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Antibiotic resistance of *Pseudomonas* species isolated from Armenian fish farms

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Antibiotic resistance became one of the major problems of human race. Extensive and unrestricted antibiotic usage in aquaculture can bring to development of antimicrobial resistance in water environments which in turn can transfer to humans via horizontal gen transfer or even directly. Therefore, there is urgent need to monitor resistance patterns in aquaculture points. *Pseudomonas* spp. are widespread in water environments hence they are suitable to use for evaluation of antibiotic resistance. The goal was to investigate occurrence of antibiotic resistance within *Pseudomonas* spp. in Armenian fish farms. Samples were taken from outlet and inlet water of different ponds of Armenia, as well as from various organs of fish by swabbing method. Identification of isolates was done according to cultural, morphological and biochemical characteristics, as well as by API-20E rapid identification system. Antibiotic susceptibility was evaluated by disk diffusion assay against 16 antibiotics. 24 *Pseudomonas* strains were isolated which belong to six species: P. *anguilliseptica*, P. *fluorescens*, P. *stutzeri*, P. *putida*, P. *aeruginosa* and P. *alcaligenes*. The obtained data showed that all isolates were resistant to at least three antibiotics. High level resistance was detected toward tetracyclines (tetracycline and oxytetracycline) and chloramphenicol within the strains isolated from fish and outlet water, whereas all strains isolated from inlet water were susceptible to mentioned antibiotics. Almost all strains had multiple antibiotic resistance index above 0.2 with the exception of two strains. Thus, high level antibiotic resistance within *Pseudomonas* spp. in Armenian fish farms was found.

Key words: *Pseudomonas* spp., antibiotic resistance, rainbow trout *Oncorhynchus mykiss*, fish pathogen, fish farm, disk diffusion assay

Introduction

Antibiotic resistance became one of serious health care problems of humanity since late 20th century [Ginovyan et al. 2015; World Health Organisation 2006]. Antimicrobial resistance of human pathogens can developed as a consequence of usage of antibiotics in human therapy and animal agriculture [CDC2013; World Health Organisation 2006]. Although the risk arisen from aquaculturing considered to be lower compared to terrestrial animal agriculturing, however in recent years particular attention was given to this issue [World Health Organisation 2006]. This is partly due to lack of data and control over usage of antibiotics in aquaculture [Romero et al. 2012; World Health Organisation 2006]. On the other hand it is well known that in many countries the usage of antibiotics in fish ponds is very

high which can bring to development of antibiotic resistance. This is in turn can transfer to human pathogens via horizontal gen transfer or even directly [Cabello 2006; Heuer et al. 2009; Marshall and Levy 2011; World Health Organisation 2006]. Therefore, it is very important to monitor and control antibiotic usage, as well as monitor antibiotic resistance in aquafarming points worldwide [World Health Organisation 2006].

Pseudomonas species are widespread in aquatic environments and are associated with both healthy and infected fishes [Allen et al. 1983; Austin and Austin 2007]. The wide prevalence of these bacteria in water environments is partly due to their ability to grow at low temperatures [Gram 1993]. They are ubiquitous in fish tissues and have been isolated from gills, skin and intestine of fishes [Lerma et al. 2014; Tripathy et al. 2007]. *Pseudomonas* species are well known opportunistic pathogens in fish farms and it is considered that they can be involved in various fish infectious diseases [Allen et al. 1983; Altinok et al. 2006; Austin and Austin 2007; Lerma et al. 2014; López-Romalde et al. 2003; Tripathy et al. 2007].

Fish farming is well developed in Armenia in the recent two decades. There are more than 200 fish farms in Armenia which were mainly located in the area of Ararat plain. The main source of water for fish farming are artesian wells [Grigoryan et al. 2014]. These fish farms all have their own wells (with a capacity of 50–100 L/s) through which water arrives under pressure [Statistical Yearbook ... 2014]. Estimations based on the reported quantity of produced fish per year in Armenia indicate that between 2005 and 2008 annual per capita consumption increased sharply from 0.3 kg to 1.8 kg [UNCTAD2011].

The antibiotic usage in Armenian fish farms have not regulated yet. The prevalence of fish infectious diseases are continuously increasing as consequence of enlargement of production volumes. Therefore the usage of antibiotics to control these diseases, as well as for prophylactic purposes, is also increasing. This can lead to development of acquired resistance toward used antibiotics. The most commonly used antibiotics were tetracyclines (oxytetracycline and tetracycline) and chloramphenicol. There was no data about possible antibiotic resistance in Armenian fish farms. Therefore, there was an urgent need to explore situation referring antibiotic resistance in Armenian fish farms.

The main goal of our research was to determine the occurrence of antibiotic resistance within *Pseudomonas* spp. isolated from different Armenian fish farms in order to obtain general image of problem in the region.

Materials and methods

Sampling. Samples were taken from skin, caudal fin, eyes, gills and internal organs of fish *Oncorhynchus mykiss* of different farms of Armenia by swabbing method from February 2015 to Jule 2015 (See Table 1). Also samples were taken from inlet and outlet waters of various fish farms. Swabs were transferred to following mediums: Pseudomonas agar base (HiMedia, M085), (Pseudomonas Agar (For Fluorescein (HiMedia, M120), Pseudomonas Isolation Agar Base (HiMedia, M406), fluorogenic mixture containing Hifluoro Pseudomonas Agar Base (HiMedia, M1469) and Aero Pseudo Selective Agar (HiMedia, M1620). Plates were incubated at 28 °C for 24–48 hours.

Identification. Identification of isolates was done according to cultural, morphological and biochemical characters, as well as by using Analytical profile index (API-20E) rapid identification system (See Table 2).

Determination of hemolytic activity. The hemolytic activity of *Pseudomonas* strains was measured on Columbia Blood agar base (HiMedia, M144) plates supplemented with 5% human red blood cells. The diameter of hemolysis produced after 24 h incubation at 30 °C was measured using a precision ruler [Santos et al. 1999].

Determination of proteolytic activity. Proteolytic activity of isolates were determined by method described by Vijayaraghavan et al. [2013]. The bacterial isolates were streaked on casein agar medium supplemented with bromocresol green (BCG) reagent (peptic digest of animal tissue (5.0 g/l), beef extract (1.5 g/l), yeast extract (1.5 g/l), sodium chloride (5.0 g/l), agar (15 g/l), casein (10 g/l) and 0. 0015% (w/v) BCG) and incubated at 37 °C for 48 h. A zone of proteolysis was detected on the casein agar plates.

Antibiotic susceptibility testing. Agar disc diffusion method [Matuschek et al. 2014) was

used to determine antibiotic susceptibility of isolated *Pseudomonas* spp. Growth medium (25 ml) (Mueller-Hinton agar medium (MHA) (HiMedia, India) was poured into Petri dishes at 50–60 °C and left to solidify under ultraviolet (UV) light (265 nm wavelength) for 15 minutes. Subsequently, a sterile cotton swab was dipped into bacterial suspensions (prepared from overnight cultures) of test strains (adjusted to turbidity of 0.5 McFarland Standard). An agar plate was inoculated by evenly streaking cotton swab over the agar medium. Then antibiotic discs were placed on the medium (maximum six discs for each Petri dish). The plates were incubated at 37 °C for 18–20 hour. Then the diameters of growth inhibition zones around the discs were measured.

Following commercial antibiotics disks were used; tetracycline (T) 30 μg, erythromycin (E) 10 μg, streptomycin (S) 25 μg, chloramphenicol (C) 30 μg, amikacin (Ak) 10 μg, neomycin (N) 30 μg, oxytetracycline (O) 30 μg, ampicillin (AMP) 10 μg, amoxicillin (AMO) 30 μg, imipenem (I) 10 μg, pefloxacin (PFLX) 5 μg, cefotaxime (CTX) 10 μg, ciprofloxacin (CIP) 5 μg), piperacillin (Pi) 100 μg, trimethoprim (TR) 5 μg, gentamicin (G) 30 μg (HiMedia, India).

Determination of multiple antibiotic resistance (MAR) index. MAR index of *Pseudomonas* isolates was determined by following formula; $MAR = {}^{a}/_{b}$, where a is the number of antibiotics toward which strains were resistant and b is the total number of tested antibiotics. The MAR index, which is higher than 0.2 (>0.2) identifies bacteria isolated from objects with higher risk of contamination, where antibiotics has been often used [Krumperman 1983].

Statistical analysis. All experiments were independently repeated at least three times. Obtained data were processed; standard deviations were calculated using GraphPad Prism 5.03 (GraphPad Software, Inc.; USA) software. One-way paired T-test was used for sample comparison in order to examine the alternative hypothesis (H1) that antibiotic resistance in *Pseudomonas spp.* isolated from outlet water of fish farms was higher than in strains isolated from inlet water *and* to compare multiple resistance of *Pseudomonas spp.* isolated from outlet and inlet water of fish farms. P-values less than 0.05 were considered as statistically significant.

Results and Discussion

Isolated *Pseudomonas* spp. *Pseudomonas* spp. are ubiquitous aquatic bacteria which are widespread in freshwater environments [Allen et al. 1983]. On the other hand some of *Pseudomonas* spp. are considered as opportunistic pathogens (including P. *aeruginosa*, P. *fluorescens*, P. *putida*, P. *stutzeri* and P. *anguilliseptica*) both for humans and animals [Peix et al. 2009]. Therefore we have chosen this species in order to monitor the level of antibiotic resistance in Armenian fish farms. During experiments 24 *Pseudomonas* spp. were isolated (See Table 1).

Isolation of *Pseudomonas* strains was done from fish farms of two main fish industry regions Masis and Armavir. Identification of isolates at species level was done by biochemical analysis (See Table 2).

According to obtained data five strains were identified as *P. anguilliseptica* (20.8%), four strains were *P. fluorescens* (16.7%), three strains were *P. stutzeri* (12.5%), five strains were *P. putida* (20.8%), five strains were *P. aeruginosa* (20.8%) and only two strains were *P. algaligenes* (8.3%). Bacterial cultures of all isolates were referenced in the microbial bank of Yerevan State University, Armenia.

Occurrence of antibiotic resistance. Antimicrobial susceptibility of *Pseudomonas* isolates were assessed toward 16 antimicrobial agents which belong to nine antibiotic classes. The obtained data showed that all isolates were resistant to at least three antibiotics (See Table 3 and Table 4).

Fish farms of both Armavir and Masis regions use artesian wells as source water. Isolates taken from inlet water of both regions had relatively low resistance compared to strains isolated from fish and outlet water (Fig. 1).

Particularly, significant difference was detected in case of three antibiotics: tetracycline, oxytetracycline and chloramphenicol ($p \leq 0.05$). These results allowed us to assume that high resistance occurring in fish farm environments was result of changes that happens in fishponds. These changes can be due to extent use of antibiotics to control infectious diseases and to disinfect water environment.

According to obtained data all *Pseudomonas* isolates were sensitive to imipenem (carbapenem class). High sensitivity of *Pseudomonas* spp. to

Table 1. Source of isolation of *Pseudomonas* strains and determination of their enzymatic activities

Isolates	Species	Sources	Region	Hemolytic activity diameter (cm)	Proteolytic activity diameter (cm)
Ps.1	P. anguilloseptica	brown trout skin	Masis	1.81±0.02	2.3±0.10
Ps.11		fry skin	Masis	1.67±0.08	2.0±0.02
Ps.16		brown trout gills	Armavir	1.70±0.02	2.2±0.10
Ps.18		eyes of rainbow trout	Masis	1.80±0.02	2.4±0.10
Ps.23		tail wound of rainbow trout	Masis	1.78±0.02	2.3±0.10
Ps.2	P. fluorescens	inlet water	Masis	1.12±0.10	1.5±0.10
Ps.3		rainbow trout gills	Masis	1.20±0.05	1.3±0.10
Ps.5		outlet water	Masis	1.07±0.12	1.2±0.10
Ps.19		wound of rainbow trout	Armavir	1.30±0.03	1.4±0.10
Ps.9	P. stutzeri	eyes of rainbow trout	Masis	0.98±0.02	1.57±0.15
Ps.13		kidney of rainbow trout fry with wounds	Armavir	0.88±0.02	1.17±0.15
Ps.22		tail wound of rainbow trout	Masis	0.99±0.02	1.60±0.15
Ps.10	P. putida	brown trout skin	Masis	1.33±0.03	2.2±0.10
Ps.14		liver of rainbow trout fry with wounds	Armavir	1.58±0.03	2.37±0.15
Ps.15		tail of rainbow trout fry with wounds	Armavir	1.73±0.03	2.57±0.21
Ps.21		wound of rainbow trout	Armavir	1.80±0.03	2.60±0.20
Ps.24		injured fin of brown trout	Armavir	1.74±0.03	2.45±0.15
Ps.4	P. aeruginosa	inlet water	Masis	1.75±0.05	1.7±0.10
Ps.7		inlet water	Armavir	1.75±0.04	1.7±0.10
Ps.12		outlet water	Armavir	1.76±0.03	1.7±0.10
Ps.17		oral cavity of brown trout	Armavir	1.74±0.04	1.3±0.10
Ps.20		wound of rainbow trout caudal fin	Armavir	1.68±0.03	1.5±0.10
Ps.6	P. alcaligenes	outlet water	Masis	1.00±0.10	1.1±0.10
Ps.8		outlet water	Armavir	0.98±0.10	1.0±0.10

Table 2. Identification of isolates based on biochemical characteristics

Test name	*Pseudomonas spp.*					
	P. aeruginosa	*P. alcaligenes*	*P. fluorescens*	*P. putida*	*P. stutzeri*	*P. anguilloseptica*
Gram stain	–	–	–	–	–	–
Motility	+	+	+	+	+	+
Fluorescent pigment on agar	–	–	+	–	+	–
Growth at:						
4 °C	–	–	–	+	–	–
30 °C	+	+	+	+	+	+
40 °C	–	–	–	–	–	–
Growth in 4% NaCl	+	–	+	+	–	–
Catalase	+	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+	+
Hydrolysis:						
Arginine	+	–(+)	+	+	–	–
Gelatin	+(–)	+(–)	+	–	–(+)	+(–)
Starch	–	–	+(–)	–	–(+)	–
Esculin	+(v)	–	–(+)	–	–	–
Indole production	–	–	–	–	–	–
Nitrate production	+	v	+	–	+	–
H2S production	–	–	–(+)	–	–	–
Methyl red	–	–	+	–	–	+
Voges Proskauer	–	–	–	–	–	–
ONPG	–	–	–	–	–	–
Citrate	+	+(–)	+	+	+(–)	+(–)
Urease	+	–	–(v)	–(v)	–(+)	–
O/F test glucose	O	O	O	O	O	O
Acid production from:						
Arabinose	+(–)	+	+	+	–	–
Fructose	v	–	+	+		–
Galactose	–	–	+	–	–	–
Glucose	+(v)	–	–	+	+	–(+)
Inositol	–	–	–	–	–	–
Lactose	–	–	–(+)	–(+)	–	–
Maltose	–	–	–(v)	–(+)	–(+)	–
Mannitol	–	–	+(v)	–(+)		–
Manose	v	–	+	–	–	–
Raffinose	–	–	–	–	–	–
Salicine	–	–	–	–	–	–
Sucrose	–	–	+(–)	–	+(–)	v
Trehalose	–	–	+	–	–	–
Xylose	+(–)	–	+	+	+	–
Sorbitol	–	–	+	–	–	–

/+/: test positive, /–/: test negative, /v/: variable reaction, /+(–)/: positive in most of strains and negative in some strains, /–(+)/: negative in most of strains and positive in some strains

Table 3. Antibiotic susceptibility of selected *Pseudomonas* isolates against commonly used antibiotics

Isolate	Species	Tested antibiotics*															
		T	E	S	C	Ak	N	O	AMP	AMO	I	PFLX	CTX	CIP	G	Pi	TR
Ps.1	P. anguillo-septica	I**	R	S	I	S	S	I	R	R	S	S	R	S	S	S	R
Ps.11		R	R	S	I	S	I	R	R	R	S	S	S	I	S	S	R
Ps.16		R	I	S	S	S	I	R	R	R	S	S	R	S	I	R	R
Ps.18		R	R	S	I	S	R	R	R	R	S	S	S	S	S	R	R
Ps.23		R	R	R	R	S	S	R	R	R	S	S	R	S	S	R	R
Ps.2	P. fluorescens	S	R	S	S	S	I	S	R	R	S	S	I	S	S	S	R
Ps.3		S	I	S	S	S	I	S	R	R	S	S	R	S	S	S	I
Ps.5		R	R	S	R	S	S	I	R	R	S	S	R	S	S	S	R
Ps.19		R	R	S	R	S	S	R	R	R	S	S	S	S	S	S	R
Ps.9	P. stutzeri	R	R	S	R	S	I	R	R	R	S	R	S	R	S	S	I
Ps.13		R	I	S	S	S	I	R	R	I	S	R	R	S	S	R	R
Ps.22		R	R	R	I	R	I	R	R	R	S	S	R	S	S	R	R
Ps.10	P. putida	R	R	S	R	S	I	S	R	R	S	S	S	I	S	S	R
Ps.14		R	I	S	S	S	I	R	R	R	S	R	R	S	S	S	R
Ps.15		R	I	S	S	S	I	R	R	R	S	R	R	S	S	R	R
Ps.21		S	R	S	I	S	I	R	R	R	S	S	R	S	S	R	R
Ps.24		R	R	R	I	S	I	R	R	R	S	S	I	S	S	R	R
Ps.4	P. aeruginosa	S	S	S	S	S	I	S	R	I	S	R	R	S	S	R	R
Ps.7		S	I	S	S	I	S	S	R	R	S	S	I	S	S	S	R
Ps.12		R	R	I	R	R	I	R	R	R	S	R	R	S	R	R	R
Ps.17		R	R	S	R	S	I	R	R	R	S	R	R	S	S	S	R
Ps.20		R	R	S	R	S	R	R	R	R	S	S	I	S	S	S	R
Ps.6	P. alcaligenes	R	R	I	R	S	S	R	R	R	S	I	I	S	S	S	R
Ps.8	P. alcaligenes	R	R	I	R	R	I	R	R	R	S	R	R	S	I	R	R

* Tested antibiotic disks; tetracycline (T) 30 μg, erythromycin (E) 10 μg, streptomycin (S) 25 μg, chloramphenicol (C) 30 μg, amikacin (Ak)10 μg, neomycin (N) 30 μg, oxytetracycline (O) 30 μg, ampicillin (AMP) 10 μg, amoxicillin (AMO) 30 μg, imipenem (I) 10 μg, pefloxacin (PFLX) 5 μg, cefotaxime (CTX) 10 μg, ciprofloxacin (CIP) 5 μg, gentamicin (G) 30 μg, piperacillin (Pi) 100 μg, trimethoprim (TR) 5 μg.

** Microbial strains were classified as sensitive (S), intermediate (I) and resistant (R).

imipenem was also shown in literature [Lerma et al. 2014].

Three penicillin class antibiotics were used: amoxicillin, ampicillin and piperacillin. Although almost all *Pseudomonas* isolates were found to be resistant to amoxicillin and ampicillin, however we are not considering this as consequence of widespread usage of these antibiotics in Armenian fishponds. First of all because there was no data about their usage, second, strains isolated from inlet water also had resistance against them. And third, resistance against these antibiotics are widespread worldwide and have been shown in many research [Akinbowale et al. 2006; Hatha et al. 2005; Nguyen et al. 2014; Saavedra et al. 2004; Saha and Pal 2002; Tripathy et al. 2007]. We found moderate resistance toward piperacillin (45.6%) including one strain which was isolated from inlet water (Ps.4). Saavedra M. J. et al. [2004] in their research detected 24% resistance against piperacillin within 100 *Aeromonas hydrophila* strains.

Table 4. Multiple Antibiotic Resistance (MAR) index of *Pseudomonas* isolates

Isolate	Species	Tested antibiotics*															
		T	E	S	C	Ak	N	O	AMP	AMO	I	PFLX	CTX	CIP	G	Pi	TR
Ps.1	P. anguilloseptica	I**	R	S	I	S	S	I	R	R	S	S	R	S	S	S	R
Ps.11		R	R	S	I	S	I	R	R	R	S	S	S	I	S	S	R
Ps.16		R	I	S	S	S	I	R	R	R	S	S	R	S	I	R	R
Ps.18		R	R	S	I	S	R	R	R	R	S	S	S	S	S	R	R
Ps.23		R	R	R	R	S	S	R	R	R	S	S	R	S	S	R	R
Ps.2	P. fluorescens	S	R	S	S	S	I	S	R	R	S	S	I	S	S	S	R
Ps.3		S	I	S	S	S	I	S	R	R	S	S	R	S	S	S	I
Ps.5		R	R	S	R	S	S	I	R	R	S	S	R	S	S	S	R
Ps.19		R	R	S	R	S	S	R	R	R	S	S	S	S	S	S	R
Ps.9	P. stutzeri	R	R	S	R	S	I	R	R	R	S	R	S	R	S	S	I
Ps.13		R	I	S	S	S	I	R	R	I	S	R	R	S	S	R	R
Ps.22		R	R	R	I	R	I	R	R	R	S	S	R	S	S	R	R
Ps.10	P. putida	R	R	S	R	S	I	S	R	R	S	S	S	I	S	S	R
Ps.14		R	I	S	S	S	I	R	R	R	S	R	R	S	S	S	R
Ps.15		R	I	S	S	S	I	R	R	R	S	R	R	S	S	R	R
Ps.21		S	R	S	I	S	I	R	R	R	S	S	R	S	S	R	R
Ps.24		R	R	R	I	S	I	R	R	R	S	S	I	S	S	R	R
Ps.4	P. aeruginosa	S	S	S	S	S	I	S	R	I	S	R	R	S	S	R	R
Ps.7		S	I	S	S	I	S	S	R	R	S	S	I	S	S	S	R
Ps.12		R	R	I	R	R	I	R	R	R	S	R	R	S	R	R	R
Ps.17		R	R	S	R	S	I	R	R	R	S	R	R	S	S	S	R
Ps.20		R	R	S	R	S	R	R	R	R	S	S	I	S	S	S	R
Ps.6	P. alcaligenes	R	R	I	R	S	S	R	R	R	S	I	I	S	S	S	R
Ps.8	P. alcaligenes	R	R	I	R	R	I	R	R	R	S	R	R	S	I	R	R

* Tested antibiotic disks; tetracycline (T) 30 μg, erythromycin (E) 10 μg, streptomycin (S) 25 μg, chloramphenicol (C) 30 μg, amikacin (AK) 10 μg, neomycin (N) 30 μg, oxytetracycline (O) 30 μg, ampicillin (AMP) 10 μg, amoxicillin (AMO) 30 μg, imipenem (I) 10 μg, pefloxacin (PFLX) 5 μg, cefotaxime (CTX) 10 μg, ciprofloxacin (CIP) 5 μg, gentamicin (G) 30 μg, piperacillin (Pi) 100 μg, trimethoprim (TR) 5 μg.

** MAR index was determined by following formula; MAR = a/b, where a is the number of antibiotics toward which strains were resistant and b is the total number of tested antibiotics.

During experiments erythromycin and chloramphenicol were tested from the macrolides with moderate resistance level 66.7% and 37.5% respectively. Resistance against erythromycin was found in one strain from inlet water isolates. Moderate resistance toward these antibiotics also reported in literature [Akinbowale et al. 2006]. It is interesting to point out that none of isolates from inlet water possessed resistance to chloramphenicol. Whereas 42.8% of isolates from fish and outlet water were resistant to chloramphenicol. There was also data about widespread usage of this antibiotic in Armenian fish farms. Therefore we can assume that occurrence of resistance to chloramphenicol was acquired as consequence of its extensive usage.

Two quinolones were used pefloxacin and ciprofloxacin with relatively low resistance levels; 33.3% and 4.2% respectively. Similar data was also reported in literature [Akinbowale et al. 2006;

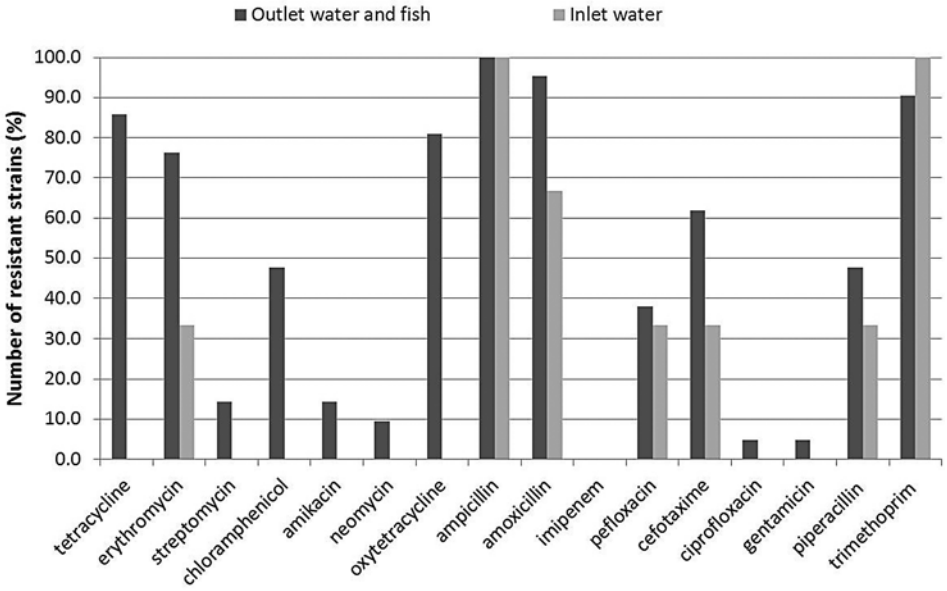

Fig. 1. Comparison of resistance patterns of *Pseudomonas* isolates from inlet water and outlet water with fish toward tested antibiotics.

Chelossi et al. 2003; McKeon et al. 1995]. In the research done by Tripathy et al. [2007] *P. aeruginosa* strains were found to be very sensitive to ciprofloxacin. It was in coincidence with our data with some minor differences. Particularly in our results Ps.9 strain was resistant and Ps.10 and Ps.11 were had intermediate sensitivity to ciprofloxacin. However all reminded strains were sensitive to this antibiotic.

Four aminoglycosides were tested and respectively low resistance was detected. The obtained data showed that there were only one resistant (Ps.12) and two (Ps.8 and Ps.16) intermediate sensitivity strains toward antibiotic gentamicin, whereas all reminded isolates were sensitive to it. Three (12.5%) of tested isolates were found resistant towards streptomycin, two (8.3%) toward neomycin and three to another aminoglycoside amikacin. Very low (2.9%) gentamicin resistance was also shown in Akinbowale et al. research [2006]. Nguyen et al. [2014] found 16.4%, 27.6% and 2.7% resistance towards gentamicin, streptomycin and neomycin respectively within 116 catfish *Pseudomonas* isolates.

One cephalosporin class antibiotic (cefotaxime) was used against which 14 (58.3%) isolates were resistant. One of inlet water strains also resistant to cefotaxime. In literature moderate resistance was found in some research as well [Akinbowale et al. 2007].

High resistance (91%) were detected to trimethoprim within tested isolates. All inlet water strains also had resistant to it. There was variable data in literature about trimethoprim resistance in water environments. Akinbowale et al. [2007] reported high resistance (95.5%) within *Pseudomonas* strains isolated from fish and sediment. Whereas only 4.8% resistance to trimethoprim was seen in other research [Akinbowale et al. 2006].

Tetracycline class antibiotics are commonly being used in Armenian fish farms to combat fish pathogens. This is mainly due to their broad range of activity and cost effectiveness [Miranda, Zemelman 2002]. In our study 75% and 70.1% resistance were found toward tetracycline and oxytetracycline respectively. All strains which had resistance toward these antibiotics were isolated from fish or outlet water. In contrast all three *Pseudomonas* strains which were isolated from inlet water found to be susceptible to tetracycline and oxytetracycline. Therefore, this can allow us to assume that resistance of *Pseudomonas* strains toward tetracyclines occurred in pools due to extensive use of tetracycline group antibiotics. Similar results have been shown in other studies. Particularly in many researches correlation between

extensive usage of tetracylnices in fishponds and development of acquired resistance by microorganisms was shown [Miranda, Zemelman 2002; Samuelsen et al. 1992].

Multiple antibiotic resistance index. The MAR indexes of all isolates were calculated. The obtained data indicated occurrence of high multidrug resistance of tested isolates (Table 4). Almost all *Pseudomonas* isolates were in high risk zone, taking into account that bacteria with >0.2 MAR index considered as highly contaminating [Krumperman 1983]. Only two strains (Ps.3 and Ps.7) had MAR index below 0.2 (0.187). Comparison of MAR index of isolates from inlet water with strains from fish and outlet water indicated existence of huge difference. All three inlet water isolates had relatively low MAR values (0.25, 0.187 and 0.12). Whereas almost all *Pseudomonas* strains isolated from fish and outlet water had very high MAR values ($p \leq 0.05$). Particularly highest MAR index (0.75) was detected in Ps.12 strain isolated from outlet water of Armvair region. MAR index of three strains (Ps.8, Ps.22 and Ps.23) were above 0.6, five strains had ≥0.5 MAR values. Six strains were had MAR index above 0.4 and three above 0.3. Only Ps.3 which was isolated from gills of rainbow trout had MAR index below 0.2.

All tested *Pseudomonas* strains found to be resistant to at least two antibiotic class. Only one (4.2%) of isolates was resistant to three antibiotic classes. Resistance to four classes was detected in 6 (25%) strains. Nine (37.5%) of isolates were resistant to five antibiotic classes, whereas three (12.5%) were resistant to six classes. Resistance to seven and eight antibiotic classes was found only one isolate for each class. High MAR values within *Pseudomonas spp.* isolated from water environment were also shown in literature [Matyar et al. 2010; Sarter et al. 2007].

Thus obtained data showed occurrence of high level multiple antibiotic resistance of *Pseudomonas* spp. isolated from fish and outlet water of Armenian fish farms comparing to strains isolated from inlet water ($p \leq 0.05$) which can be as consequence of their extensive antibiotic exposure.

Conclusion

Thus, obtained data indicated occurrence of high level antibiotic resistance and multidrug resistance of *Pseudomonas* spp. in Armenian fish farms. In case of three antibiotics (tetracycline and oxytetracycline and chloramphenicol) which were widely used in Armenian fishponds resistance was found only within strains isolated from outlet water and fish tissue. Whereas all strains isolated from inlet water susceptible to these antibiotics. This can lead to speculation that *Pseudomonas* strains acquired resistance as result of extensive exposure to these antibiotics. Taking into account existence of high level antibiotic resistance there is an urgent need for regulation and control over usage of antibiotics in Armenian fish farms. First of all because of high transfer risk of resistance to humans. And secondly, high antibiotic resistance within *Pseudomonas* spp. can lead to severe economic losses as many of isolated resistant strains were considered as fish pathogens.

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Устойчивость видов рода *Pseudomonas*, изолированных из радужной форели рыбоводческих хозяйств Армении

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Постоянное и неограниченное использование антибактериальных препаратов в области аквакультуры способно привести к распространению устойчивых к антибиотикам штаммов бактерий в водных экосистемах, откуда они могут попасть в организм человека непосредственно или через горизонтальный перенос генов. В связи с этим регулярный мониторинг устойчивых к антибиотикам патогенных и условно-патогенных штаммов является необходимым при выращивании аквакультуры. Род *Pseudomonas spp.* широко распространен в водных экосистемах. Целью представленной работы является оценка устойчивости видов и штаммов рода *Pseudomonas spp.* к широкому спектру антибиотиков в рыбоводческих хозяйствах Армении. Отбор образцов артезианской воды проводили в точках при входе в пруды, для выращивания радужной форели и выходе, с кожных покровов форели и внутренних органов методом мазков. Идентификацию изолированных штаммов проводили методом изучения макро- и микроскопических характеристик. Для идентификации изолированных штаммов использовано биохимическое тестирование и быстрые системы идентификации API 20E. Изучена устойчивость изолированных штаммов рода *Pseudomonas* относительно 16 антибиотиков, согласно рекомендациям CLSI (Clinical Laboratory Standards Institute, 2007).Выделено и идентифицировано 24 штамма из рода *Pseudomonas*, которые относятся к шести видам: *P. anguilliseptica*, *P. fluorescens*, *P. stutzeri*, *P. putida*, *P. aeruginosa* и *P. alcaligenes*. Полученные данные показали, что все штаммы проявили устойчивость по меньшей мере к трем антибиотикам. Высокая устойчивость была обнаружена у штаммов, изолированных из артезианской воды и форели относительно антибиотиков из тетрациклиновой группы (тетрациклин и окситетрациклин) и хлорамфеникола. Почти у всех штаммов множественный индекс устойчивости к антибиотикам превышал 0,2.

Ключевые слова: *Pseudomonas* spp., радужная форель *Oncorhynchus mykiss*, устойчивость к антибиотикам, патогенные бактерии, рыбоводческие хозяйства, диск-диффузный метод.