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Studies on Epizootiology and Pathogenicity of *Staphylococcus epidermidis* in Tilapia (*Oreochromis* spp.) Cultured in Taiwan

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Shih-Ling Huang, Wei-Cheng Chen, Mei-Chuan Shei, I-Chiu Liao and Shiu-Nan Chen (1999) Studies on epizootiology and pathogenicity of *Staphylococcus epidermidis* in Tilapia (*Oreochromis* spp.) cultured in Taiwan. *Zoological Studies* **38**(2): 178-188. This paper describes the epizootiology of a disease of tilapia with clinical signs that include white nodules and microscopic granulomatous formations. Diseased fish showed splenomegaly with diffusion of numerous white nodules. The most severe lesions were presented in the spleen and anterior kidney. Morphological, biological, and biochemical characteristics of microorganisms isolated from the diseased tilapia were examined and classified by Baird-Parker's biochemical subgrouping scheme. Strain LK0728 identified as *Staphylococcus epidermidis*, was used in a challenge test. Histopathological changes were similar to those seen in naturally infected fish. This is the 1st report on the isolation of staphylococci pathogenic to tilapia and on the confirms the etiological agent causing mass mortality in tilapia from 1992 to 1996 in Taiwan.

Key words: Granuloma, *Staphylococcus epidermidis*, Pathogenicity, Electron-dense particles.

Since 1992, mass mortalities of unknown etiology have occurred in pond-cultured tilapia in Taiwan, and have resulted in significant economic losses. Diseased tilapia were first observed in freshwater ponds in eastern and southern Taiwan, and have since spread all over the island area, in freshwater, brackish water, and seawater ponds. Although most of the moribund fish were normal in appearance, the internal organs such as spleen or anterior kidney contained numerous nodules. Several introduced bacterial pathogens have introduced severe disease in tilapia, including Gram-positive bacteria, *Streptococcus* sp. (Miyazaki et al. 1984); Gram-negative bacteria, i.e., *Aeromonas hydrophila* (Amin et al. 1985, Leung et al. 1994), *Pseudomonas fluorescens* (Miyazaki et al. 1984), *Edwardsiella tarda* (Plumb and Sanchez 1983, Kaigge et al. 1986), and *Vibrio vulnificus* (Sakata and Hattori 1988); and a Rickettsia-like microorganism (RLO) (Chern and Chao 1994). To identify pathogens, isolation and histopathology are routinely used to examine diseased fish. In the present studies, during 1992 to 1996, in more than 60% of cases, *Staphylococcus epidermidis* was the dominant pathogenic species isolated from the tilapia. It is therefore considered necessary to investigate the pathogenicity of this bacteria.

S. epidermidis was first reported as a fish pathogen by Kusuda and Sugiyama in 1981. A severe epizootic caused by this organism occurred in farmed yellowtail (*Seriola quinquiradiata*) and red sea bream (*Chrysophrus major*) in Japan from July 1976 to Sept. 1977 (Kusuda and Sugiyama 1981, Sugiyama and Kusuda 1981). Typical signs of the disease consisted of exophthalmia, congestion, and ulcerations on the tail.

In the present study, we attempt to describe the isolation and characterization of the bacteria from moribund tilapia. A challenge test was also performed as a confirmation of pathogenicity against experimental fish. This is the 1st report on the isolation and confirmation of *Staphylococci* pathogenic to tilapia.

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MATERIAL AND METHODS

Sampling

From 1992 to 1996, mass mortalities in pond-cultured tilapia occurred annually. Our observations showed that the disease was species specific, it occurred in all species of tilapia or its hybrids but not in other fish polycultured with tilapia. The disease was also observed in tilapia cultured in seawater, brackish-water or freshwater ponds. Surveys were performed during 1992 to 1996 on severe epizootics involving granulomatous formation occurring in monocultured and polycultured ponds of tilapia in Taiwan. Most tilapia samples with clinical signs were obtained from fish farms where mass mortality had occurred.

Investigations of the potential pathogens, bacteria, and other pathogens, including a rickettsia-like organism and viruses in the diseased tilapia, were as described below.

Detection of viruses and rickettsia-like organisms

To detect viruses or rickettsia-like organisms, internal organs including spleen, anterior kidney, and other organs from diseased tilapia were processed for light and transmission electron microscopic examination using methods described by Fryer et al. (1990), Rodger and Drinan (1993), and Wen et al. (1994).

Isolation and identification of bacteria

Internal organs including spleen, anterior kidney, trunk kidney, liver, skeletal muscle, heart, brain, stomach, intestine, mesentery, gonad, and eye from moribund fish were inoculated aseptically onto different culture media. Brain heart infusion agar (BHIA, Difco), brain heart infusion broth (BHIB, Difco), Tryptone soy agar (TSA, Difco), Columbia agar (CA, Difco), and MacConkey agar (Difco) were used for the isolation of bacteria. Inoculated media were incubated at 28 °C for 7 d. Bacterial colonies were obtained by streaking and re-streaking onto fresh media as described above, and identified by using morphological, physiological, and biochemical tests as described by Evans and Niven (1950), Baird-Parker (1963 1965), Schleifer and Kloos (1975), and Schleifer (1986).

The strain LK0728 was originally isolated in July 1993 from the spleen of diseased tilapia in Lukang, Changhua, located in west-central Taiwan. The biological characteristics were determined and challenge tests of strain LK0728 were performed in this study. Other bacterial strains from different sources of tilapia in cultured ponds, including 2 strains from Lukang (LK1205 and LK1221), one from Hualien (HL0106), and one from Kaoshiung (KH0110) were also used in this study. *Staphylococcus epidermidis* CCRC 10783 was purchased from CCRC (Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan) as the standard strain.

Antimicrobial susceptibility

Sensitivity of bacteria against antimicrobial agents was determined by using disk diffusion on Muller-Hinton agar. Dosages of antimicrobial agents were as follows: amikacin (30 μg), ampicillin (10 μg), bacitracin (0.04 units or 10 units), cephalosporin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), furazolidone (100 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (2 μg), lysostaphin (10 units), methicillin (5 μg), neomycin (5 μg), novobiocin (5 μg or 30 μg), oxacillin (1 μg), oxytetracycline (30 μg), penicillin G (10 units), polymyxin (300 units), streptomycin (10 μg), sulfadiazine (300 μg), and tetracycline (30 μg), respectively (Koneman et al. 1988).

Histopathological observations

Light microscopy

Tissue samples from gills, spleen, anterior kidney, trunk kidney, liver, skeletal muscle, heart, brain, stomach, intestine, mesentery, gonad, eye, fin, and gas bladder from moribund and apparently healthy fish were removed and fixed in 10% neutral buffered formalin and processed for sectioning as described by Humason (1979). Sections (4-6 μm) were stained using haematoxylin and eosin (H and E) and Giemsa's stain techniques, respectively. All samples were then studied under a light microscope (Olympus BH-2) (Ribelin and Migaki 1975).

Semi-thin sections, 1 μm thick, were stained with toluidine blue and then processed for light microscopic observations.

Transmission electron microscopy (TEM)

Tissues, including the kidney and spleen collected from naturally and experimentally infected tilapia, were processed for TEM observations. Tissue blocks were minced at a thickness of 0.5-1 mm, fixed in 2.5% glutaraldehyde for 2 h and post-fixed with 1% osmium tetraoxide for 1 h, at 4 °C. The sections were then dehydrated in a graded series of

ethanol and absolute acetone, and embedded in Spurr's resin (Spurr 1969). Ultra-thin sections were cut with an ultramicrotome (Sorvall MT 5000) and stained with uranyl aetate and lead citrate according to procedures described by Frasca and Parks (1965). The sections were then examined by using a Hitachi H-600 electron microscope.

Preparation for negative staining

In preparation for negative staining, a drop of bacterial suspension was placed onto Formvar-carbon-coated copper grid and stained for 5-10 s using 2% phosphotungstic acid (pH 7.0). The section was then observed by using a Hitachi H-600 electron microscope at different magnifications.

Experimental animals

Healthy experimental tilapia obtained from a tilapia farm located at the Taiwan Fisheries Research Institute, Lukang Station, were kept in 1000-liter tanks with aeration for 1 wk prior to the experiment. During the course of the experiment, fish were fed commercial tilapia food.

Collection of bacteria and its supernatant

Collection of bacteria was performed by using 6-cm discs of dialysis membrane (molecular weight cut-off of 6000~8000, Spectra/Por 1) overlaid on BHIA and by spreading 0.2-0.3 ml of overnight-cultured broth with a sterilized glass rod. After a 24-h incubation at 30 °C, the bacteria were collected by washing the dialysis membrane in 5 ml saline.

The bacterial suspension was adjusted to 1.34×10^9 cfu/ml by plate counting methods as described by Messer (1979). The suspension was then diluted into 1.34×10^3, 1.34×10^4, 1.34×10^5, 1.34×10^6, 1.34×10^7, or 1.34×10^9 cfu/ml with normal saline, respectively.

Bacterial supernatant was obtained by adjusting the bacterial suspension to a density of 3.24×10^9 cfu/ml and centrifuging the suspension at 5000 rpm for 20 min at 4 °C (Hitachi 05PR22). The supernatant was collected by filter-sterilization using a 0.22-μm filter.

Challenge

The challenge tests for healthy fish (*Oreochromis aureus*) against LK0728, HL0106, KH0110, LK1205, and LK1221 were performed using 8 groups of fish. Each group consisted of 10 fish weighing 60-70 g reared in an aquarium with a water temperature of 28 °C. Experimental fish were injected intraperitoneally (i.p.) with 0.5 ml of a bacterial suspension at a density ranging from 1.34×10^3 to 1.34×10^9 cfu/ml, respectively. Fish of the supernatant group were injected i.p. with 0.5 ml of filter-sterilized supernatant, and the control fish were similarly injected i.p. with 0.5 ml of saline.

Bacterial isolation was also performed with moribund fish of each group. The predominant bacterial growth was identified in accordance with morphological, physiological, and biochemical characteristics.

RESULTS

An epidemiological survey was performed in the present study. Totally 159 cases were examined using biological techniques. Cumulative cases of bacterial pathogen number 143 (90%), including 100 cases of *S. epidermidis* and 43 cases of Gram-negative bacteria. About 16 cases (10%, including 9 cases of mixed infection with *S. epidermidis*) belonged to a suspicious rickettsia-like organism. In particular, no case related to rickettsia-like organisms was observed during 1995-1996. This survey showed that *S. epidermidis* was the dominant species found in moribund tilapia. It is suggested that the mass mortality of cultured tilapia in Taiwan were caused by *S. epidermidis*.

Clinical symptoms

Clinical symptoms observed from the tilapia challenged with strain LK0728 were similar to those found in naturally infected pond-cultured tilapia. Clinical signs and gross pathology related to this disease varied. The majority of diseased fish showed no external clinical signs or abnormalities and only a few cases revealed exophthalmia, lesions in the epidermis and fins, or distended abdomen containing ascites fluid.

Moribund fish showed lethargic and abnormal whirling swimming activities on the water surface or at the bottom of ponds. Heavily infected fish could survive, unless the water quality had seriously deteriorated.

Internal signs

Distribution of pathological symptoms in selected tissues of infected fish is listed in table 1. Typical lesions, marked with white nodules and microscopic granulomatous formation, shown in figure

1, were observed in heavily infected fish. The most severe signs in diseased fish were spleen hypertrophy, spleen and the anterior kidney containing numerous white or yellowish nodules. Heavily infected fish also revealed ring-shaped lesion foci that can be used as an observable symptom for disease diagnosis. Similar lesions were also present in the liver, trunk kidney, gonad, stomach, intestine, and mesentery but not in the heart or brain. In addition, the gills of some severely infected fish showed irregular nodules.

Isolation of the pathogen

Gram-positive cocci could usually be isolated from fish with typical symptoms of granuloma. The bacterium was oval to spherical shaped with an average diameter of 0.65 μm. Its cells occurred predominantly in pairs or clusters producing a slime that helped the cells adhere to the agar surface. The cell was also demonstrated to be catalase and Gram positive, with no flagella or cilia and thus it was immotile.

When the bacteria was cultured in BHIA, white colonies formed. It was also found that the colonies were smooth, raised, glistening, circulars and entire. Sizes of the colonies varied from 0.1 to 1 mm in diameter on BHIA after 24 h incubation at 30 °C. With increasing culture periods or elevated temperatures, colonies became stickier.

Morphological, physiological, and biochemical characteristics of the isolated bacteria LK0728, HL0106, KH0110, LK1205, LK1221, and CCRC 10783 were also determined in this experiment, and the results are shown in tables 2, 3, and 4. The bacteria LK0728, HL0106, KH0110, LK1205, LK1221, and CCRC 10783 are coagulase-negative (bound coagulase-negative, free coagulase-negative), Latex agglutination-negative, mannitol (aerobic)-positive, and acetoin-positive. Optimal temperatures for growth ranged from 10 °C to 45 °C. The cells were able to grow in a medium containing NaCl at concentrations up to 15% (Table 2). LK0728 was able to produce acid aerobically and anaerobically from glucose, fructose, galactose, inulin, maltose, mannose, ribose, sucrose, or trehalose. In contrast, no acid was produced aerobically or anaerobically from lactose, melibiose, raffinose, rhamnose, xylose or xylitol (Table 5). The susceptibilities of LK0728, HL0106, KH0110, LK1205, and LK1221 against selected drugs were tested, and the results are listed in table 6.

Five isolates (LK0728, HL0106, KH0110, LK1205 and LK1221) possessed similar characteristics as follows: acid production from glucose under anaerobic conditions; non-resistance to lysostaphin (10 unit); production of acid from glycerol aerobically in the presence of 0.4 μg/ml erythromycin; inhibition by 100 μg furazolidone; negative to a modified oxidase test; non-inhibition by 0.04 unit bacitracin, etc. (Table 4). According to the description by Baird-Parker (1963 1965) and in Bergey's manual (Schleifer 1986), the 5 isolates were cataloged as Group 1 (*Staphylococcus* Rosenbach), subgroup VI bacterium, and classified as *S. epidermidis* (Schleifer and Kloos 1975). In addition, LK0728 also produced a positive reaction on DNase, bile esculin, starch, casein, tyrosine, urease, xanthine, egg yolk, EY tellurite, nitrate, hippurate, alkaline phosphatase, and MR tests, and negative reactions to the gelatin, tributyrin, lysine, ornithine, or ONPG tests.

Histopathological study

Histopathological observations in all suspected positive tissues, including the spleen, anterior kidney, trunk kidney, liver, gonad, mesentery, stomach, and intestine, were performed, and the results are listed in table 1. The most severe pathological reactions were noted in the spleen and anterior kidney. Focal necrosis, showing numerous diffuse granulomas with necrotic centers, was observed in spleen parenchyma and kidney interstitium (hematopoietic tissue) (Fig. 2). Focal necrotic loci spreading

Table 1. Pathogenicity in selected target tissues of tilapia infected by LK0728, *Staphylococcus epidermidis*

Tissue	Granuloma	Electron-dense particles
Brain	–[a]	+
Gill	–	–
Muscle	–	+
Liver	++	++
Spleen	+++	+++
Anterior kidney	+++	+++
Heart	–	–
Pancreas	++	++
Trunk kidney	++	++
Stomach	+	++
Intestine	+	++
Mesenterium	+	+
Gonad	+	+
Eye	–	–
Fin	–	–
Gas bladder	–	–

[a]The above observations were performed with light microscope and TEM.
Key: +++ severe; ++ moderate; + slight; – negative reaction.

Table 2. Morphological and biological characters of isolates and *Staphylococcus epidermidis* CCRC type strain

Characteristic	HL0106	LK0728	KH0110	LK1205	LK1221	CCRC10783	Bergey's manual
Cell form				spherical cocci			
Bact. diameter (μm) (avg.)	0.65	0.65	0.65	0.65	0.65	0.65	
Colony diam. on BHIA (mm)	0.5	0.5	0.6	0.5	0.6	0.5	
Colony edge on BHIA				entire			
Colony pigment	W	W	WY	W	W	W	–
Gram stain	+	+	+	+	+	+	+
Motility	–	–	–	–	–	–	–
Growth on:							
BHIA	+	+	+	+	+	+	
TSA	+	+	+	+	+	+	
MacConkey agar	–	–	–	–	–	–	
Nutrient agar	+	+	+	+	+	+	
Baird-Parker agar	+	+	+	+	+	+	
Columbia agar	+	+	+	+	+	+	
Simmon citrate	–	+	–	+	+	–	
Mannitol salt agar	+	+	+	+	+	+	
0% NaCl nutrient agar	+	+	+	+	+	+	
5% NaCl nutrient agar	+	+	+	+	+	+	
10% NaCl nutrient agar	+	+	+	+	+	+	w
15% NaCl nutrient agar	(+)	+	(+)	(+)	+	+	–
Growth at:							
10 °C (BHIA)	+	+	(+)	+	(+)	+	
15 °C (BHIA)	+	+	+	+	+	+	– w
40 °C (BHIA)	+	+	+	+	+	+	+
45 °C (BHIA)	(+)	(+)	+	(+)	+	+	

Key: W, white; WY, whitish yellow; +, positive reaction; (+), weakly positive; w, weak reaction; – , negative reaction; – w: negative to weak reaction.

Table 3. Biochemical characters of isolates and *Staphylococcus epidermidis* CCRC type strain (I)

Characteristic	HL0106	LK0728	KH0110	LK1205	LK1221	CCRC10783	Bergey's manual
Catalase	+	+	+	+	+	+	+
Modify oxidase test	–	–	–	–	–	–	–
Hugh-Leifson test	F	F	F	F	F	F	
MR	+	+	+	+	+	+	
VP	+	+	+	+	+	+	+
Hydrolysis of:							
Starch	+	+	+	(+)	–	–	
Tyrosine	+	+	(+)	+	–	–	
Xanthine	+	+	+	+	–	–	
Hippurate	+	+	+	+	(+)	+	
Bile esculin	+	+	–	–	–	–	
Tributyrin	–	–	–	+	–	+	
Gelatin	+	–	–	–	+	–	
Clearing of:							
Casein	–	+	+	–	+	–	
Egg yolk	+	+	–	–	–	–	
EY tellurite	+	+	–	–	–	–	
Decarboxylation of:							
Arginine	+	+	–	–	–	–	+w
Lysine	–	–	+	–	–	+	
Ornithine	–	–	–	–	–	–	
Nitrate	+	+	+	+	+	+	+w
TSI	A/K	A/K	A/K	A/K	A/K	A/K	
ONPG	+	–	–	–	–	–	
Hemolysine	α	α	α	α	α	α	d

Key: +, positive reaction; (+), weakly positive; F, fermentation; A/K, acid/alkaline; +w, positive to weak reaction; d, 11-89 strains positive; α-hemolysin, partial lysis of the erythrocytes surrounding a colony.

Table 4. Biochemical characters of isolates and *Staphylococcus epidermidis* CCRC type strain (II)

Characteristic	HL0106	LK0728	KH0110	LK1205	LK1221	CCRC10783	Bergey's manual
Anaerobic growth-thioglycollate	+	+	+	+	+	+	+
Aerobic growth-thioglycollate	+	+	+	+	+	+	+
PYR hydrolase	–	–	–	–	–	–	
DNase	+	+	+	+	+	+	– w
Alkaline phosphatase	–	+	–	+	+	–	+
Urease	+	+	+	–	+	+	+
Lipase (spirit blue agar)	–	–	–	–	–	–	
Bound coagulase (rabbit)	–	–	–	–	–	–	–
Free coagulase (rabbit)	–	–	–	–	–	–	–
Latex agglutination	–	–	–	–	–	–	
Resistance to lysostaphin (10 unit)	–	–	–	–	–	–	–
Inhibition by furazolidone (100 μg)	+	+	+	+	+	+	+
Inhibition by bacitracin (0.04 unit)	–	–	–	–	–	–	–
Production of acid from 1% glycerol aerobically in the presence of 0.4 μg erythromycin	+	+	+	+	+	+	+

Key: +, positive reaction; –, negative reaction; – w, negative to weak reaction.

Table 5. Utilization of carbohydrates of isolates and *Staphylococcus epidermidis* CCRC type strain

Carbohydrate (aerobic/anaerobic)	HL0106	LK0728	KH0110	LK1205	LK1221	CCRC10783	Bergey's manual
Glucose	+/+	+/+	+/+	+/+	+/+	+/+	+/+
D-Fructose	+/+	+/+	+/+	+/+	+/+	+/+	d/
D-L-Ramnose	–/–	–/–	–/–	–/–	–/–	–/–	
D-Xylose	–/–	–/–	–/–	–/–	–/–	–/–	–/
D-Mannose	+/+	+/+	+/+	+/+	–/–	+/+	(+)/
D-Galactose	+/+	+/+	+/+	+/+	+/+	+/+	d/
D-Lactose	–/–	–/–	+/+	–/–	–/–	–/–	–/
Maltose	+/+	+/+	+/+	+/+	+/+	+/+	+/
D-Trehalose	+/+	+/+	–/–	–/–	+/+	–/–	–/
Melibiose	–/–	–/–	–/–	–/–	–/–	–/–	
Sucrose	+/+	+/+	+/+	+/+	+/+	+/+	+/
D-Raffinose	–/–	–/–	–/–	–/–	–/–	–/–	–/
Inulin	+/+	+/+	+/+	+/+	+/+	+/+	
Glycerol	+/–	+/–	+/–	+/–	+/–	+/–	
D-Mannitol	+/–	+/–	+/–	+/–	+/–	+/–	d/–
D-Ribose	–/–	–/–	–/–	–/–	–/–	–/–	d/
Xylitol	–/–	–/–	–/–	–/–	–/–	–/–	–/

Key: +, positive reaction; –, negative reaction; ( ), delay reaction; d, less than 90% of strains positive or negative.

Table 6. Susceptibility of isolates against various antibiotics

Drug (dosage)	HL0106	LK0728	KH0110	LK1205	LK1221
Amikacin (30 μg)	+	+	+	+	+
Ampicillin (10 μg)	+	+	+	+	+
Bacitracin (10 unit)	+	+	+	+	+
Cephalosporin (30 μg)	+	+	+	+	+
Chloramphenicol (30 μg)	+	+	+	+	+
Erythromycin (15 μg)	+	+	+	+	+
Gentamycin (10 μg)	+	+	+	+	+
Kanamycin (30 μg)	+	+	+	+	+
Lincomycin (2 μg)	+	+	+	+	+
Methicillin (5 μg)	+	+	+	+	+
Neomycin	+	+	+	+	+
Novobiocin (30 μg)	+	+	+	+	+
Oxacillin (1 μg)	+	+	+	+	+
Oxytetracycline (30 μg)	+	+	+	+	+
Penicillin G (10 unit)	+	+	+	+	+
Polymyxin (300 unit)	+	+	+	+	+
Streptomycin (10 μg)	+	+	+	+	+
Sulfadiazine (300 μg)	–	–	–	–	–
Tetracycline (30 μg)	+	+	+	+	+

Key: +, sensitive; –, resistant.

throughout the entire infected tissue were also observed. The infected pancreas, liver, mesentery and gonad, showing focal necrosis and granuloma changes were also observed. However, the severity of abnormalities in described tissue was less than that in the anterior kidney and spleen. Very few lesions were observed in the hearts of infected fish (Fig. 3).

Infected fish not only showed necrotic symptoms in infected tissue but also revealed numerous electron-dense particles in many infected tissues including the spleen (Fig. 4), muscle (Fig. 5), brain, kidney, pancreas, liver, intestine, stomach, mysentery, gonad, and skeletal muscle (Table 1). The occurrence of phagocytosis could be observed in most phagocytes in the spleen, liver, and kidney, respectively (Figs. 6, 7).

Fig. 1. Natural infection; typical lesions, marked white nodules and microscopical granulomatous formation, as observed in heavily infected fish. AK: anterior kidney, S: spleen.

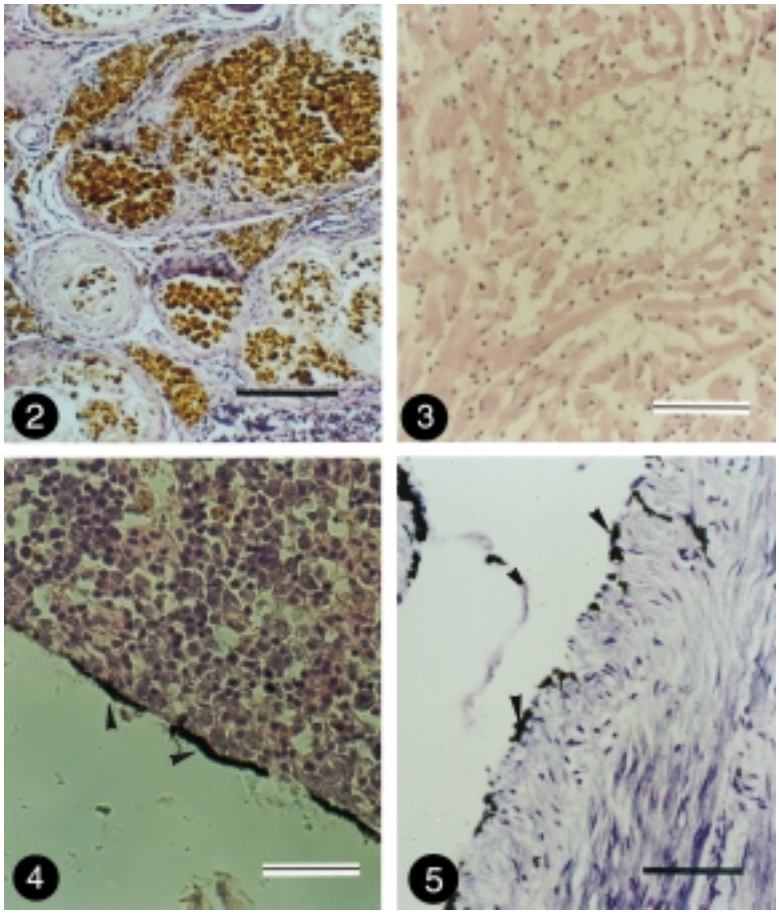

Fig. 2. Natural infection; numerous granuloma with necrotic centers scattered through the spleen parenchyma. H and E stain, bar: 100 mm.
Fig. 3. Natural infection; slight lesions as observed in heart of infected fish. H and E stain, bar: 25 μm.
Fig. 4. Natural infection; numerous small particles as observed in connective tissue of spleen. Arrowhead: small particles, Giemsa stain, bar: 50 μm.
Fig. 5. Natural infection; a large amount of small particles in connective tissue of muscle. Arrowhead: small particles, Giemsa stain, bar: 100 μm.

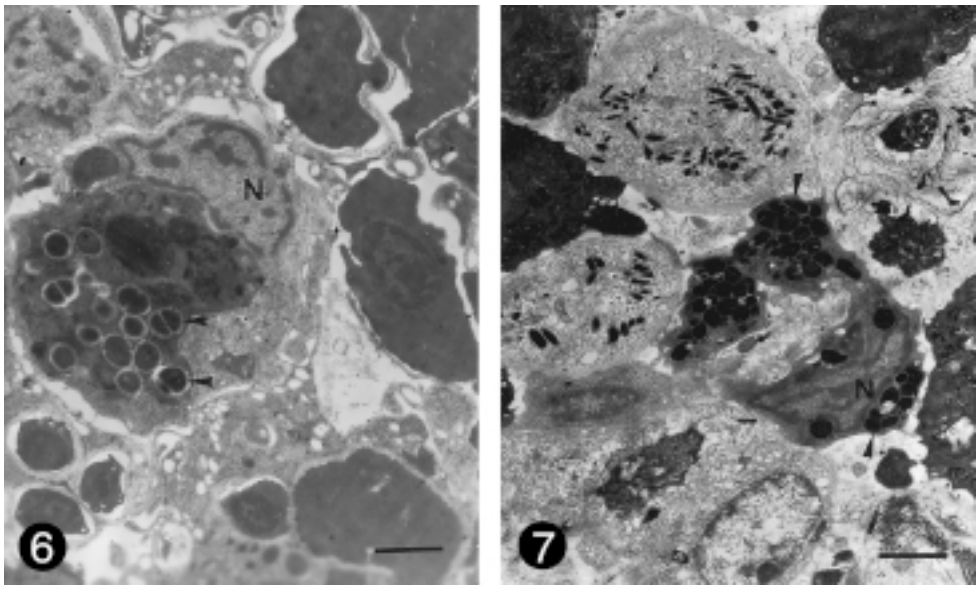

Fig. 6. Natural infection; phagocytosis in the spleen of infected fish. Arrowhead: binary fission of coccus, N: nucleus, bar: 2 μm.
Fig. 7. Natural infection; electron-dense particles phagocytosed by phagocytes in the spleen of infected fish. N: nucleus of phagocyte, arrowhead: electron-dense particles, bar: 2 μm.

Challenge

To confirm the reproducibility of the disease in host fish, a challenge test was performed, and a positive result was obtained in the experimentally inoculated healthy tilapia. Results are listed in table 7. Observations of challenged fish were extended to 45 d. When tilapia were experimentally injected with 1.34×10^7 or 1.34×10^9 cfu/ml bacteria, a total of 6 fish died in each group. Seven fish died in the group receiving bacterial supernatant. Of the remaining groups, fish survived when a dose of 1.34×10^3, 1.34×10^4, 1.34×10^5, or 1.34×10^6 cfu/ml was inoculated into the fish, respectively.

During the initial 2 wk of the infectivity trial, the bacteria could be re-isolated from those groups. However, after 3 wk after infection, it was difficult to isolate bacteria from the infected fish. In comparison, no bacterium was recovered from the control fish or the supernatant-inoculated group.

Focal necrosis and granuloma formation were observed in spleen parenchyma after 4 wk by challenged with 0.5 ml 1.34×10^7 cfu/ml (Fig. 8). Bacteria could be re-isolated from groups receiving bacteria suspension injection. Characterizations of the re-isolated bacteria were the same as those of the isolated *S. epidermidis* from naturally infected tilapia. Clinical symptoms and histopathological changes of the experimental fish induced by bacterial infection (HL0106, KH0110, LK1205 and LK1221) were similar to those of naturally infected fish (data not shown).

Table 7. Challenge test to tilapia (*Oreochromis aureus*) by LK0728, *Staphylococcus epidermidis* (from 24 July to 10 Sept. 1996)

Amount of bacteria (cfu/ml)	Injection dose	No. of tested fish	Time after inoculation (day)									
			1	2	3	5	10	15	20	30	45	Total
Control	0.5 ml	10	0	0	0	0	0	0	0	0	0	0
1.34×10^3	0.5 ml	10	0	0	0	0	0	0	0	0	0	0
1.34×10^4	0.5 ml	10	0	0	0	0	0	0	0	0	0	0
1.34×10^5	0.5 ml	10	0	0	0	0	0	0	0	0	0	0
1.34×10^6	0.5 ml	10	0	0	0	0	0	0	0	0	0	0
1.34×10^7	0.5 ml	10	0	1	0	0	1	2	2	0	0	6
1.34×10^9	0.5 ml	10	1	0	1	2	1	1	0	0	0	6
Supernatant	0.5 ml	10	1	0	1	1	2	1	1	0	0	7

According to the results described above, we suggest that *S. epidermidis* was the pathogen that caused mass mortality in pond-cultured tilapia in Taiwan.

DISCUSSION

Staphylococcus epidermidis as a fish pathogen was first reported by Kusuda and Sugiyama (1981). During July 1976 to Sept. 1977, the bacteria caused mass mortality in farmed yellowtail (*Seriola quinquiradiata*) and red sea bream (*Chrysophrys major*) in Japan (Kusuda and Sugiyama 1981). In this paper, Gram-positive cocci were isolated from diseased tilapia. The 5 bacterial strains (LK0728, HL0106, KH0110, LK1205, and LK1221) in tilapia from different areas evidently possessed similar biological and biochemical characteristics as described in tables 2-5, and were identified as *S. epidermidis*.

During 1992 to 1996, the isolation of bacteria should have been performed at the beginning of infection using the direct puncture technique. Isolation of the bacteria is comparatively difficult when the infection becomes chronic or 3 wk after infection using the same technique. This may be caused by the majority of bacteria being phagocytosed by macrophages as shown in figure 6. Definite isolation is possible by culturing the bacteria on BHIA using ground or minced tissues.

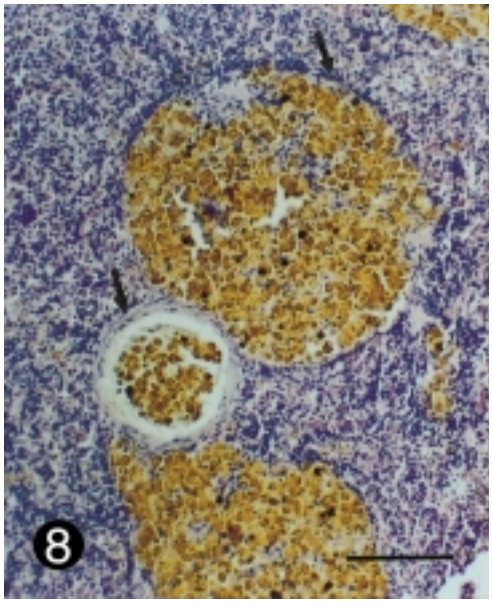

Fig. 8. Artificial infection; focal necrosis and granuloma changes present in the spleen parenchyme 4 wk after inoculation of *S. epidermidis* at a dose of 0.5 ml 1.34×10^7/ml. Arrow: epithelioid cell of circular structure, H and E stain, bar: 100 μm.

Challenge tests were performed using bacterial suspensions and filtered-sterilized supernatants by i.p. injection. Results indicated that the supernatant and densities of bacteria greater than 1.34×10^7 caused 60% mortality of experimental fish; in contrast, no mortality was observed in the control fish. The bacteria could be re-isolated from the fish groups after bacterial suspension injection. Characterizations of the re-isolated bacteria were the same as those of *S. epidermidis* isolated from naturally infected tilapia. Clinical symptoms and histopathological changes of the experimental fish induced by bacterial infection were similar to those of naturally infected fish.

Results of the challenge test showed that tilapia were damaged by viable bacteria or their exudates, and were characterized by histopathogical changes and presence of electron-dense particles in various tissues when viable bacteria or its supernatant were inoculated. Determining the definite factors involved in *S. epidermidis* causing mass mortality in tilapia requires further investigation. It is therefore concluded that *S. epidermidis* may cause mass mortality in cultured tilapia in natural environments in Taiwan.

Heavily infected fish initially showed no external clinical signs or abnormalities and only a few cases revealed exophthalmia, or lesions in the epidermis and fins. In contrast, Kusuda and Sugiyama (1981) showed that typical signs in yellowtail (*S. quinquiradiata*) and red sea bream (*C. major*) caused by *S. epidermidis* included exophthalmia, congestion, and ulcerations on the tail. Although, clinical symptoms may vary between diseased tilapia and diseased yellowtail and red sea bream, high mortalities caused by *S. epidermidis* may result in identical a significant economic losses among these kinds of fish.

In the present study, pathological reactions were found in the spleen, anterior kidney, trunk kidney, pancreas, liver, intestine, stomach, mesentery, gonad, and muscle, etc. The most severe pathological changes were noted in the spleen and anterior kidney (Table 1). In addition, large amounts of electron-dense particles were observed in the brain, spleen, kidney, pancreas, liver, intestine, stomach, mesentery, gonad, and skeletal muscle by TEM examination, but predominantly in the spleen and anterior kidney. The relationship between pathological reaction induced by *S. epidermidis* and electron-dense particles in various tissues is still unknown, and needs further investigation.

In the histopathological study performed on the tissues of infected fish, electron-dense particles

were observed in the internal tissues; the nuclear ultrastructural reactions and the particles were suspected to be expression of apoptosis by Kerr (1965) and Kerr et al. (1972). Moreover, TO-2 cells (tilapia ovary, Chen et al. 1983) revealed severe nuclear changes with many electron-dense particles in the affected cells when incubated with filtered supernatant from the spleen or from *S. epidermidis*-cultured medium (data not shown). An investigation of apoptotic bodies induced by the bacterium is in progress.

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吳郭魚罹患表皮葡萄球菌之特性研究

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研究 1992 年至 1996 年間養殖吳郭魚之流行性病害，典型的病害特徵包括在各組織器官中形成多發性白色結或肉芽腫，病變以脾臟及前腎最嚴重，從罹病魚可以分離出革蘭氏陽性球菌，依據細菌之形態學、生物學及生物化學等特質，將細菌鑑定為表皮葡萄球菌 *Staphylococcus epidermidis*，病原性試驗係以分離菌株 LK0728 *S. epidermidis* 進行，試驗魚之組織病變包含肉芽腫病變、組織傷及出現高電子密度顆粒等，病變發生情形均與自然罹病之吳郭魚很類似，本文係為第一篇吳郭魚遭受表皮葡萄球菌 *Staphylococcus epidermidis* 侵害的病害報告。

關鍵詞：肉芽腫，表皮葡萄球菌，病原性試驗，高電子密度顆粒。

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