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簡 報 Notes

A New Coccus Disease of Cultured Bullfrog

新發現養殖牛蛙球菌症

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In addition to the well known septicemic disease (redleg disease) of bullfrog caused by *Aeromonas hydrophila*, there are at least 9 species of gram negative and 3 species of gram positive bacteria had been found to be pathogenic to bullfrog[3]. But all these species found not only in diseased bullfrog, but also from apparently healthy ones and also from enviromental waters. They caused diseases only under some undefinite yet ecological parameters[1,3,4]. Among the gram positive bacteria isolated from bullfrog, *Staphylococcus epidermidis* and one species of *Corynebacterium* were classified as pathogenic, while *Streptococcus* was regarded as not pathogenic[3]. In the recent two or three years, the cultured bullfrog in Taiwan were found to be suffered from a gram positive coccus infection, great damage was encountered, in some cases mortality up to almost 100% within weeks had been noticed.

The moribund bullfrog usually show symptoms of darken skin coloration, with enlargement of abdomen, due to hydropsis and or inflammation of alimentary tract. Paralysis of appendages, occasionally with unbalanced body posture was also noticed in advanced cases. Fresh smear of the body fluid and visceral organs include liver, kidney, blood, intestine, brain and spinal cord usually could find a gram positive cocci, arranged in long or short chains, or in less cases arranged in pairs or single (Fig. 1). Dimeter of the coccus is about 0.8 μ. SEM viewing reveals that the cocci are somewhat coryneform (Fig. 2). A prelimenary screening examination of the isolate, indicate that the organism is a nonhaemolytic, bacitracin sensitive, none Lancefied group B *Streptococcus sp.* and was confirmed by CDC[6], Atlanta. Similar organism had been reported isolated in four cases, but none were of bullfrog origin[5,6].

The new epidemics of cultured bullfrog is thought to be chronic but it could be a potential sources of mass mortality in commercial production of bullfrog. The sources of this organism and its pathogenecity to other aquatic animals is under investigating.

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Fig. 1. *Streptococcus* sp. isolate from cultured bullfrog (10×100).

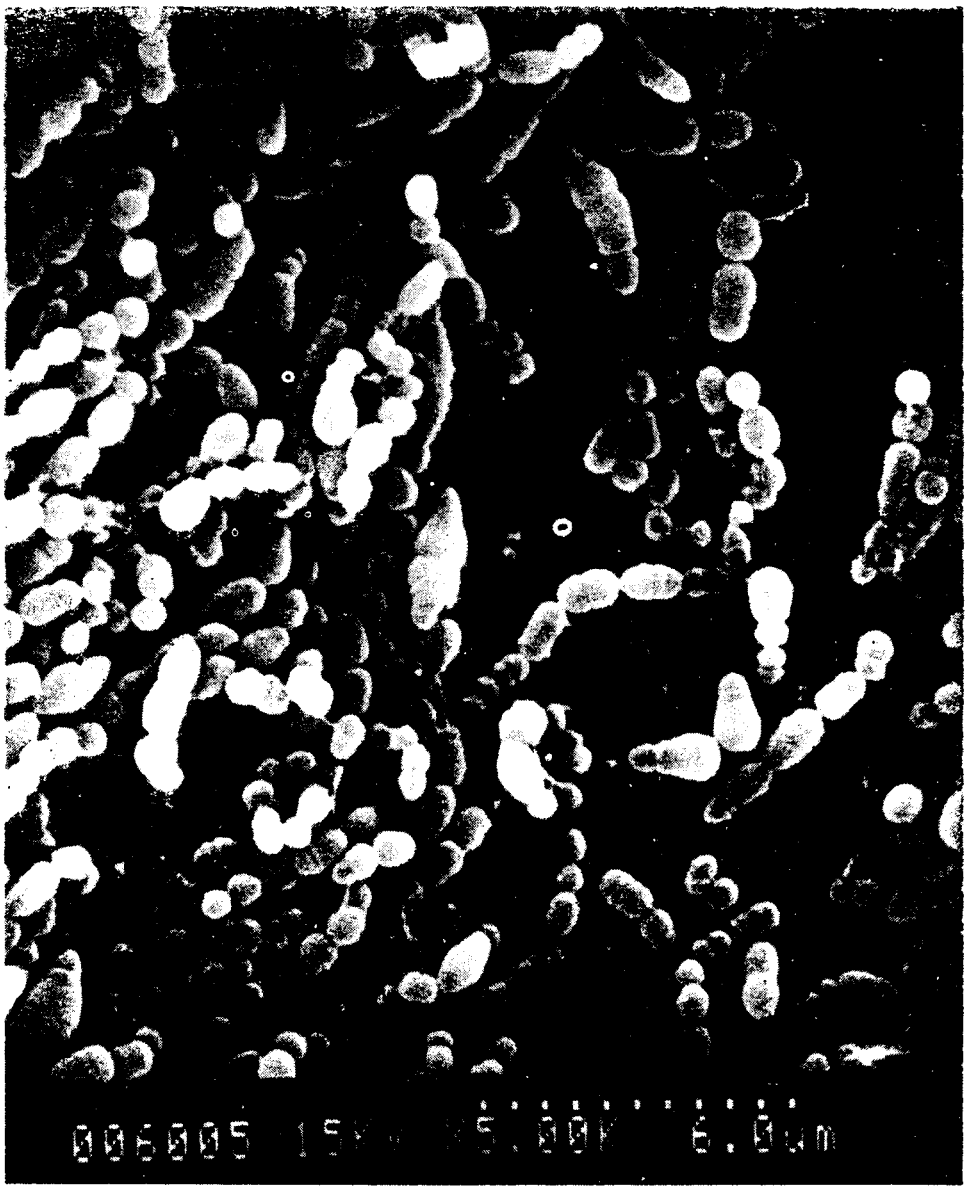

Fig. 2. SEM of *Streptococcus* sp. isolate from Bullfrog. (×7000)

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Characteristics of Non-hemolytic Streptococci Isolated from Infected Captive Bullfrog (*Rana catesbeiana*)

養殖牛蛙 (*Rana catesbeiana*) 分離非溶血性鏈球菌之性狀

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Summary

Twenty two strains of Streptococci were isolated from infected bullfrog (*Rana catesbiena*) and tadpoles, during the period of middle 1980 to late 1984. Morphological, biochemical as well as serological characters tested of all strains were closely related, but not identical.

All isolates did not react with Lancefield A,B,C,D,E and G antisera, did not hemolysis rabbit or sheep erythrocytes. Few strains are varied in dimeter of colony size, abilities of growth in 65°C (30 min), in 10°C and 45°C, in PH 9.6, and in 0.1% methylene blue milk. Some strains are also different in bacitracin sensitivity, hydrolsis of esculin in the presence of 40% bile and degradation of mannitol, trehalose and salicin. All other characters tested are unanimous in all strains.

Four selected isolates, of which two are bacitracin sensitive, two are not sensitive are all highly pathogenic to bullfrog in artificial infection experiment, but they do not cause any noticeable symptom or produce lethal effect on tilapia or common carp or small mouse. Their lethal effet on tigrinaris frog varied among strains. *Streptococcus facium* (ATCC-19434) and *Streptococcus faecalis* (ATCC-19433) were not able to infect all the experimental animals at all.

Introduction

Streptococcal infection in lower vertebrates have rarely been reported before 1970's, only the following two papers had been collected: *Streptococcus fecalis* infection in rainbow trout((Hoshina, Sano & Morimoto 1958), and r-hemolytic *Streptococci* infection in golden shiners (*Notemigonus crysoleucas*) (Robinson & Meyer 1966). But, reports of epizotics incidences in fishes caused by *Streptococci* increased dramatically in the past decade (Barham et al., 1979; Cook et al., 1975; Kaige et al., 1984; Kusuda et

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al., 1978; Kusuda et al., 1982; Kusuda et al., 1976; Minami, 1979; Minami et al., 1979; Pappalardo et al., 1982; Plumb et al., 1974; Shimitzu, 1982).

Although Streptococci were found in the gut of bullfrog (Amborski et al., 1974; Carr et al., 1976; Glorioso et al., 1974b). They were considered to be non pathogenic. In the present study of bullfrog diseases in Taiwan since 1980, the authors found that the gram positive cocci was involved in most cases of the mass mortality observed, while, the red-leg symptom caused by the notorious motile aeromonasis was noticed only in minor cases. The causative gram positive agent was isolated and preliminarily identified as a r-hemolytic, not Lamcefield group B, bacitracin sensitive *Streptococcus sp.* (Chung and kou, 1984). With further study on the characteristics of these isolates, they are not only heterogeneous of several types but also differing from all isolates of ichthyic origin reported elsewhere (Kitao, 1982; Kusuda et al., 1978; Robinson et al., 1966; Wilkinson et al., 1973).

The present study describes the morphological, biochemical and serological characteristics of these isolates. Discussion on the possible origin of these isolates and their potential infectivity to bullfrog, tigrinaris frog, common carp, tilapia and mouse were included.

Materials and Methods

Sources of isolates

All strains were isolated from apparently diseased bullfrog or tadpole(Chung & Kou, 1984), from the frog farms in Ping-tung and I-lang counties during the period fromthe middle of 1980 through late 1984. Isolation and culture of the strains were according to the usual bacteriological method: Trypticase Soy Agar (TSA, Difco), Brain Heart Infusion agar (BHI, Difco) and Streptosel agar (BBL) (1968) were used for primary isolation of bacteria. The inoculated agar plates were incubated at 30°C for 24-48h, punctiform and pin-head size colonies growth on each agar plate were transfered and subcultured in the respective medium. The pure isolates were then transfered to BHI agar incubated at 30°C for 24-48h. All characteristics of the isolates were determined by using these young cultures. *Streptococcus fecalis* (ATCC-19433) and *Strep. facium* (ATCC-19434) were choosed as reference strains in performance tests.

For the purpose to clarify the origin of the gram positive cocci, the formulated pellet feed and fly larva used as frog feed were examined in three efforts. The latter had been used only during the metamorphosis stage and is presently replaced by the formulated pellet diet. The feeds were sampled from three frog farms in epidemics. 5g of pellet feed or 20 fly larvas were blendered separately with 10 ml. volume of sterilzed 0.85% NaCl solution which is further diluted 10, 10^2 and 10^3 times, 0.1 ml of the diluents were inoculated onto a BHI agar plate, bacterial identification procedures were identical to that described in the previous section.

Bacteriological tests

Gram stain reaction of all isolates were checked by Ryu's KOH method (Ryu, 1940). Morphology and motility of the bacteria were examined at the magnification of 1250X from a gram stained specimen by using a light objective and from a hanging drop prepation by using a phase contrast objective respectively. The strain KA-41 was also examined by using TEM and SEM.

Classification of the Streptococci were based on Bergey's manual of determinative bacteriology 8th ed (Buchanan & Gibbons, 1974). Biochemical characterization of the isolates was made according to the methods describribed in Mac Faddin (1980) unless otherwise indicated.

All isolated strains were inoculated onto MacConkey agar and Tomato juice agar (Difco) enriched with 5% cholerae growth factor (CGF) to test the ability of these organisms to grow on the two media. Difco sensitivity disc was used for the study on susceptibility of these isolates to 11 antibiotics.

Serological analysis

An antiserum of strain KA-41 was obtained from a rabbit which was injected intravenously with formain killed cells. A microplate agglutination method was used to examine the serological status of the isolates, and the two reference enterococci, *A. hydrophila* and *E. tarda* were also used to test the cross reaction.

Lancefield groups of the isolates were determined by the capillary precipitin technique, the antigen used for this determination were prepared by both autoclave method of Rantz and Randoll (1955) and Hot HCl method of Lancefield (1933). The antisera of group A, B, C, D, E and G (Difco) were used in the tests.

Pathogenecity tests

Six Streptococcal strains were selected for infection experiments. The selected strains were cultured on BHI agar at 30°C and harvested after 24h incubation. The concentration of the bacterial suspension was first estimated by comparison with MacFaland's standard solution No. 9, while the exact colony forming unit (CFU) was determined by standard plate count method.

Infection experiments were performed by injection, dipping and feeding methods:

(*1*) Injection method and determination of LD_{50}

LD_{50} of strain KA-41 to bullfrog was determined by intraperitoneal injection of 5 different does (9×10^8, 9×10^7, 9×10^6, 9×10^5 and 9×10^4 CUF in 0.1ml/100g B. W.). 10 bullfrogs were used for each dosage, while only 5 bullfrogs were used in the control group. The control group were injected with 0.9% saline solution (0.1ml/100g B. W.) instead of bacterial suspension. LD_{50} for 7 days period was computed by using probit analysis.

In addition to determination LD_{50} of strain KA-41 to bullfrog, this strain and the

following five strains: KA-45 KA-46, KA-40 and *Strep. fecalis* (ATCC-19433), *Strep. facium* (ATCC-19434) were used to test their infectivity and lethal effect to the following five kind of animals: bullfrog (*Rana catesbieana*), native frog (*Rana tigrinaris* var.) small white mouse (I. C. R), common carp (*Cyprinus carpio* L.) and tilapia (*Oreochromis niloticus*). The method of intramuscular (i. m.) injection was employed for the two fish species and that of intraperitoneal (i. p.) was used in frog and mouse. The injection dose was 9×10^8 CFU in 0.1ml/100g B.W.. 10 animals of each species were used for each strain, except that only 5 mice were used instead of 10.

(2) Dipping and feeding metbods.

In dipping method, 10 bullfrogs were dipped for 30 min. in a 1l bacterial suspension at the concentration of 9×10^8 CFU/ml. of strain KA-41. In feeding method, the same strain was introduced into 10 bullfrog's stomach at the dose of 9×10^8 CFU/100g B. W., through a 1c.c. disposable syringe with a 5cm long polyethylene tube (I.D.=1.5mm) attached.

After administration of bacteria, each frog was stocked in a separate 5 l plastic container, with small amount of tapwater in it. The two kinds of fish was each stocked in a 40 l glass aquarium each containing 15 l well aerated tapwater. All experimental sets were maintained at ambient temperature (21-28°C). The performance was observed for 7 days, during which period no feeding was attempted, except mouse was feeded with commercial powder feed.

Results

Sources of isolates

22 strains of gram positive cocci were isolated from apparently diseased bullfrog and tadpoles in the warmer seasons (June through November) of years. All isolates grow well on BHI agar or blood agar. Under the incubation condition at 30°C for 24h, two type of colonies were observed: 6 strains formed small pin head, convexed opaque colonies, about 1 mm in dimeter, the other 16 strains formed pin point semitranslucent colonies less than 1 mm in dimeter. All isolates grow very poorly on the TSA plate, colonies were noticed only after 36-48h incubation at 30 C.

In the three efforts of trying to isolate the gram positive cocci from the two kinds of bullfrog feeds, no any similar organism was found.

Bacteriological characteristics

All isolates are nonmotile and coccal to lenticular in form (Fig 1). The dimeter of the bacterial cell is $0.4\text{-}0.8\times0.5\text{-}1.0\mu$ in size. The cell are single, double or arranged in short chain, in a few cases a long chain up to more than 40 cells may also be found from a fresh smear preparation from infected frogs (Fig 2). A thin slime layer covering the bacterial surface could be observed clearly by TEM (Fig 3), but not by light microscope under a 1250× magnification.

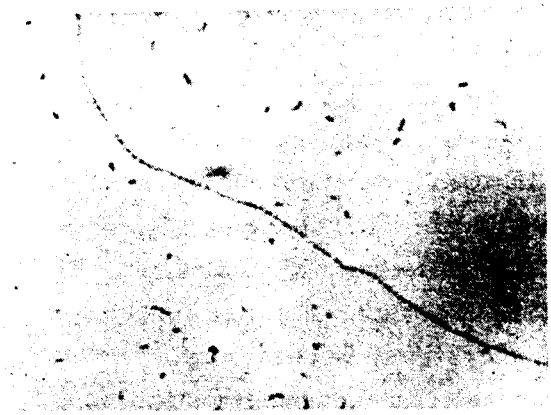

Fig. 1. Very long Chain of *Streptococcus sp*. Smear from infected bullfrog (1250X).

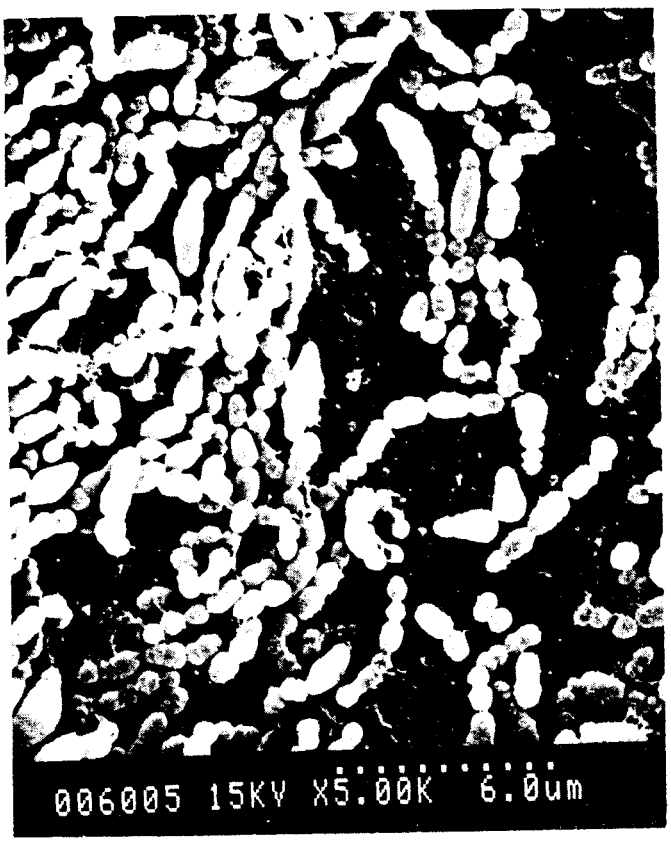

Fig. 2. S. E. M. of Streptococcus isolated from infected bullfrog (5000X).

Fig. 3. T. E. M. of Streptococcus isolated from infected bullfrog Showing the thin Slime layer around the cell.

None of the isolates were able to grow on MacConkey agar or Tomato juice agar enriched with 5% CGF (Table 1), except that two strains were found to form visible colonies on the MacConkey agar after a four days incubation.

Biochemical and serological characteristics of the isolates and the reference strains are summarized in Table 2 and Table 3. All strains were catalase and cytochrome oxidase negative, and fermentatively metabolize glucose. The fermentative reaction could only be detected by a staph OF method (Mac Faddin 1980), because all isolates were either growth feable or not growth at all in a Hugh-Leifson O-F medium. All isolates were poor or not growth able in the MR/VP broth, negative in citrate assimilation, lysine and ornithine decarboxylation, only three isolates were able to produce arginine dihydrolase. All isolates were also innert in sugar degradation, only degraded glucose and sucrose, except that 3 isolates were able to hydrolyze mannitol, trehalose and salicin in additions to glucose and sucrose. The six bacitracin sensitive strains all showed punctiform colony. Only three strains among the isolates were able to grow on a streptosel agar (based on macroscopical examination). None of the isolates were able to grow on SF or KF medium.

Antibiogram test of susceptibility and resistant to 11 kinds of antibiotics, revealed that all isolates were highly sensitive to only penicillin (Table 4). All strains were resistant to Streptomycin, Neomycin, Kanamycin, Gentamycin, Nalidixic acid and colistin. Sensitivity of the isolates to Tetracycline, Cholorotetracycline, Oxytetracycline, Erythromycin and Oleandomycin varied among the strains.

Table 1. Morphological and culture characteristics of streptococci isolared from bullfrog and ATCC reference streptocoeei.

	22 strains of Streptococci isolated from bullfrog	(*Streptococcus faecalis* ATCC-19433)	*Streptococcus faecium* (ATCC-19434)
Gram stain/viscosity in 3% KOH	+/+	+/+	+/+
Cell morphology	coccus to lenticular	coccus	coccus
Cell size	0.4-0.8x0.5-1.0u		
Growth on:			
Trypticase Soy agar	+[a]	++	++
Brain Heart Infusion	++[b]	++	++
Blood agar	++	++	++
Todd Hewitt agar	++	++	++
Streptosel agar	3/22[c]	+	+
MacConkey agar	2/22	+	+
Tomato Juice agar +5% CGF	—[d]	—	—
SF medium	—	+	+
KF medium	—	+	+
Bile esculin (Difco)	5/22	+	+
Growth in 6.5% NaCl	2/22	+	+
in 0.1%MB milk	3/22	+	+
Growth at PH 9.6	3/22	+	+
at 10 C	2/22	+	+
45 C	2/22	+	+
65 C(30 min)	—		+
Growth under anaerobic condition (with Co_2)	+	+	+
Motility	—	—	—

a. +: growht or positive, b. ++: goodgrowth, c. No. of positive strain/No. of tested strains d. —: negative

Table 2. Biochemical and serological characteristics of streptococci isolated from bullfrog and ATCC reference streptococci.

	22 strains of Streptococci isolated from bullfrog	*Streptococcus faecalis* (ATCC-19433)	*Streptococcus faecium* (ATCC-19434)
Catalase	−[a]	−	−
Cytochrome oxidase	−	−	−
OF test in			
Hugh Leifson medium	NG[b]	F	F
Staph OF	F[c]	F	F
Indole production	−		
Methyl Red	4/22[d]	+	+
Voges Proskauer	−	−	−
Hydrogen sulfide	−	−	−
Nitrate reduotion	−		
Citrate (Simmons)	−	+	−
Starch hydrolysis	−		
Hippurate hydrolysis			
Arginine dihydrolase	2/22	+	+
Lysine decarboxlase	−	−	−
Ornithine decarboxylase	−	−	−
Bacitracin senstive (Taxo A)	6/22	−	−
Hemolysis			
sheep erythrocyte	r		
rabbit erythrocyte	r		
Lancefield group	Not A,B,C,D,E ofG	D	D

a: −, negative, b: NG, not growth or feable growth, c: F, Fermentation,
d: No. of positive strain/No. of tested strain.

Table 3. Utilization of carbohydrates of streptococci isolated from bullfrog and ATCC reference streptococci.

	22 strains of Streptococci isolated from bullfrog	*Streptococcus faecalis* (ATCC-19433)	*Streptococcus faecium* (ATCC-19434)
Acid from			
D-Arabinose	−[a]	−	+
D-Xylose	−		
Galactose	−		
Dextose	+[b]	+	+
Sucrose	21/22[c]	+	+
Lactose	−	+	+
Mannitol	2/22	+	+
Trehalose	3/22	+	+
Sorbitol	−	+	−
Salicin	3/22	+	+
Glycerin	−	+	−
Inositol	−		
Raffinose	−		
Maltose	−		
Gas from carbohydrate	−	−	−

a: − Negative reaction
b: + Positive
c: No. of positive reaction strains/No. of tested strains

Table 4. Antibiogram of resistance and susceptibilitly to antibiotics of streptococci isolated from bullfrog.

Antibiotic	S[a]	R[b]
Penicillin	+	
Streptomycin		+
Neomyoin		+
Kanamycin		+
Gentamycin		+
Tetracycline	14/22[c]	9/22
Chlortetracycline	17/22	6/22
Oxtetracycline	14/22	9/22
Erythromycin	16/22	7/22
Oleandomycin	17/22	6/22
Nalidixic acid		+
Colistin		+

a: S:Susceptible
b: R:Resistant
c: No. of sensitive or resistant strains/No. of tested strains

Serological analysis

The antiserum of strain KA-41 prepared from a rabbit, showed a poor agglutinate titer. The titer of this antiserum against itself antigen was 320. The titer of antigens of all isolates against this antiserum fell in the range of 80-320. The two reference enterococcus and *A. hydrophila* and *E. tarda* did not agglutinate with the antiserum above the 20 times dilution (Table 5).

Capillary precipittin grouping test indicated that all isolates did not react to Lancefield A, B, C, D, E and G group antisera.

Pathogenecity test

All six strains used in the infection experiments by injection method were unable to induce any noticeable infection in tilapia, common carp and small white mouse. However, the tested four bullfrog isolates were able to induce severe infection in experimental bullfrogs and caused 100% mortality in 7 days. In contrary they showed lower infectivities to tigrinaris frog, induced only 20% to 70% mortalities (Table 6). The LD_{50} of strain KA-41 on bullfrog on a seven days period was 3.72×10^{5} CFU/100g B. W. ranging between 1.28×10^{5} and 1.07×10^{6} CFU/100g B. W. The infection of the strain KA-41 on the bullfrog by the methods of forced feeding and dipping administration produced 10% and 60% mortalities respectively.

Table 5. Agglutination of different sources streptococci and *A. hydroplia* and *E. tarda* against antiserum of *Streptococcus sp.*

Antigens	Dilution							control
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	
KA-46,AB-29,AB-30,AB-31, KA-21,KA-22,AA-25,KA-41, KA-45	+++	+++	+++	+++	++	+	−	−
KA-5,KA-6,JA-17,JA-19, KA-20,AA-26,KA-42,KA-44	+++	+++	++	+	±	−	−	−
KA-4,JA-16,JA-18, KA-23	++	+	+	±	−	−	−	−
Strepto.facium (ATCC-19433)	+	±	−	−	−	−	−	−
Strepto .fecalis (ATCC-19434)	−	−	−	−	−	−	−	−
A. hydrophila	−	−	−	−	−	−	−	−
E. tadra	−	−	−	−	−	−	−	−

Table 6. Infection experiments of six strains of different sources streptococci on bullfrog and tigrinaris frog by I. P. injection.

Strain No.	Inoculum (CFU/100g animai)	Mortality	
		Bullfrog[a]	Tigrinaris frog[b]
KA-11	9×10^{8}	10/10[c] (100)[d]	3/10 (30)
	9×10^{7}	10/10 (100)	−[e]
	9×10^{6}	8/10 (80)	−
	9×10^{5}	7/10 (70)	−
	9×10^{4}	2/10 (20)	−
	9×10^{3}	0/10 (0)	−
KA-45	9×10^{8}	10/10 (100)	7/10 (70)
KA-46	9×10^{8}	10/10 (100)	5/10 (50)
KA-40	9×10^{8}	10/10 (100)	2/10 (20)
Strepto. faecalis (ATCC-19433)	9×10^{8}	0/5 (0)	−
Strepto. faecium (ATCC-19434)	9×10^{8}	0/5 (0)	−
Control	0	0/5 (0)	0/5 (0)

a: Body weight 110-300g, b: 12-17g, c: No. of death/No. of test,
d: mortality e: not test

Discussion

Despite the fact that epizotics incidences of streptococcal infection in fishes had been reported numerously during the past decade, as stated in the introduction, there are only three species had been identified as fish pathogens: *S. faecalis*, *S. facium*, and *S. equisimillis*. The last species was only identified as *S. equisimillis* similar organism and not exactly defined yet (Ohnishi & Jo,. 1981). All other ichthyic origin streptococci that had been isolated were not belong to any known defined species yet. In addition to the biochemical characteristics, the haemolytic characteristics and antigenic structures of the fishes and frog sources streptococci also varied with the human and animal sources strains. The haemolytic characteristics and Lancefield grouping criteria are both essential in in differentiate the pathogenic status of human and animal streptococci. But, both characteristics seem irrelated to the infective nature of the ichthyic and the bullfrog orgin strains. Wilkinson et al (1973) Robinson & Meyer (1966) and Plumb et al (1974) all reported Lancefield groupable, non haemolytic streptococcicosis of fresh water and marine fishes, but Ohnishi & Jo (1981), Rasheed & Plumb (1984) and Yasunaga (1983) etc stated both α and β hemolytic, all are Lancefield ungroupable Streptococci that caused epidemics in cultured fishes.

All isolates in this study were very similar to each other but not identical with respects to the cultural, biochemical and serological characteristics. They are also similar in biochemical characteristics to that of the isolates of Kitao et al (1981). However varied in the methyl red reaction, arginine dihydrolase production and bacitracin sensitivity. The sugar degradation ability were more innert in the present isolates.

The haemolytic reaction and the infected host species of the isolates in this study was also completely different from that of kitao etal (1981) The antibiogram study indicated that many of the isolates were resistant to antibiotics that were highly effective to inhibit streptococci of the ichthyic origin (Katae. 1982; Kitao et al,. 1983), This finding is significantly important to chemotherapeutical application in bullfrog disease control and upgrade the human hygenic problem.

The restlts of infection experiment showed that the infectivity raised by the oral administration was far lower as compared with that produced by the dipping method. This implies that the causative agent invaded the host not through the digestive tract. Similar results were also reported (Kusuda et al., 1981; Kusuda & Komatsu., 1978; Rasheed & Plumb., 1984). It is more likely that the streptococcicosis of the fish and the bullfrog were water borne or occured by direct contact.

All the four bullfrog isolated *Streptococcus spp* induced infection on bullfrog and produced 100% mortality in a condition of no noticed stress present, they are considered to be obligative pathogen of highly virulent to bullfrog. The pathogenecity of the tested strains on bullfrog seemed not effected by serially subcultures.

In the present study, these pathogenic organisms were not found from the currently used frog feed, so the origin of these organism is not known yet. Mtnami (1979),

and Yasunaga (1983) both reported that fish pathogenic streptococci had been isolated from fresh and frozen marine fishes such as sardine etc used for diet of cultured yellowtail. Marine fish also had been used as frog diet before the artificial pellet feed was developed. Occurrence of the streptococci in the dietary marine fishes remains to be investigated.

中 文 摘 要

1980年中至1984年底間由罹病牛蛙及其蝌蚪（參閱前報）中分離出 22 株鏈球菌，其形態學，生化學及血清學上之特性幾乎完全一致，僅在菌落之大小，對溫度之耐性，生長於 pH9.6 及 0.1 % methyleme blue milk 之能力，對於 bacitracin 之敏感性，在40% bile salt 中水解 esculin 之能力，以及分解 mannitol, trehalose 與 Salicin 之反應等特性上，菌株間出現差異性。

所有22株 Streptococci 均爲非溶血性而且非屬於 Lancefield group 中之 A, B, C, D,E 或 G 中之任一 group ，同時，病原性試驗顯示 4 株分離菌（bacitracin sensitive 及 resistant 各 2 株）對牛蛙有極高之病原性，對於虎皮蛙（*Rana tigrinars* Var.）之病原性則菌株間有顯著差異。對於鯉魚、吳郭魚及小白鼠則全無病原性。*Strep. faecalis* 及 *Strep facium* 則對以上各試驗動物均無致病性。

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