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Bacterial flora from a healthy freshwater Asian sea bass (*Lates calcarifer*) fingerling hatchery with emphasis on their antimicrobial and heavy metal resistance pattern

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ABSTRACT

This paper describes bacterial flora, the antimicrobial and heavy metal resistance pattern of bacteria isolated from a commercial freshwater Asian sea bass (*Lates calcarifer*) fingerling hatchery in Terengganu, Malaysia. This study was carried out to provide information on bacterial flora which can be isolated from water samples of freshwater Asian sea bass as well as its antimicrobial and heavy metal resistance pattern. This information may useful for fish farmers for treatment and prophylactic purposes. In the present study, bacteria were isolated from 26 freshwater sea bass fingerling tank water samples using Tryptic Soy Agar (TSA), Mac Conkey Agar, Thiosulphate Citrate Bile Salt Agar (TCBS), Eosin Methylene Blue Agar (EMB), Glutamate Starch Pseudomonas Agar (GSP), Xylose Lysine Deoxycholate Agar (XLD) and Baird Parker Agar. Bacterial identification was conducted using conventional biochemical tests and confirmed with a commercial bacterial identification kit. The antimicrobial resistance pattern of isolated bacteria was established using a disk diffusion method whereas the heavy metal resistance pattern (mercury, cadmium, chromium and copper) was determined using two fold agar dilution method. The results of the present study showed that *Aeromonas* spp. (n = 16), *Escherichia coli* (n = 20), *Edwardsiella tarda* (n = 8), *Pseudomonas* spp. (n = 20), *Salmonella* spp. (n = 24) and *Vibrio* spp. (n = 20) were successfully isolated and identified. The total colony forming unit (CFU) of bacteria on Tryptic Soy Agar (TSA), Mac Conkey Agar, Thiosulphate Citrate Bile Salt Agar (TCBS), Eosin Methylene Blue Agar (EMB), Glutamate Starch Pseudomonas Agar (GSP), Xylose Lysine Deoxycholate Agar (XLD) and Baird Parker Agar ranged from 1×10^5 to 2.6×10^7 CFU/mL. Overall, the multiple antibiotic resistance (MAR) index indicated that water samples were no under high risk exposure to the tested antibiotics. The antibiotic susceptibility test of bacterial isolates to 17 antibiotics (oxolinic acid 2 µg, ampicillin 10 µg, erythromycin 15 µg, lincomycin 15 µg, oleandomycin 15 µg, amoxicillin 25 µg, sulphamethoxazole 25 µg, chloramphenicol 30

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µg, doxycycline 30 µg, florfenicol 30 µg, flumequine 30 µg, fosfomycin (50 µg), kanamycin 30 µg, nalidixic acid 30 µg, tetracycline 30 µg, nitrofurantoin 50 µg and spiramycin 100 µg) showed that 75.2% were reported as sensitive cases whereas 19.4% and 5.4% were resistant and intermediately sensitive cases. More than 90% of the bacterial isolates were sensitive to chloramphenicol, kanamycin, oxolinic acid, florfenicol, nitrofurantoin, flumequine, fosfomycin, tetracycline and doxycycline. On the other hand, all bacterial isolates were resistant to all tested heavy metals except for copper (13.8%).

Key words: freshwater Asian Seabass, *Lates calcarifer* fingerling, antibiotic, heavy metal

Introduction

Nowadays, worldwide demand for fish is increasing rapidly and has led to fully exploited or overexploited wild fisheries (DELGADO et al., 2003). Therefore, the aquaculture industry is expanding rapidly world wide as well as in Southeast Asia (CHANG et al., 2001) to solve this problem. More new species have been introduced in to this sector which are suitable for human consumption as well as used to supply other related business activities (GARZA-GIL et al., 2009). One of the important species is Asian sea bass (*Lates calcarifer*). Asian sea bass, also known as barramundi, is an anadromous fish which can adapt to both freshwater and seawater environments and is widely cultured and marketed especially in Southeast Asia (PATERSON et al., 2003). Asian sea bass is a highly commercial fish species and popular seafood in Malaysia. Therefore, many sea bass hatcheries and farms have been established to produce Asian sea bass to meet local and overseas market demand. However, so far baseline information on antibiogram and bacterial species associated with Asian sea bass culture in Malaysia is still lacking. Therefore, this study was conducted to reveal the antibiogram and bacterial species associated with Asian sea bass culture. The results of the present study can give us insight information into the level and species of bacteria that may be found in Asian sea bass cultures as well as their antibiogram and heavy metal resistance pattern.

Materials and methods

100 mL of water samples were collected from 26 freshwater Asian sea bass fingerling tanks. This hatchery is located 200 m from the South China Sea, in Terengganu, Malaysia. The water parameters of the sampling site were measured using a pH meter (YSI, USA). The temperature, dissolved oxygen, pH and salinity of the sampling sites were 25 ^{o}C, 7.1 mg/L, 7.2 and 0.02 ppt, respectively.

One mL of each water sample was serially diluted in sterile physiological saline and plated on seven media; Tryptic Soy Agar (TSA), Mac Conkey Agar, Thiosulphate Citrate Bile Salt Agar (TCBS), Eosin Methylene Blue Agar (EMB), Glutamate Starch Pseudomonas Agar (GSP), Xylose Lysine Deoxycholate Agar (XLD) and Baird Parker Agar.

All the inoculated media were incubated at room temperature for 24 to 48 h. The bacterial colonies that grew on the selective media were selected for the identification

test. The obtained bacterial isolates were identified using conventional biochemical tests (HOLT et al. 1994) and confirmed with a commercial identification kit (BBL, USA). The selected bacterial isolates were then stored in 20% glycerol and kept at -80 °C as stock

The present isolates were cultured in tryptic soy broth (TSB) (Oxoid, England) at room temperature for 24 h. The bacterial cells were then centrifuged at 14,500 rpm for 5 min by using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted to a 10^6 colony forming unit (CFU) by using saline and monitored with a Biophotometer (Eppendorf, Germany) before being swabbed on the prepared Mueller Hinton agar (Oxoid, England). The antibiotic susceptibility test was conducted according to the Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Oxoid, England) (BAUER et al., 1966). The antibiotics tested including oxolinic acid (2 µg), ampicillin (10 µg), erythromycin (15 µg), lincomycin (15 µg), oleandomycin (15 µg), amoxicillin (25 µg), chloramphenicol (30 µg), doxycycline (30 µg), florfenicol (30 µg), flumequine (30 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), tetracycline (30 µg), nitrofurantoin (50 µg), fosfomycin (50 µg) and spiramycin (100 µg) (Oxoid, England). Interpretation of the resulted inhibition zones as sensitive (S), intermediary sensitive (I) and resistant (R) was made according to the standard provided by the manufacturer. Finally, the antimicrobial susceptibility of the isolates present was determined according to National Committee for Clinical Laboratory Standards (ANONYM, 2006).

The MAR index (multiple antibiotic resistance) of the present isolates against the tested antibiotics was calculated based on the formula as follows KRUMPERMAN (1985) and SARTER et al., (2007):

MAR index (multiple antibiotic resistance) = $X/(Y \times Z)$

X = total of antibiotic resistance case;

Y = total of antibiotic used in the study;

Z = total of isolates.

A MAR index value of equal or less than 0.2 meant that those antibiotics were seldom or never used for the animal in terms of treatment whereas a MAR index value higher than 0.2 means the animal have received high risk exposure to those antibiotics.

The heavy metal resistance test was carried out as described by MIRANDA and CASTILLO (1998) Bacterial tolerance to four elements of heavy metal, i.e. mercury (Hg^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}), was determined by the agar dilution method. Overnight bacterial suspension was spread onto plates of TSA medium incorporated with different concentrations of $HgCl_2$, CdCl, $K_2Cr_2O_7$ and $CuSO_4$ (Fluka, Spain). By two-fold dilutions, concentrations of both Cd^{2+} and Cr^{6+} ranged from 100 to 400 µg/mL while concentrations of Hg^{2+} and Cu^{2+} ranged from 25 to 400 µg/mL and 2400 to 600 µg/mL, respectively. For the purpose of defining metal resistance, the isolates were

considered as resistant if growth was obtained at a concentration of 100 µg/mL Cd^{2+} and Cr^{6+} and 600 µg/mL Cu^{2+} (ALLEN et al., 1977). The operational definition of tolerance as used in this study was based on positive bacterial growth when the concentration of heavy metals was above the stated concentration for resistance.

Results

Table 1. Total colony forming unit (CFU) of bacteria isolates (*Aeromonas* spp., *Escherichia coli*, *Edwardsiella tarda*, *Salmonella* spp., *Pseudomonas* spp. and *Vibrio* spp.) obtained from seabass fingerling hatchery

Tank	*Salmonella* spp. (CFU/mL)	*Vibrio* spp. (CFU/mL)	*Escherichia coli* (CFU/mL)	*Aeromonas* spp. (CFU/mL)	*Edwardsiella tarda* (CFU/mL)	*Pseudomonas* spp. (CFU/mL)
1	10^5	2×10^5	10^5	10^5	10^5	10^5
2	10^5	10^5	10^5	10^5	10^5	10^5
3	10^5	10^5	4×10^6	10^5	10^5	10^5
4	1.2×10^6	4×10^5	3.4×10^6	10^5	4.7×10^6	10^5
5	10^5	10^5	1×10^7	10^5	10^5	10^5
6	10^5	10^5	10^5	10^5	10^5	10^5
7	10^5	10^5	2.2×10^7	10^5	10^5	10^5
8	10^5	10^5	10^5	10^5	10^5	10^5
9	10^5	10^5	10^5	1×10^5	1.2×10^5	10^5
10	10^5	10^5	10^5	10^5	10^5	10^5
11	10^5	10^5	10^5	10^5	10^5	10^5
12	10^5	10^5	10^5	10^5	10^5	1.3×10^6
13	10^5	10^5	10^5	10^5	10^5	10^5
14	10^5	10^5	10^5	10^5	10^5	10^5
15	2.3×10^7	4.7×10^6	2.6×10^7	10^5	1.8×10^7	10^5
16	10^5	10^5	2×10^5	10^5	3.6×10^6	10^5
17	10^5	9.1×10^6	10^5	10^5	10^5	10^5
18	10^5	10^5	1.14×10^7	10^5	10^5	3×10^5
19	10^5	10^5	10^5	10^5	10^5	10^5
20	10^5	10^5	3.2×10^6	10^5	10^5	10^5
21	10^5	10^5	10^5	10^5	10^5	10^5
22	10^5	10^5	10^5	10^5	10^5	10^5
23	10^5	10^5	10^5	10^5	10^5	10^5
24	10^5	10^5	10^5	10^5	10^5	4×10^6
25	10^5	10^5	10^5	10^5	10^5	10^5
26	10^5	10^5	10^5	10^5	10^5	10^5

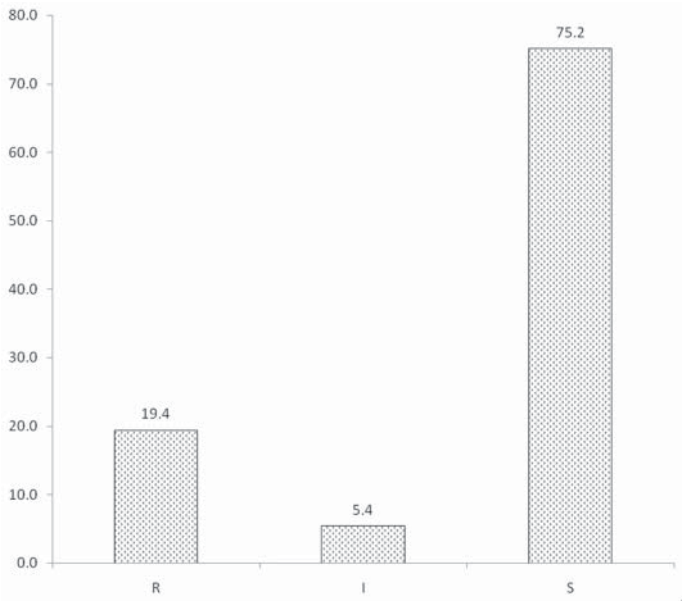

Fig. 1. Total percentage (%) sensitivity of 17 antibiotics against bacteria isolated from sea bass fingerling hatchery

Table 2. Multiple antibiotic resistance (MAR) value of bacterial isolates from freshwater Sea bass (*Lates calcarifer*) fingerling hatchery

Bacterial isolate	MAR value
Total bacterial isolates	0.19
Aeromonas spp.	0.36
Escherichia coli	0.08
Edwardsiella tarda	0.32
Salmonella spp.	0.23
Pseudomonas spp.	0.37
Vibrio spp.	0.08

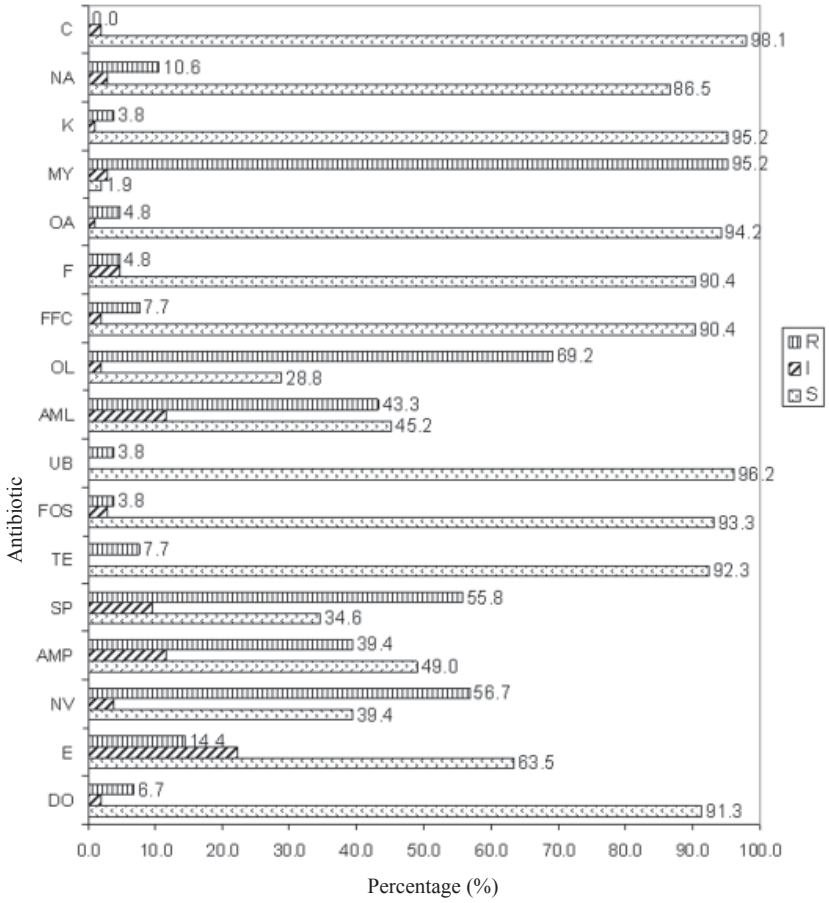

Fig. 2. Total percentage (%) sensitivity of 17 antibiotics against bacteria isolated from sea bass fingerling hatchery

No histamine producing bacteria were isolated in the present study. The total colony forming unit (CFU) of bacteria isolates (*Aeromonas* spp., *Escherichia coli*, *Edwardsiella tarda*, *Salmonella* spp., *Pseudomonas* spp. and *Vibrio* spp.) in the present study ranged from 1×10^5 to 2.6×10^7 CFU/mL (Table 1). The antibiotic susceptibility test of bacterial isolates to 17 antibiotics (oxolinic acid 2 µg, ampicillin 10 µg, erythromycin 15 µg, lincomycin 15 µg, oleandomycin 15 µg, amoxicillin 25 µg, sulphamethoxazole 25 µg, chloramphenicol 30 µg, doxycycline 30 µg, florfenicol 30 µg, flumequine 30 µg, fosfomycin (50 µg),

kanamycin 30 µg, nalidixic acid 30 µg, tetracycline 30 µg, nitrofurantoin 50 µg and spiramycin 100 µg) showed that 75.2% were reported as sensitive cases, whereas 19.4% and 5.4% were reported as resistant and intermediately sensitive cases (Fig. 1). All of *Vibrio* spp. in the present study were found sensitive to the tested antibiotics except for lincomycin and nalidixic acid in which none of *Vibrio* spp. strains were sensitive to lincomycin and only 20% of *Vibrio* spp. were sensitive to nalidixic acid. Florfenicol, flumequine and nitrofurantoin were found can control all strains of *Pseudomonas* spp. On the other hand, they were resistant to oleandomycin, amoxicillin and lincomycin. Flumequine, oxolinic acid and kanamycin were found to be able to control the growth of all strains of isolated *E. tarda* and *E. coli* but lincomycin and oleandomycin failed to do so. Nalidixic, kanamycin, flumequine, oxolinic acid, erythromycin and nitrofurantoin successfully inhibited the growth of all strains of *Aeromonas* spp. in the present study, while they were found resistant to oleandomycin and lincomycin. Overall, more than 90% of the bacterial isolates were sensitive to chloramphenicol, kanamycin, oxolinic acid, florfenicol, nitrofurantoin, flumequine, fosfomycin, tetracycline and doxycycline (Fig. 2). The multiple antibiotic resistance (MAR) index indicated that water samples were not under high risk exposure to the tested antibiotics (Table 2). All bacterial isolates were found resistant to mercury, cadmium and chromium. On the other hand, 86.2% of bacterial isolates were sensitive to copper.

Discussion

This is the first report on bacteria species found in freshwater Asian sea bass (*L. calcarifer*) hatcheries in Malaysia. It is very important to monitor bacterial disease causative agents such as *Aeromonas* spp., *Escherichia coli*, *Edwardsiella tarda*, *Salmonella* spp., *Pseudomonas* spp. and *Vibrio* spp. in which these bacteria may become serious or potential risk factors. A lack of awareness of types and levels of bacteria existing in an aquaculture system may cause the whole system to come under a potentially high risk of being devastated by bacterial diseases. The level of bacteria associated in the water sample of this hatchery in the present study was found high compared to other studies. For instance, the bacteria level in the rearing tank of Vendace (*Coregonus albula*) ranged from 10^3 to 10^4 CFU/mL (ZMYSLOWSKA et al., 2001). On the other hand, a high level of *Vibrio anguillarum* was observed in the study of MIZUKI et al. (2006), in which the concentration of *V. anguillarum* in the Japanese flounder, *Paralichthys olivaceus*, hatchery ranged from 10^5 to 10^6 CFU/mL. The authors claimed that bacteria existing in the fish's intestinal tract may have produced an antibacterial substance which killed pathogenic bacteria in the fish. In the present study, no abnormal behaviors or clinical signs were observed among the cultured Asian sea bass fingerlings in the present study. Furthermore, the fish farmer claimed that no disease outbreak had occurred since the hatchery started to

operate. Therefore, we may conclude that freshwater Asian sea bass in the present study are resistant to bacterial disease, maybe due to probiotics which exist in the intestinal tract of the fish. However, further study should be carried out before we come to a conclusion. In addition, no other scientific report on bacteria in freshwater Asian sea bass hatcheries has been recorded. Therefore, it is quite difficult to make a comparison in terms of levels and bacteria species in Asian sea bass hatcheries.

Chloramphenicol, kanamycin, oxolinic acid, florfenicol, nitrofurantoin, flumequine, fosfomycin, tetracycline and doxycycline were found capable of being used as antimicrobial agents for treatment and prophylactive purposes for the culture fish since more than 90% of the bacterial isolates were sensitive to these antibiotics. However, the Malaysian government has banned the use of chloramphenicol, oxolinic acid, nitrofurantoin and tetracycline in Malaysian aquacultures. Therefore, we suggest that fish farmers can use kanamycin, florfenicol, flumequine, fosfomycin and doxycycline as antimicrobial agents in their fish farms. Among 5 types of antibiotics (kanamycin, florfenicol, flumequine, fosfomycin and doxycycline), flumequine was found the most suitable antibiotic to be used in this hatchery as well as this area for aquatic health management, since this antibiotic shows the highest percentage of sensitive cases recorded in the present study. However, residues of this antibiotic may remain in the sediment and the surroundings after application. Therefore, this antibiotic has side effect directly on the external bacteria population within the aquatic ecosystem (LALUMERA et al., 2004). Therefore, fish farmers can use other antibiotics such as kanamycin, florfenicol, fosfomycin and doxycycline alternately, instead of using flumequine continually.

Overall, the multiple antibiotic resistance (MAR) index indicated that water samples were not under high risk exposure to the tested antibiotics and no histamine producing bacteria were isolated in the present study. Therefore, this area, as well as this hatchery were found suitable for aquaculture activity. There is very little information on heavy metal resistance patterns of bacteria from aquaculture sites. Therefore it is quite difficult to make a comparison of the heavy metal pattern of the present bacteria isolates to other studies. High heavy metal resistance cases were observed among the present bacterial isolates could be the result of heavy metal contamination with fertilizer, which contains heavy metal residues, since the hatchery in our study is in an agricultural areas. Thus, this may contribute to heavy metal resistance among the bacteria in the present study.

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WEI, L. S., N. MUSA, W. WEE: Bakterijska flora zdravoga mlađa azijskoga lubina (*Lates calcarifer*) s naglaskom na otpornost na antibiotike i teške metale. Vet. arhiv 80, 411-420, 2010.

SAŽETAK

Opisana je bakterijska flora te otpornost na antibiotike i teške metale bakterija izdvojenih iz mlađa slatkovodnoga azijskoga lubina (*Lates calcarifer*) na mrijestilištu u Terengganu u Maleziji. Istraživanje je provedeno sa svrhom da se odredi bakterijska flora u uzorcima vode na ribogojilištu azijskoga lubina kao i otpornost izdvojenih bakterija na antibiotike i teške metale. Rezultati mogu biti od koristi za uzgajivače riba pri liječenju i provođenju preventivnih mjera. Bakterije su bile izdvojene iz uzoraka vode uzetih iz 26 bazena. Za izdvajanje su bili upotrijebljeni triptoza sojin agar (TSA), MacConkeyev agar, tiosulfatni citratni agar sa žučnim solima (TCBS), agar s eozin-metilenskim modrilom (EMB), gutamat-škrobni agar za uzgoj pseudomonasa (GSP), ksiloza-lizin deoksikolatni agar (XLD) i Baird-Parkerov agar. Bakterije su bile identificirane pomoću uobičajenih biokemijskih testova, a njihova identifikacija bila je potvrđena pomoću komercijalnih kompleta. Otpornost na antibiotike bila je određivana difuzijskim postupkom, a na teške metale (živu, kadmij, krom i bakar) postupkom dvostrukih razrjeđenja na agaru. Izdvojene su i identificirane sljedeće vrste: *Aeromonas* spp. (n = 16), *Escherichia coli* (n = 20), *Edwardsiella tarda* (n = 8), *Pseudomonas* spp. (n = 20), *Salmonella* spp. (n = 24) i *Vibrio* spp. (n = 20). Ukupan broj bakterijskih kolonija na TSA, TCBS, EMB, GSP, XLD i Baird-Parkerovom agaru kretao se od $1x10^5$ do $2,6x10^7$ CFU/mL. Višestruka otpornost bakterija na antibiotike pokazala je da su uzorci vode bili izloženi visokom riziku od zagađenosti s testiranim antibioticima. Osjetljivost bakterijskih izolata bila je pretražena na 17 antibiotika (oksolinsku kiselinu 2 µg, ampicilin 10 µg, eritromicin 15 µg, linkomicin 15 µg, oleandomicin 15 µg, amoksicilin 25 µg, sulfametoksazol 25 µg, kloramfenikol 30 µg, doksiciklin 30 µg, florfenikol 30 µg, flumekvin 30 µg, fosfomicin 50 µg, kanamicin 30 µg, nalidiksičnu kiselinu 30 µg, tetraciklin 30 µg, nitrofurantoin 50 µg i spiramicin 100 µg). Pokazalo se da je 75,2% izolata bilo osjetljivo dok je 19,4% bilo otporno, a 5,4% umjereno osjetljivo na antibiotike. Više od 90% bakterijskih izolata bilo je osjetljivo na kloramfenikol, kanamicin, oksolinsku kiselinu, florfenikol, nitrofurantoin, flumekvin, fosfomicin, tetraciklin i doksiciklin. S druge strane, svi bakterijski izolati bili su otporni na sve upotrijebljene teške metale osim na bakar (13,8 %).

Ključne riječi: slatkovodni azijski lubin, *Lates calcarifer*, mlađ, antibiotici, teški metali