Turk. J. Vet. Anim. Sci.
30 (2006) 337-341

Short Communication

In-vitro Antimicrobial Susceptibility of Pathogenic Bacteria in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum)

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Received: 13.09.2005

Abstract: MIC_{50} and MIC_{90} values of ciprofloxacin, trimethoprim-sulphamethoxazole, tetracycline and erythromycin for 39 fish pathogenic bacterial strains (*Yersinia ruckeri*, *Enterococcus seriolicida*, and *Aeromonas salmonicida*) were determined using the E-test and agar dilution method. The MIC_{50} value of ciprofloxacin was approximately 1 mg/l for all fish pathogenic isolates. All fish pathogenic isolates showed elevated susceptibility to ciprofloxacin.

Key Words: *Yersinia ruckeri*, *Enterococcus seriolicida*, *Aeromonas salmonicida*, MIC, E-test, agar dilution method

Gökkuşağı Alabalıkları (*Oncorhynchus mykiss* -Walbaum)'nda Patojenik Bakterilerin İn-vitro Antibiyotik Duyarlılıkları

Özet: Balık patojeni olan 39 bakteriyel suşun (*Yersinia ruckeri*, *Enterococcus seriolicida*, *Aeromonas salmonicida*) siprofloksasin, trimethoprim-sulfamethaksazol, tetrasiklin ve eritromisine ait MIC_{50} and MIC_{90} değerleri E-test ve agar dilusyon metodu kullanılarak araştırıldı. Siprofloksasin'in MIC_{50} değeri tüm suşlar için yaklaşık olarak 1 mg/l olarak tespit edildi. Tüm izolatlar, in–vitro ortamda Siprofloksasin'e % 100 oranında duyarlılık göstermiştir.

Anahtar Sözcükler: *Yersinia ruckeri*, *Enterococcus seriolicida*, *Aeromonas salmonicida*, MIC, E-test, agar dilusyon metodu

Turkey has great potential for the fisheries industry because of its location. Having access to an excellent quality freshwater resource improves fish farming hatchery operations.

The major bacterial diseases in rainbow trout (*Oncorhynchus mykiss*, Walbaum) in Turkish freshwater hatcheries are the following: enteric redmouth disease (ERM), caused by *Yersinia ruckeri* (1); furunculosis, caused by *Aeromonas salmonicida* (2); and streptococcosis, caused by *Enterococcus seriolicida* (3).

Furunculosis is one of the major enzootic fish farm diseases in the world (2). Mortality rates may be as high as 90% and the disease can cause major economical losses in rainbow trout hatcheries. Yersiniosis can be peracute, acute or chronic in fish (1). Streptococcosis is an emerging animal pathogen that has been isolated from cattle, various fish species, and humans. With the development of intensive aquaculture, enterococcal infections of fish have become a major problem worldwide (3,4). These diseases have spread rapidly throughout Turkey and have also been diagnosed in many European countries (5,6).

Control of these diseases by the use of therapeutic antimicrobials has been possible in some situations; however the development of drug-resistant strains is an increasing problem (7). The risk of selecting for drug-resistant fish-pathogenic bacteria, some even causing disease in humans, remains probably the most critical concern in fish health (8). The correct selection of

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antimicrobial agents should decrease economic losses and more importantly decrease the risk to public health.

Several testing methods, including disc diffusion, broth microdilution, agar dilution and the E-test (AB Biodisk, Solna, Sweden), have been used to determine the in vitro susceptibilities of pathogenic bacteria to antimicrobial agents (9). The Clinical Laboratory and Standards Institute (CLSI, formerly the NCCLS) Subcommittee on Veterinary Antimicrobial Susceptibility Testing has standardized the agar dilution susceptibility testing method for bacteria that grow at warmer temperatures (i.e. 37 °C). In this study, the E-test was used to determine the antibiotic susceptibilities of fish-pathogenic bacteria. This method is less labor intensive, less time consuming and more practical than the agar dilution test for antibiotic susceptibility testing (10).

The aim of this study was to compare the E-test and agar dilution method and to evaluate the MIC_{50} and MIC_{90} values of various antibiotics against clinical isolates of fish pathogenic strains.

Antimicrobial susceptibilities of 3 strains of *Aeromonas salmonicida*, 10 strains of *Yersinia ruckeri* and 26 strains of *Enterococcus seriolicida* were investigated in this study. The strains were collected from clinical cases from 2000 to 2004, and were preserved by freezing at -80 ºC at the Adnan Menderes University Department of Microbiology.

Strains were revived on tryptic soy agar (TSA) and incubated at 25-28 °C (*Aeromonas salmonicida* and *Yersinia ruckeri*), and at 37 °C (*Enterococcus seriolicida*) for 24 h (11). Cultures were stored at 4 °C until experimental use.

The susceptibility of the 39 fish pathogenic bacterial strains to 4 antibiotics was measured. The following antimicrobials were tested: trimethoprim-sulphamethoxazole, erythromycin, tetracycline and ciprofloxacin. The antimicrobial susceptibility of isolates was investigated by means of the E-test System (AB Biodisk, Solna, Sweden). The E-test uses plastic strips. One side of the strip contains a concentration gradient of the antimicrobial agent and the other contains a numeric scale that indicates the drug concentration in μg ml^{-1} (12). Mueller Hinton agar plates (Oxoid, Basingstoke, UK) 4-mm thick were inoculated using a swab that had been submerged in a bacterial suspension standardized to match the turbidity of a 0.5 McFarland standard. The surface of the plate was swabbed in 3 directions to ensure a complete distribution of the inoculum over the entire plate. Within 20 min of inoculation, the antimicrobial agent strips were applied and the plates were incubated for *Y. ruckeri* and *A. salmonicida* (25-28 °C, 24 h) and for *E. seriolicida* (37 °C, 24 h) (13). All the tests were completed in duplicate. After incubation, the plate was examined and an elliptical zone of growth inhibition was observed around the strip. The minimal inhibitory concentration (MIC) was read from the scale on the strip at the intersection of the growth with the E-strip.

The agar dilution method was performed to determine MICs for *Y. ruckeri* and *A. salmonicida* (25-28 °C, 24 h) and for *E. seriolicida* (37 °C, 24 h) by using trimethoprim-sulphamethoxazole (Hoffman-La Roche, Inc.), tetracycline, erythromycin, ciprofloxacin (Sigma, St Louis, MO, USA) according to CLSI methods (14). Serial 2-fold dilutions were tested for antibiotic MIC determinations (from 0.03 to 256 mg/ml).

The comparison of MICs between agar dilution and the E-test for 39 fish pathogenic bacteria is shown in Table 1. MIC ranges, MIC_{50}, MIC_{90} and resistance rates are given in Table 2. An isolate was declared resistant based on CLSI breakpoints developed for mammalian pathogens at 37 °C. Currently, there are no resistance breakpoints available for any aquaculture pathogens in any fish species. Due to the lower optimal growth temperature ranges of aquatic bacteria (25-28 °C), the standard CLSI breakpoints developed for mammalian pathogens at 37 °C seems useless. Therefore, new methods regarding CLSI breakpoints for fastidious aquatic pathogens need to be developed for the future.

Yersinia ruckeri strains were 0% resistant to ciprofloxacin for the E-test, but they were 30% resistant to ciprofloxacin when the agar dilution method was used.

Enterococcus seriolicida was 0% resistant to ciprofloxacin for both methods utilized. The resistance rates of tetracycline and erythromycin were higher when using the agar dilution method, relative to the E-test (Table 2).

Aeromonas salmonicida strains were 0% resistance to ciprofloxacin for both methods utilized. All the results are given in Table 2.

The MIC_{50} value of ciprofloxacin was approximately 1 mg/l for all fish pathogenic isolates.

Globally the number of fish hatcheries is increasing. Hatching high numbers of fish together may sometimes cause outbreaks and economic losses. For rainbow trout,

Table 1. Comparison of antimicrobial MICs between agar dilution and the Etest for 39 fish pathogenic bacteria.

Bacterial Strains	Antimicrobial Agents	Methods	Number of isolates with MIC (mg/l) of														MIC_{50}	MIC_{90}
			0.03	0.06	0.13	0.25	0.50	1	2	4	8	16	32	64	128	256		
Enterococcus seriolicida (n=26)	Trimethoprim+ Sulphamethaxazole	AD			2	3	2	5	5	1		4	4				1	16
		E			1	2	5	4	3	5		3	2	1			2	16
	Tetracycline	AD			1	1	4	2	3	4	2	4	5				4	16
		E			2	2	5	3	5	2	3	1	3				2	16
	Erythromycin	AD					1	1	3	5	4	6	3	3			8	32
		E				2	1	3	5	2	2	4	4	1	1	1	4	32
	Ciprofloxacin	AD			1	3	5	5	8	4							1	2
		E			4	6	3	7	2	4							0.5	2
Aeromonas salmonicida (n=3)	Trimethoprim+ Sulphamethaxazole	AD						1		2							1	4
		E					2			1							0.5	4
	Tetracycline	AD										2	1				16	32
		E										2	1				16	32
	Erythromycin	AD									NT							
		E									1		1	1			32	64
	Ciprofloxacin	AD					2	1									0.5	1
		E				1	1	1									0.5	1
Yersinia ruckeri (n=10)	Trimethoprim+ Sulphamethaxazole	AD				1	3		1		4	1					2	8
		E				2	1			2	2	2		1			4	16
	Tetracycline	AD					1		5	2		1	1				2	16
		E			1	1	3			2		2	1				0.5	16
	Erythromycin	AD									NT							
		E			1	1	2	1	1		2	1	1				1	16
	Ciprofloxacin	AD					2	2	1	2	2	1					2	8
		E				2	1	3	4								1	2

AD, Agar Dilution. E, E-test. NT, not tested. MIC_{50}, minimal inhibitory concentration including 50% of the strains; MIC_{90}, minimal inhibitory concentration including 90% of the strains.

Table 2. The MIC ranges, MIC_{50}, MIC_{90} values and resistance rates.

Bacterial Strains	Antimicrobial Agents	Methods	MIC range (mg/l)	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Resistance %
Enterococcus seriolicida (n=26)	Trimethoprim+ Sulphamethaxazole	AD	0.13-32	1	16	31
		E	0.13-64	2	16	23
	Tetracycline	AD	0.13-32	4	16	53
		E	0.13-32	2	16	27
	Erythromycin	AD	0.50-64	8	32	86
		E	0.13-64	2	16	50
	Ciprofloxacin	AD	0.13-4	1	2	0
		E	0.13-4	0.5	2	0
Aeromonas salmonicida (n=3)	Trimethoprim+ Sulphamethaxazole	AD	1-4	1	4	67
		E	0.50-4	0.5	4	33
	Tetracycline	AD	16-32	16	32	100
		E	16-32	16	32	100
	Erythromycin	AD	No data			
		E	8-64	32	64	100
	Ciprofloxacin	AD	0.50-1	0.5	1	0
		E	0.25-1	0.5	1	0
Yersinia ruckeri (n=10)	Trimethoprim+ Sulphamethaxazole	AD	0.25-8	2	8	50
		E	0.25-64	4	16	70
	Tetracycline	AD	0.50-16	2	16	40
		E	0.13-32	0.5	16	30
	Erythromycin	AD	No data			
		E	0.13-32	1	16	40
	Ciprofloxacin	AD	0.50-8	2	8	30
		E	0.25-2	1	2	0

furunculosis, yersiniosis and streptococcosis are considered the most important diseases by several researchers (15).

Many fish farms release effluent from ponds without treatment. Various amounts of antibiotic residues may still be present in the effluent following antibiotic therapy (16). Numerous studies suggest a correlation between findings of increased bacterial resistance levels on and around inland fish farms and the use of antimicrobial agents at the farms (17,18).

Previous investigations on bacterial infections in Turkish freshwater rainbow trout hatcheries have focused on disease outbreaks from a single pathogen (15,19). Kirkan et al. (15) identified *Yersinia ruckeri* in rainbow trout obtained from the Aegean region of Turkey. *Yersinia ruckeri* strains were only found to be susceptible to streptomycin. In contrast, all *Yersinia ruckeri* strains presented resistance to penicillin, chloramphenicol, kanamycin, erythromycin, and ampicillin. In another study, the same investigators (19) identified *Aeromonas salmonicida* in rainbow trout obtained from a Turkish hatchery farm. They showed that *A. salmonicida* strains were susceptible to streptomycin and ciprofloxacin, but resistant to penicillin, erythromycin and cefuroxime sodium.

In our study, all *A. salmonicida* and *E. seriolicida* strains isolated from rainbow trout samples were susceptible to ciprofloxacin for both methods. However, *Y. ruckeri* strains were 30% resistant to ciprofloxacin with the agar dilution method. No differences in MICs were found between the E-test and agar dilution methods in the resistance percentages of *E. seriolicida* strains.

All strains had approximately the same MIC_{50} values for ciprofloxacin. *Y. ruckeri* strains had increased resistances to trimethoprim-sulphamethaxazole with MIC values of between 0.25 and 64.0 mg/l (MIC breakpoints ≥4/76 resistant) (20). Of 13 *E. seriolicida* strains (50%) were resistant to erythromycin (MIC values 0.13-64 mg/l) for the E-test. Based on the results of agar dilution method for *E. seriolicida* strains, a total of 23 strains

(86%) were found to be resistant to erythromycin (MIC breakpoints ≥8 resistant) (20) (Table 2).

The use of antimicrobial drugs in aquaculture has been under tight scrutiny in the past 2 decades (8), mainly due to the social and ecological significance of fish farming. Reasons at the forefront include a potential toxicity of drugs or their metabolites in consumers. This has been addressed through the definition of maximal residue limits (MRLs) (12). Presently, the risk of selecting drugs that may select for not only drug-resistant fish pathogenic bacteria but also human health pathogens has lead to a rising concern over worldwide fish health practices. Medical authorities are especially vigilant in this domain, and the resulting debates have led legislators to dictate requirements, clearly limiting the veterinary use of antimicrobial drugs in aquaculture (8). Some products have been prohibited from use in food producing animals in some countries (i.e. fluoroquinolones in the USA), and others have been replaced with chemical species restricted for use in non-food animals.

In conclusion, the results obtained from this study show that, for the control of fish bacterial diseases, the selection of correct antimicrobial agent is very important. Thus, an antibiotic with a minimal residue limit should be selected to protect human health from potential hazards caused by contaminated fish meat.

References

1. Rucker, R.: Redmouth disease of rainbow trout (*Salmo gairdneri*). Bull. Off. Int. Epizoot., 1966; 65: 825-830.
2. Wiklund, T., Dalsgaard, I.: Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species, a review. Dis. Aquat. Organ., 1998; 32: 49-69.
3. Eldar, A., Ghittino, C., Asanta, L., Bozzetta, E., Goria, M., Prearo, M., Bercovier, H.: *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. Curr. Microbiol., 1996; 32: 85-88.
4. Elliott, J.A., Collins, M.D., Pigott, N.E., Facklam, R.R.: Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns. J. Clin. Microbiol., 1991; 29: 2731-2734.
5. Meier, W.: Enteric redmouth disease: outbreak in rainbow trout in Switzerland. Dis. Aquat. Organ., 1986; 2: 81-82.
6. Rintama¨Ki, P., Valtonen, E.T., Frerichs, G.N.: Occurrence of *Yersinia ruckeri* infection in farmed whitefish, *Coregonus peled* Gmelin and *Coregonus muksun* Pallas, and Atlantic Salmon, *Salmo salar* L., in Northern Finland. J. Fish Dis., 1986; 9: 137-140.
7. Munro, A.L.S., Hasting, T.S.: Furunculosis, Bacterial Disease of Fish (Eds by Inglis V., Roberts J.R., Bromage N.R.), Blackwell Scientific Publications, Oxford, 1993; pp 122-142.
8. Gustafson, R.H., Bowen, R.E.: Antibiotic use in animal agriculture. J. Appl. Microbiol., 1997; 83: 531-541.
9. Huang, M.B., Baker, C.N., Banerjee, S., Tenover F.C.: Accuracy of the E-test for determining antimicrobial susceptibilities of staphylococci, enterococci, *Campylobacter jejuni* and Gram-negative bacteria resistant to antimicrobial agents. J. Clin. Microbiol., 1992; 30: 3243-3248.
10. Ng, L, K., Sawatzky, P., Galas, M., Rodrigues Ddos, P., Heitmann, I., Fernandez, S., Bolmstrom, A.: Can E-test be used to determine *Vibrio cholerae* susceptibility to erythromycin? Antimicrob. Agents Chemother., 2003; 47: 1479-1480.
11. Holt, J.G., Krieg, N.R., Sneath, P.H.A., Stanley, J.T., Williams, T.S.: Bergey's Manual of Determinative Bacteriology. William & Wilkins, Baltimore, Maryland, USA, 1994; pp. 190-191.
12. Bolmström, A.: Susceptibility testing of anaerobes with E-test. Clin. Infect. Dis., 1993; 16 (Suppl. 4): 367–370.
13. Walsh, T.R, Bolmström, A., Qwärnström, A., Gales, A.: Evaluation of a new E-test for detecting metallo-beta-lactamases in routine clinical testing. J. Clin. Microbiol., 2002; 40: 2755-2759.
14. Clinical Laboratory Standards (CLSI): MIC Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. NCCLS document M7 A6. 2003.
15. Kirkan, S., Goksoy, E.O., Kaya, O.: The isolation of *Yersinia ruckeri* from rainbow trouts in Aydin region. J. Pendik Vet. Microbiol., 2000; 31: 27-30.
16. Smith, P., Donlon, J., Coyne, R., Cazabon, D.: Fate of oxytetracycline in a freshwater fish farm: influence of effluent treatment systems. Aquaculture, 1994; 120: 319-325.
17. McPhearson, R.M., Depaola, A., Zywno, S.R., Motes Jr., M.L., Guarino, A.M.: Antibiotic resistance in Gram-negative bacteria from cultured Catfish and aquaculture ponds. Aquaculture, 1991; 99: 203-211.
18. Spanggaard, B., Jorgensen, F., Gram, L., Huss, H.H.: Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture, 1993; 115: 195-207.
19. Kirkan, S., Göksoy, E.O., Kaya, O.: Isolation and antimicrobial susceptibility of *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss*) in Turkey hatchery farms. J. Vet. Med. B, 2003; 50: 339-342.
20. Clinical Laboratory Standards (CLSI): Minimal inhibitory concentration (MIC) breakpoints for Enterobacteriaceae and Enterococcus spp. M100-S13, 2004.