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Isolation and Characterization of Bacterial Pathogens from Eels (*Anguilla japonica*) Cultured in Korea.

Hah, Yung-Chil*, Soon-Woo Hong*, Hee-Bok Oh*,
John L. Fryer and John S. Rohovec****
(* Dept of Microbiology, Seoul National University)
(** Dept of Microbiology, Oregon State University, Corvallis, Oregon 97331)

養殖 뱀장어(*Anguilla japonica*)에 感染하는 細菌의 分離同定 및 그 病原性에 관하여

하영칠* · 홍순우* · 오희복* J.L. Fryer,** J.S. Rohovec**
(*서울대 미생물학과, **오레곤 주립대 미생물학과)

ABSTRACT

Twenty two cultures of pathogenic bacteria were isolated from cultured eels (*Anguilla japonica*) from Asan Hatchery. The bacteria were characterized by their biochemical properties, serological relationships, infectivity to gold fish and susceptibility to various antimicrobial compounds. Fourteen of 22(64%) cultures were identified as *Edwardsiella tarda*, five (23%) as *Aeromonas hydrophila* and three (14%) as *Vibro anguillarum*. *Edwardsiella tarda* isolates proved to be the main cause of the disease in cultured eels. They were serologically homogeneous and their virulency to gold fish was higher than any of the other groups of bacteria tested. The virulence of 3 isolates were low in gold fish exposed to the bacteria by the waterborn route. Ten strains were tested for their susceptibility to 12 antimicrobial compounds and were resistant to from one to six drugs: in particular, tetracycline derivatives and sulfisoxazole.

INTRODUCTION

Since Bergman (1909) first described *Vibrio anguillarum* to be the cause of the 'red pest' disease of eels from the Baltic Sea, over 20 species of pathogenic bacteria have been reported in connection with diseases of marine and freshwater fish. The presence of disease has emerged as a major limiting factor in the development of commercial aquaculture in many part of the world.

From August to October 1983, investigations were made on the bacterial disease of cultured eels at the Asan Hatchery. Though the mean mortality was only 3%, the eels were suffering some kinds of bacterial infection. The diseased fish examined exhibited several clinical signs: externally, numerous diffuse hemorrhages were present in the skin, particularly on the vental surface and around the base of the fins. One to several surface lesions were also observed in many fish. Internally, the kidney was typically enlarged in some fish.

The purpose of the present work was to provide information for the etiology and pathology of bacterial diseases among populations of cultured fish. This paper documents the first isolation of *Edwardsiella tarda*, *Vibrio anguillarum* and *Aeromonas hydrophila* from cultured eels (*Anguilla japonica*) in Korea.

MATERIALS AND METHODS

Sources of Bacteria

* 본 논문은 한국과학재단의 연구비에 의해 연구된 것 임.

Cultures of pathogenic bacteria were isolated from diseased eels. Fish at each location were examined bacterial isolates cultured by streaking kidney tissue on brain heart infusion Agar (BHIA: Difco) and incubating at 20°C for up to one week.

Reference strains of *E.tarda* and *V.anguillarum* LS-174 and *V.ordalli* MSC-275 were used for comparison tests of biochemical and serological properties.

Characterization

The isolates were characterized by morphological, biochemical and serological properties. Cell morphology and motility were determined by microscopic examination of Gram stains and wet mounts of 24 hr broth cultures incubated at 20°C. Colony appearance on BHI agar was examined after 3 to 4 days of incubation at 20°C.

The requirement for NaCl was tested by incubating one loopful of 24 hr broth culture in nutrient broth with and without the addition of NaCl(w/v). Growth at different incubation temperature were determined by inoculating one loopful of 24 hr broth cultures into BHI broth (BHIB:Difco) and each tube was incubated at selected temperatures for one week.

Gelatin hydrolysis was carried out by growing cultures in BHIB with gelatin-charcoal disk.

Biochemical tests were performed by the methods outlined in the Manual of Methods for General Bacteriology (2). Certain of the biochemical test were confirmed by inoculating API 20 E.

Infectivity

To define the virulence of primary isolates, laboratory tests were conducted using the gold fish as test animal. Fish weighing 3-5g were injected intraperitoneally with 0.1ml of inocula. The inocula was prepared by suspending bacterial cell from 24 hr culture grown on BHIA. Cells from each strain were suspended 0.85% NaCl solution. Bacterial concentration of the inocula was adjusted to about 10^9 cell/ml. The test fish were placed in static aquariums at 15-20°C, and observed for two weeks. The strains that do not kill any fish nor show any signs of disease were classified as avirulent and excluded from further study. The strains of high virulence were further tested and LD_{50} values were determined for the gold fish.

To estimate LD_{50} values a series of ten fold dilutions of bacterial suspension were prepared ranging 10^4 through 10^8 cells/ml. The number of bacterial cells in the inocula was measured by the standard plate count method. Five gold fish weighing 3-5g were injected intraperitoneally with 0.1ml of each dilution and observed for one week. Water temperature was maintained at 15-18°C during the tests.

The invasiveness of organisms to gold fish was tested by waterborne exposure. Gold fish held in aquariums at 15°C were exposed to cultures by the waterborne route. Bacteria grown in BHIB at 20°C were added directly to the aquaria to give final concentrations ranging from about 10^6 to 10^9 cells per ml of aquarium water. After a 15min exposure, fish were transfered to aquaria with fish pathogen free fresh water and observed up to 2 weeks.

Susceptibility to Antimicrobial Compounds

The reaction to antibiotics was determined by placing sensi-discs of the antibiotics (BBL) on Müeller-Hinton Agar plates streaked with the test organism. Ten strains were tested for susceptibility to oxytetracycline (30μg), tetracycline (30μg), streptomycin (10μg), novobiocin(5μg), chloramphenicol(30μg), neomycine(30μg), nalidixic acid(30μg), methicillin(5μg), nitrofurantoin(300μg), erythromycin(15μg), kanamycin (30μg) and sulfisoxazole(2μg).

Serological Analysis

Immune sera were obtained from rabbits injected intraveneously with saline-washed suspensions of cells in successive doses of 0.5, 1.0, 1.5, and 2.0ml every fourth day. The rabbits

were led from the heart 1 week after the last injection. A concentration of 1 : 5,000 sodium azide was added to each sera and stored in −70°C. The antigen consisted of a suspension of saline-washed cells which had been heated in boiling water for 2hr to destroy the flagella and adjusted to final concentration of 10^9 cells/ml.

The serological relationships among isolates and a reference strain were examined by the slide agglutination procedure including control tests with saline and normal rabbit serum. Adsorbed antisera were prepared by mixing equal amounts of a dense suspension of heterogenous antigen which cross-reacted with known antiserum. The mixed suspension was incubated at 50°C for 30 min. and for 5hr at 4°C. The antigen and adsorbed antibody were removed by centrifugation (3,000xg for 10 min).

RESULTS

Isolation of Bacteria

Twenty-two isolates of pathogenic bacteria were obtained from the examination of 46 eels. They were composed of 14 *E.tarda* (64%), 3 *V.anguillarum* (14%), and 5 *A. hydrophilla* (23%). One fish was infected with each of the three Gram negative bacteria.

Seven fish infected with *E.tarda* exhibited severe diffuse hemorrhage on the ventral body surface and the lower part of vent were swollen with hemorrhages as a consequence of enlarged kidney. One to several lesions on the body surface without signs of hemorrhages were observed in remaining 7 fish infected with *E. tarda*. *Edwardsiella tarda* was isolated in pure culture and was the dominant colony type from fish with clinical signs of disease. This suggests that hemorrhages are formed in acute or sub-acute case, and in chronic case non-hemorrhagic lesions are observed.

Characterization

Edwardsiella tarda was slow growing and producing colonies less than 1mm in diameter after 48hr at 20°C on BHIA. They were yellowish in color, raised and had entire edge. Biochemical properties of the *E. tarda* isolates were identical to one another and to reference strain except the production of acid from mannitol (Table 1). They were motile by flagella, cytochrome oxidase negative, fermented glucose and produced H_2S in Triple Sugar Iron Agar (TSIA) slants.

Organisms of *V.anguillarum* were small Gram negative rods displaying a slight curvature and motile by a single polar flagellum. Colonies of *V.anguillarum* on BHIA were 1~3mm in diameter, translucent, slightly raised and mucoid. They were cytochrome oxidase positive and sensitive to 0—129 and novobiocin (5μg). Three isolates were identical in their biochemical properties to one another. Detailed reactions and properties of the *V.anguillarum* isolates are presented in Table 1. The organism was unable to ferment sucrose and mannitol and to produce indole. Nybelin subdivided *V.anguillarum* into two types (18). Type A, var. typica, produced acid but no gas from sucrose and mannitol and produced indole. Type B, var. anguillicida, was differentiated by its inability to ferment these sugars and to produce indole. The vibrios isolated in this study seem to be related to Type B, var. anguillicida.

A total of 5 cultures were assigned to the motile Aeromonads based on the following criteria; Gram negative rod, motile, fermenation of glucose, cytochrome oxidase neagtive, resistance to 0—129 and lysine decarboxylase negative. Further characters of these strains are given in Table 1. Five isolates of motile Aeromonads consisted of 3 different biochemical types, showing remarkable discrepancy in their salt tolerance, urease activity, ability to hydrolyse starch, tween 80 and to ferment certain sugars. The biochemical characters of type I were identical to ideal phenotype of *A. hydrophila* biovar.

Table 1. Cultural and physiological properties of isolates obtained from diseased eels at Asan Hatchery.

Characteristics	Group of isolates					Reference strian
	Aeromonas hydrophila			*Vibrio anguillarum* (3)	*Edwardsiella tarda* (14)	*Edwardsiella tarda*
	Type I(1)*	Type II(2)	Type III(2)			
Motility	+	+	+	+	+	+
Oxidase	+	+	+	+	−	−
O/F	F	F	F	F	F	F
Vibriostat(0-129)	−	−	−	+	NT	NT
Novobiocin(5ug)	R	R	R	S	NT	NT
Growth at 37°C	+	+	+	+	+	+
〃 42°C	+	+	+	+	+	+
Growth in 0% NaCl	+	+	+	+	+	+
〃 6% NaCl	−	−	+	−	−	−
〃 pH 9.0	+	+	+	+	−	NT
Gas from glucose	+	+	+	−	+	+
Catalase	+	+	+	+	+	+
Growth in KCN	+	+	+	NT	−	−
Nitrate reduction	+	+	+	+	+	+
Methylred reaction	+	+	+	−	+	+
Voges-Proskauer	+	+	+	−	−	−
Indole production	−	−	−	−	+	+
Citrate, Simmon's	+	+	+	−	−	−
Gelatin liquifaction	+	+	−	−	−	−
Casein hydrolysis	+	+	+	−	−	NT
Starch hydrolysis	+	+	−	+	−	NT
Aesculin hydrolysis	+	−	−	−	−	−
Urease	−	−	+	+	−	−
Phenylalanine	−	−	−	−	−	−
H_2S from TSI	−	−	−	−	+	+
Growth on McConky	+	+	+	+	+	+
Arginine hydrolysis	+	+	−	+	−	−
Lysine decarboxylase	−	−	−	−	+	+
Ornithin decarboxylase	+	+	−	−	+	+
β-galactosidase	+	+	−	+	−	−
Lipase (Tween 80)	+	+	−	NT	−	−
Acid from glucose	+	+	+	+	+	+
〃 inositol	−	−	−	−	−	−
〃 lactose	−	−	+	−	−	−
〃 sucrose	−	+	+	−	−	−
〃 maltose	+	+	+	+	+	NT
〃 mannitol	+	+	+	+	+	+
〃 mannose	+	+	+	−	−	NT
〃 trehalose	+	+	+	+	+	NT
〃 adonitol	−	−	−	−	−	−
〃 salicin	+	−	−	−	−	−
〃 arabinose	+	−	+	−	−	−
〃 raffinose	−	+	+	−	−	−
Growth on 4% selenite	NT	NT	NT	NT	+	+

*( )number of isolates.

hydrophila. The remaining two types were also identified as *A. hydrophila.*

Infectivity

Edwardsiella tarda, *V. anguillaum* and A. hydrophila isolated from eels were pathogenic for experimental gold fish when injected intraperitoneally. The 50% lethal dose of each of the isolates for gold fish were determined and are shown in Table 2. The LD_{50} for E. tarda was 6.73×10^5, which is 5 to 50 times higher than those of *V. anguillarum* and *A. hydrophila*. There was no significant differences in invasiveness of between the three species of test organisms for experimental fish by waterborne exposure. The mortalities and minimum concentration for waterborne infection are presented in Table 3.

Susceptibility to Antimicrobial Compounds

Results of the antimicrobial sensitivity of 3 *E.tarda*, 2 *V. anguillarum* and 5 isolates represen ting three types of *A. hydrophila* are shown in Table 4. *Edwardsiella tarda* and *V. anguillarum* isolates each showed identical response to the antibiotics tested, whereas the susceptibility of *A. hydrophila* isolates were variable. *Vibro anguillarum* was characteristically sensitive to Novobiocin. *Edwardsiella tarda* isolates were resistant to 6 antibiotics, or half of those tested. All of the isolates from eel except *V. anguillarum* were resistant to tetracycline derivatives and sulfisoxazole. Eight isolates (80%) of cultures tested, were resistant to erythromycin.

Table 2. The LD_{50} of bacterial isolates obtained from diseased eels determined in gold fish[a]

Identified species	LD_{50}(CFU)
Edwardsiella tarda	6.73×10^5
Vibrio anguillarum	3.88×10^6
Aeromonas hydrophila	2.86×10^7

a: average weight; 4.1g

Table 3. Invasiveness of bacterial isolates obtained from diseased eels and tested with gold fish[a]

species strain	mortality (%) within 2 weeks	minimum concentration for invasiveness (CFU/ml of water)
Edwardsiella tarda	0 to 60	1.97×10^6
Vibrio anguillarum	0 to 20	2.85×10^7
Aeromonas hydrophila	0 to 33	3.11×10^6

a: average weight; 3.9g

Serological Analysis

Table 4. Susceptibility of of eel isolates to selected antimicrobial compounds in agar diffusion method.*

Drugs	Conc. (ug)	*V. anguillarum*		*A. hydrophila*			*E. tarda*		
		03−E18S	03−E17L2	Type 1	Type 2	Type 3	02− E1	02−E7	03−E1
Oxytetracycline	30	S	S	R	R	R	R	R	R
Tetracycline	30	S	S	I	R	R	R	R	R
Streptomycin	10	S	S	S	S	S	S	S	S
Novobiocin	5	S	S	R	R	R	R	S	R
Chloramphenicol	30	S	S	R	S	S	S	S	S
Neomycin	30	S	S	S	S	S	S	S	S
Nalidixic acid	30	S	S	S	S	I	S	S	S
Methicillin	5	R	R	R	R	R	R	R	R
Nitrofurantoin	300	S	S	S	S	R	S	S	R
Erythromycin	15	R	I	R	S	S	R	R	R
Kanamycin	30	S	S	S	S	S	S	S	S
Sulfisoxazole	2	S	S	R	R	R	R	R	R

S: sensitive R: resistant I: intermediate

*: Müeller-Hinton Agar is used for diffusion method

Antisera against 2 *E. tarda* isolates and the reference strain *E. tarda* were made from rabbits. All 14 isolates agglutinated with 2 of the immunesera without any difference in their activity. Agglutination adsorption studies were carried out in order to further examine the antigenic relationship among 14 *E. tarda* isolates. Each of two antisera was adsorbed with the remaining heterogenous strains. When these adsorbed sera were tested again against each homologous antigen, both of the homologous strains failed to produce agglutination. We could find no heterogeneity in any of the serological properties among *E. tarda* isolates. The reference strain of *E. tarda*, however, was not agglutinated with prepared immune sera against 2 isolates from eels and none of the isolates was agglutinated with specific antisera for reference strain. It is concluded that there is no serological relationship between reference strain of E. tarda and isolates obtained from eels.

DISCUSSION

The bacteria isolated from kidney of diseased eels were identified as *E. tarda*, *V. anguillarum* and *A. hydro*-phila by their biochemical nature. Agglutination reaction revealed that there was no serological relationship between *E. tarda* isolates and the reference strain used. *E. tarda* was classified into 4 serogroups by Park et al. (4). In order to define the serological nature of the eel isolates, comparision study with known types of *E. tarda* is needed.

Edwardsiella tarda has been previously reported to be pathogenic to eels (4), rainbow trout and chinook salmon (1). And there were numerous reports concerning the isolation of *V. anguillarum* from various kinds of marine and certain freshwater fish throughout the world. Though bacterial pathogens may cause catastrophic mortalities, very little attention has been given to the isolation and characterization of bacterial fish pathogen for the control of bacterial diseases in Korea. In this study, *E. tarda*, *V. anguillarum* and *A. hydrophila* were isolated and characterized for the first time from eesl cultured in Korea.

In spite of numerous reports of vibriosis, the basis for the pathology of the organism is poorly understood. It has been noted that *V. anguillarum* can be devided into at least 3 serogroups, and their relatedness to pathogenecity discussed (18). Determination of the serotype and pathology of the organism isolated in this work is the subject of additional research.

Though high numbers of cells were required to produce 50% lethal effect, *E. tarda* was the most virulent of the strains tested. The high ratio of isolation (64%) and LD_{50} dose suggest *E. tarda* was dominant and a major representative pathogen in the eel farm investigated.

The mortalities observed after waterborne exposure of gold fish to dilutions of test organisms were not linear enough to estimate 50% lethal dose by waterborne route of infection. It suggests that all the test organisms were only slightly invasive under these experimental condition.

All of the isolates tested were resistant to at least one of six drugs. Appearance of a *A. hydrophila* strain resistant to chloramphenicol is interesting since this drug was often used for the treatment of bacterial diseases in the eels of the farm investigated.

적 요

아산 양어장에서 양식되고 있는 뱀장어(*Anguilla japonica*)로 부터 22주의 병원성 세균을 분리하여 그 생화학적 성질, 혈청학적관계, 금붕어에 대한 감염성 및 여러 항생물질에 대한 감수성을 시험하였다.

22균주중 14주 (64%)는 *Edwardsiella tarda*, 5주 (23%)는 *Aeromonas hydrophila*, 기타 3균주 (14%)는 *Vibrio anguillarum*으로 동정되었다.

3가지 분리균중에서 *E. tarda*는 동일한 혈청형으로 그 분리율 및 금붕어에 대한 감염성이 가장 높았다. 이에 따라 *E. tarda*가 조사된 양어장의 뱀장어에서 발생하는 세균성 질병의 주 병원체로 판명 되었다.

세 분리균주의 균액을 수조에 풀어 금붕어에 접촉시켰을 때 그 감염율은 세 균주 모두 상대적으로 낮았다.

10주의 분리균을 택해 12가지 항생물질에 대해 감수성 검사를 한 결과 하나에서 여섯가지 약제에 대해 저항성을 나타냈으며, 그 중 tetracycline 유도체와 sulfisoxazole에 대한 저항성이 현저하였다.

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