (= Arabinose util.)	—	+	d (.09)	—	—
OXI*4 (= Cytochrome oxidase)	+	+	+	—	+
NO_2 (= Nitrate reduction)	+	d (.56)	+	+	—
GAS (= ditto)	—	d (.14)	—	—	—

+: all strains positive, —: all strains negative, d ( ): either positive or negative (frequency of occurrence of positive reaction), (L): linear, (W): wiggling, F: fermentative.

*1 WALTERS and PLUMB (1978).

*2 Glucose not utilized.

*3 Judged after 48 hrs of incubation at 25°C.

*4 Same as cytochrome oxidase test.

Table 4. Results of rapid slide agglutination tests against various eel pathogens with rabbit anti-ET-7617 serum

Bacterial antigen		Reaction with rabbit anti-ET-7617 serum	
Species	No. of strains tested	Neat	Diluted 1:5
Present vibrio	24	+	+
Vibrio anguillarum	7	—	—
Aeromonas hydrophila	11	d (.09)	—
Edwardsiella tarda	18	—	—
Pseudomonas anguilliseptica	20	—	—

+: all strains positive, —: all strains negative, d ( ): either positive or negative (frequency of occurrence of positive reaction).

Table 1. Results of rapid slide agglutination tests against various organisms with rabbit anti-ET–517, KV–1, and ET–7617 sera

Bacterial antigen			Antiserum against								
			ET–517			KV–1			ET–7617		
Species	Strain no.	Source	Neat	Diluted 1:5	Diluted 1:10	Neat	Diluted 1:5	Diluted 1:10	Neat	Diluted 1:5	Diluted 1:10
	ET–517	Eel	+	+	+	+	+	+	+	+	+
	ET–519	do.	+	+	–	+	+	+	+	+	–
	ET–7605	do.	+	+	+	+	+	–	+	+	–
	ET–7606	do.	+	+	+	+	+	+	+	+	+
	ET–7607	do.	+	+	+	+	+	–	+	+	+
	ET–7616	do.	+	+	+	+	+	+	+	+	+
	ET–7617	do.	+	+	+	+	+	+	+	+	+
Vibrio sp. isolated from diseased eels	ET–7618	do.	+	+	+	+	+	–	+	+	+
	ET–7619	do.	+	+	+	+	+	+	+	+	+
	ET–7717	do.	+	+	+	+	+	+	+	+	+
	KV–1	do.	+	+	+	+	+	+	+	+	+
	KV–2	do.	+	+	+	+	+	+	+	+	+
	KV–3	do.	+	+	+	+	+	+	+	+	+
	ES–7601	do.	+	+	+	+	+	+	+	+	+
	ES–7602	do.	+	+	+	+	+	+	+	+	+
	HM 1–1	do.	+	+	–	+	+	+	+	+	+
	HM 1–3	do.	+	+	+	+	+	+	+	+	+
	ATCC 19264	Cod	–	–	–	–	–	–	–	–	–
	LS–174	Coho salmon	–	–	–	–	–	–	–	–	–
	V1669A	do.	–	–	–	–	–	–	–	–	–
	MSC–275	do.	–	–	–	–	–	–	–	–	–
V. anguillarum	PB–15	Ayu	–	–	–	–	–	–	–	–	–
	PT–7601	do.	–	–	–	–	–	–	–	–	–
	ET–506	Eel	–	–	–	–	–	–	–	–	–
	ET–208	do.	–	–	–	–	–	–	–	–	–
	HT–7601	Yellowtail	–	–	–	–	–	–	–	–	–
V. fischeri	ATCC 7744	Dead squid	–	–	–	–	–	–	–	–	–
	ATCC 15382	Cod	+	–	–	+	–	–	+	–	–
V. parahaemolyticus	ATCC 17802	Shirasu food poisoning	–	–	–	–	–	–	–	–	–
V. alginolyticus	V374	Marine sample	–	–	–	–	–	–	–	–	–
V. cholerae	NIH35A3	Outbreak of cholera	–	–	–	–	–	–	–	–	–
Vibrio sp.*1	K–3	Ayu	–	–	–	–	–	–	–	–	–
Vibrio sp.*2	RF–4	Rainbow trout	–	–	–	–	–	–	–	–	–
	RST–1	do.	–	–	–	–	–	–	–	–	–
	RF–7602	do.	–	–	–	–	–	–	–	–	–
Aeromonas salmonicida subsp. *salmonicida*	NCMB 1102	Salmon	–	–	–	–	–	–	–	–	–
subsp. *masoucida*	NCMB 2020	Japanese salmon	–	–	–	–	–	–	–	–	–
A. hydrophila	ET–2	Eel	+	–	–	+	–	–	+	–	–
	ET–4	do.	–	–	–	–	–	–	–	–	–
Edwardsiella tarda	ET–78039	Eel	–	–	–	–	–	–	–	–	–
	ET–78043	do.	–	–	–	–	–	–	–	–	–
	ET–7601	Eel	–	–	–	–	–	–	–	–	–
Pseudomonas anguilliseptica	ET–2	do.	–	–	–	–	–	–	–	–	–
	NE–2	do.	–	–	–	–	–	–	–	–	–
	ET–7413	do.	–	–	–	–	–	–	–	–	–
P. fluoresens	ATCC 13525	—	–	–	–	–	–	–	–	–	–
P. putida	ATCC 12633	—	+	+	–	+	+	–	–	–	–
Pasteurella piscicida	K–1	Yellowtail	–	–	–	–	–	–	–	–	–
Escherichia coli	WP2	—	+	+	+	+	+	+	–	–	–

*1 KUSUDA (1965).

*2 OHNISHI and MUROGA (1976, 1977).