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Short communication

RESISTANCE PATTERNS OF *AEROMONAS SALMONICIDA* TO COMMON ANTIMICROBIAL AGENTS

Eshetu Yimer[1]

National Animal Health Research Centre
PO Box 181689, Addis Ababa, Ethiopia

ABSTRACT: The antibiotic sensitivity profiles of a collection of *Aeromonas salmonicida* isolates obtained from a range of geographical locations in Scotland was investigated. The investigation was part of a collaborative study to characterise *A. salmonicida* on a range of parameters which include cytotoxicity, virulence, autoagglutination and outer membrane protein profiles. Twenty eight isolates, including *A. salmonicida* FCS strain and *A. salmonicida* 1102, were subjected to antimicrobial sensitivity testing of 11 antimicrobial agents by a disc diffusion method and an agar dilution method to determine minimum inhibitory concentrations (MIC's). The semi-quantitative results of the disc test were confirmed and a more precise value obtained by the second method. Seven different sensitivity patterns were detected. All the 12 isolates which were resistant to oxolinic acid were found to be resistant to the 5 antimicrobial agents oxytetracycline, oxolinic acid, sulphamethoxazole and the potentiated-sulphonamides Romet-30 and co-trimoxazole.

Key words/phrases: *Aeromonas salmonicida*, antimicrobial, disc diffusion, minimum inhibitory concentration, resistance

INTRODUCTION

Aeromonas salmonicida is the causative organism of furunculosis, a haemorrhagic septicaemic disease of salmonids. Improved husbandry techniques and environmental conditions have been applied to control furunculosis

[1] Current address: Ethiopian Health and Nutrition Research Institute, PO Box 1242, Addis Ababa, Ethiopia

(McCarthy and Roberts, 1980). However, when there are outbreaks it is essential to use chemotherapy to control the disease.

A number of antimicrobials are in current use in aquaculture in the different parts of the world. These include oxytetracycline and ormethoprim-sulphadimethoxine (Romet-30) approved by Federal Drugs Administration (FDA) for use in aquaculture in USA (Stamm, 1989) and oxytetracycline, potentiated-sulphonamides, oxolinic acid and amoxicillin for use in Great Britain (V. Inglis, personal communication). The utilization of antimicrobials to the entire sick and normal fish population by fish culturists has been suggested as the main cause of the emergence of drug resistance among fish bacterial pathogens (Hahnel and Gould, 1982). Partial inappatence of fish due to disease may result in fish taking sub-inhibitory doses of drugs with the feed and could contribute to the emergence of low level resistance. Cross resistance to the quinolones was recorded in laboratory induced mutants and reduced susceptibility to oxytetracycline of the quinolone-developed mutants (Stamm, 1989). Transfer of tetracyclines and sulphonamides drug resistance determinants were recorded in *A. salmonicida* (Aoki *et al.*, 1983).

The aims of this study were:
- to determine the antimicrobial sensitivity profiles of a collection of *A. salmonicida* isolates;
- to analyze the frequency and nature of single and multiple antibiotic resistance; and
- to investigate the stability of antibiotic resistance.

MATERIALS AND METHODS

Bacteria

A total of 28 strains of *A. salmonicida* were used in this study. The bacteria were from the Institute of Aquaculture and had been isolated from outbreaks of furunculosis in salmon in different parts of Scotland during the years 1989 and 1990. They had been transferred for further purification and stored in 'protect' beads at -70° C.

For the purpose of reference *Escherichia coli* NTCC 10418 known sensitive and *Pseudomonas aeruginosa* ATCC 27853 known resistant were included. In addition, *A. salmonicida* 1102 known non pathogenic (National Collection of

Marine Bacteria, Aberdeen) and *A. salmonicida* FCS strain known pathogenic (Marine Harvest, Scotland) were also included in this study.

Antimicrobials

The following antibiotic solutions were separately prepared at concentrations of 160 $\mu g(ml)^{-1}$ to determine the MIC's: Oxolinic acid, Oxytetracycline, Amoxicillin, Romet-30 (potentiated sulphonamide), Chloramphenicol and Florfenicol.

Determination of antimicrobial sensitivity by the disc diffusion method

The antibiotics used for the disc diffusion method included: Co-trimoxazole-25 μg (containing Sulphamethoxazole-23.75 μg and Trimethoprim 1.25 μg); Furazolidone 50 μg; Sulphamethoxazole 25 μg; Nitrofurantoin 100 μg and Enrofloxacin 5 μg, all obtained from the Oxoid Unipath Limited Hampshire, England, in addition to the drugs used for the determination of MIC's *i.e.*, Oxolinic Acid 2 μg, Oxytetracycline 30 μg, Amoxicillin 10 μg, Potentiated Sulphonamide (Romet-30) 25 μg, Chloramphenicol 10 μg and Florfenicol 30 μg. The antibiotics sensitivity patterns of the different isolates of *A. salmonicida* were determined according to Acar and Goldstein (1986). The zone diameter for individual antibiotics were translated as susceptible, intermediate and resistant with reference to an interpretative chart (NCCLS, 1990b).

Determination of minimum inhibitory concentrations (MIC's)

To determine the MIC's a modification of the approved standard agar plate doubling dilution method of the US National Committee for Clinical Laboratory Standards (NCCLS, 1990a) was used.

RESULTS

Determination of antibiotic sensitivity

The isolates of *A. salmonicida* tested for antibiotic sensitivity patterns by the disc test exhibited seven different resistance patterns based on the number of antimicrobials resisted (Table 1).

All the 12 isolates of *A. salmonicida* which were resistant to oxolinic acid were also resistant to oxytetracycline. The isolates having an intermediate sensitivity

to oxolinic acid by the disc test were found to be resistant to the drug by the MIC test.

The results of the disc test indicated that almost two-third of the isolates were resistant to oxytetracycline, half to oxolinic acid, three-quarter to sulphamethoxazole, one-quarter to co-trimoxazole and potentiated-sulphonamide (Romet-30). All of the isolates which were resistant to Romet-30 also showed similar resistance to co-trimoxazole and sulphamethoxazole. From the results obtained, each isolate was resistant to at least one antimicrobial agent except *A. salmonicida* FCS strain. Six out of the 28 isolates, which represent 21.42%, were found to be resistant to the 5 antimicrobials oxytetracycline, oxolinic acid, sulphamethoxazole and the potentiated-sulphonamides Romet-30 and co-trimoxazole. Intermediate results for the disc test were obtained in furazolidone, oxolinic acid and sulphamethoxazole while in all the rest of the antimicrobials the zone diameters were within the limits of either the resistant or sensitive range.

Table 1. The resistance patterns of *A. salmonicida* isolates.

Antimicrobial agents	Disc content in μg	Sensitive	Intermediate	Resistant
Oxolinic acid	2	13	1	14
Oxytetracycline	30	9	0	19
Romet-30	25	21	0	7
Co-trimoxazole	25	21	0	7
Sulphamethoxazole	25	7	0	25
Nitrofurantoin	100	25	0	3
Furazolidone	50	26	2	0
Amoxicillin	10	28	0	0
Chloramphenicol	10	28	0	0
Florfenicol	30	28	0	0
Enrofloxacin	5	28	0	0

Determination of minimum inhibitory concentrations (MIC's)

The isolates were classified into two groups as sensitive and resistant to oxolinic acid as determined by the MIC's ranging from 0.02–0.08 $\mu g(ml)^{-1}$ and 1.25–5 $\mu g(ml)^{-1}$, respectively (Table 2).

Two-third of the isolates were resistant to oxytetracycline and three of the isolates were not inhibited from growth at 80 $\mu g(ml)^{-1}$ of the drug. The distribution of the resistance pattern for oxolinic acid and oxytetracycline appeared to be bimodal with the majority of the isolates occupying the resistant portion of the graph for oxytetracycline.

Table 2. Distribution of the MIC values of the 28 isolates.

MIC values $\mu g(ml)^{-1}$	Antimicrobial agents					
	AML	CAF	298/30	OA	OT	Romet-30
0.005						
0.01						
0.02				1		
0.04				10		
0.08	2			1	3	
0.15	21	1			6	
0.30	5	15	8			
0.60		12	20			1
1.25				3		20
2.5				10		1
5				3		4
10						
20					5	
40					9	
80					2	2
>80					3	

From the total of the 28 isolates 16 were resistant to oxolinic acid, 19 to oxytetracycline, 7 to Romet-30 while all the isolates were found to be sensitive to amoxicillin, chloramphenicol and florfenicol with MIC values ranging from 0.08–0.3, 0.15–0.6 and 0.3–0.6 $\mu g(ml)^{-1}$ for each of the drugs, respectively. The MIC's of the two closely related drugs chloramphenicol and florfenicol were within the same range.

Multiple resistance was recorded in 6 of the isolates. They were found to be resistant to 5 antimicrobials oxytetracycline, oxolinic acid, sulphamethoxazole and potentiated sulphonamides (Romet-30 and co-trimoxazole).

DISCUSSION AND CONCLUSION

There was not much difference between the disc and agar dilution tests except for the two isolates B90153 (3) and B90118 (2) which were sensitive and intermediate by the disc test, respectively and both resistant by the MIC test. Although the disc method gives consistent and reliable results when performed carefully, the agar dilution method of determining MIC's test gave clear cut end points and was more accurate in differentiating the sensitivity patterns of those isolates of *A. salmonicida* that have intermediate or partly sensitive results by the disc test.

Precise interpretive standards based on the disc diffusion method of Bauer *et al.* (1966) such as used in testing antimicrobial susceptibility in human pathogens have not been developed for fish pathogens where standards are still being developed (Hahnel and Gould, 1982). This study supports the findings of Inglis *et al.* (1991) and makes a contribution to such standards.

Resistance of *A. salmonicida* to oxolinic acid in this study was similar to that of laboratory induced resistant mutants (Hastings and Mckay, 1987) but was higher than the findings of Aoki *et al.* (1983) where 61 of 129 *A. salmonicida* strains were resistant to this drug.

All the isolates in this study were sensitive to the fluoroquinolone enrofloxacin and results of field trials by Bowser *et al.* (1990) supported further evaluation of enrofloxacin as a candidate compound for use in aquaculture.

Resistant to oxytetracycline was high and the pattern was similar in both tests. The three most resistant isolates in the case of oxytetracycline were not inhibited by 80 $\mu g(ml)^{-1}$ which meant that their MIC was at least 500 fold higher than the sensitive isolates. Oxytetracycline is widely used in Scotland (Richards *et al.*, 1991) and this may be associated with the high level of resistance.

The results of the antibiotic sensitivity to the two chemically closely related antibiotics chloramphenicol and florfenicol indicated that all the 28 isolates were sensitive to both tests. Florfenicol is a broad spectrum antibacterial similar to chloramphenicol, with greater *in vitro* potency against pathogenic organisms than chloramphenicol (Adams *et al.*, 1987). It has high activity against chloramphenicol-resistant strains of bacteria and this should make it a good

antibiotic to replace chloramphenicol which has been banned from use in food animal medicine.

Amoxicillin was licensed for use in aquaculture in the United Kingdom in 1990 (V. Inglis, personal communication) and all the isolates tested were susceptible. Although it has not been used sufficiently to exert a selection pressure, 4 out of 209 strains of *A. salmonicida* recently isolated from salmon in Scotland were found to be resistant (Richards *et al.*, 1991) representing an intrinsic resistance.

The marked improvement in the anti-bacterial activity conferred on sulphonamides, when linked to trimethoprim, clearly indicated their potential for use in the treatment of fish bacterial diseases (McCarthy *et al.*, 1974).

All the 12 isolates which were resistant to oxolinic acid were also found to be resistant to oxytetracycline reflecting the development of cross resistance. From the total of 20 isolates showing multiple drug resistance 15 had a shared resistance to oxytetracycline and sulphamethoxazole. The recorded multiple resistance in six of the isolates is a matter of considerable concern.

There is no major difference between the disc diffusion method and the agar dilution method of antimicrobial sensitivity testing if the bacterial lawns are prepared carefully for the disc method. Antimicrobial sensitivity testing should precede the use of antimicrobial in fish farms.

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