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Research Article

Co-Selection of Mercury- And Antibiotic-Resistance in Hatchery-Reared Salmonids

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Abstract

Exposure of environmental bacteria to pollutants, including heavy metals, antibiotic residues and detergents, can contribute to the development and spread of bacterial antibiotic resistance. Co-selection of resistance to known environmental pollutants, such as mercury, has the potential to increase the antibiotic resistance profile in bacteria. In this study, we have isolated and characterized 44 apparently distinct mercury-resistant bacteria from three different hatchery-reared, salmonid species (brook, lake and rainbow trout). Bacteria representing 14 genera were identified through partial 16S rDNA sequencing, with the genera of eight of the isolates belonging to γ-Proteobacteria. This work suggests that environmental exposure to mercury or other heavy metals or pollutants are co-selecting the occurrence of antibiotic resistance in at least some commensal bacteria in the hatchery fish. Additionally, we have identified potential probiotic bacteria that harbor resistances, suggesting care must be taken moving forward with the use of such bacteria.

Keywords: Antibiotic Resistance; Co-Selection; Mercury; Salmonids

Introduction

The animal gastrointestinal tract harbors a wide variety of commensal bacteria as part of the normal microbiota, and is a common entry point for pathogenic bacteria. Commensal bacteria may gain nutrition from the diet of the host organism, and they function in providing innate immunity and essential metabolites for the host [1]. It is well established that the gut microbiota contributes to the overall health of the host [2-4]. Microbiota may also be exposed to a variety of environmental toxic compounds, such as heavy metals and other pollutants, through the diet of the host organism or through water.

The toxicity of mercury in its various forms and its ubiquity in the global environment has been well-documented [5-8]. Since mercury bioaccumulates, concentrations of mercury in tissues tend to increase with increasing trophic levels. Predatory fish have been shown to accumulate mercury [9]. In previous work, our labs have shown that mercury accumulates in brook trout [10].

In this study, three species of hatchery-reared salmonids were investigated to identify mercury-resistant gastrointestinal bacteria. These fish were collected from fish hatcheries in Maine and Illinois, and the bacteria were isolated from presumptively healthy fish. Potentially pathogenic bacteria (including *Aeromonas* and *Yersinia* spp.) and putative beneficial probiotic bacteria (*Carnobacterium* spp.) were isolated from these fish samples. Antibiotic resistance profiles, using commercial MIC plates and spot MIC testing, indicate that there are in some cases many antibiotic resistance phenotypes within some of these isolates. We speculate that the exposure to environmental mercury, through atmospheric precipitation or food source, may be indirectly co-selecting for the persistence and possible horizontal gene transfer of antibiotic resistance traits in these commensal bacteria.

Materials and Methods

Fish were maintained by the hatchery staff and fed commercial diets. Fish were caught with a large dip net from a raceway containing fish of a specified age (Table 1) and humanely killed through either immersion in MS-222 or physical percussion and stored on ice. Fish were aseptically dissected along the ventral

midline within 2 hrs of capture and the entire intestine of each fish was excised, from the pyloric sphincter to anus, using a sterile scalpel.

Ingests were scraped from the intestines and combined from each type of fish (isolation was performed at different times) and 1g of ingesta was added to 9 mL of sterile 0.1% peptone diluents or 1X PBS in a 50 mL screw cap tube. These solutions were agitated for 5 sec using a Vortex® mixer. Serial dilutions ranging in concentrations from 10^{-2} to 10^{-4} were prepared, and spread onto tryptic soy agar (TSA) plates amended with 50 µM $HgCl_2$. Plates were incubated aerobically at room temperature for up to 14 days. Purified cultures were characterized for Gram staining, morphology and motility (data not shown).

Hatchery	Location	Species	Approximate age of fish (number fish sampled)	Year	Number of isolates (Gram - / Gram +)
Dry Mills Hatchery	Gray, ME	Brook trout (*Salvelinus fontinalis*)	10 months (6)	2002	20/12
Governor Hill Fish Hatchery	Augusta, ME	Lake trout (*Salvelinus namaycush*)	6 years (2)	2000	6/5
Jake Wolfe Fish Hatchery	Manito, IL	Rainbow trout (*Oncorhynchus mykiss*)	6 & 10 months (6)	2003-2004	10/7

Table 1: Origin and age of salmonid species sampled. This table provides information regarding the origin of the salmonid fish sampled and the approximate age at the time of collection.

Antibiotic Sensitivity Profiles

The activity of antimicrobial agents against each mercury-resistant isolate were assessed by the minimum inhibitory concentration (MIC) method. MICs were determined using the Sensititre™ dried susceptibility panels (MG and MJ) according to manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH).

DNA Isolation, PCR and Sequencing

Total DNA was isolated from bacterial colonies by resuspension of pellets in 50 µL of 1% Triton X-100, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0 and incubated at 99°C for 5 min [11]. PCR reactions using 16S primers [12] without the GC clamp were used to amplify a central region of the 16S rRNA gene. Sequencing results of the partial 16S rRNA products were analyzed using the nucleotide BLAST program [13].

Results

A total of 60 HgCl2 resistant-isolates were collected from three fish species in different experiments. A small region of the 16S rRNA gene was amplified and sequenced from each of the isolates. Based on partial sequencing (see supplementary file), bacteria were identified as shown (Tables 2, Table 3).

Genus	Isolate	Cell wall synthesis	Protein synthesis	Nucleic acid synthesis	Folate synthesis	$HgCl_2$ (µM)
Gamma Proteobacteria						
Acinetobacter sp.						
	BT3B	FAZ, CEP				1000
	LTC1, LTD2					500
	LTE1					≥1000
	RTNov60	MEZ, TIM, FAZ, CEP, FOX			SXT, SUL	250

Shewenella sp.						
	RTNov18	AUG, FAZ, CEP				1000
	RTNov62	AMP, A/S, AUG, FAZ, CEP, FOX			SUL	≥1000
	RTNov94				SUL	250
Pseudomonas sp.						
	BT4B1, BT4B2	AMP, A/S, TIM AUG, FAZ, CEP, FOX, FUR	NIT		SXT, SUL	500
	BT1B	AMP, A/S, TIM AUG, FAZ, CEP, FOX, FUR	NIT		SXT, SUL	500
	BT2I	AMP, A/S, TIM, AUG, FAZ, CEP, FOX, FUR	NIT		SXT, SUL	500
	RTJuly7				SUL	≥1000
Aeromonas sp.						
	BT2D	AMP, A/S, TIM, FAZ, CEP				1000
	BT3A, BT3B, BT3C					250
	BT3H	AMP, A/S, TIM, AUG, FAZ, CEP, FOX, FUR	NIT, TET		SXT, SUL	≥1000
	LTK	AMP, A/S, FAZ, CEP				250
	RTNov34	AMP, MEZ, A/S, TIM				≥1000
	RTNov50	AMP, A/S			SUL	500
	RTNov69	A/S	NIT	NOR, LOM	SXT, SUL	1000
	RTNov93	AMP, A/S, TIM			SXT, SUL	250

Table 2a: Maximal antibiotic resistance of Gram negative bacteria as determined by Sensititre™ plate dilution MIC analysis. (Antibiotic abbreviations for Tables 2 and 3 are: amikacin (AMI), amoxicillin/clavulanic acid (AUG), ampicillin/sulbactam (A/S), ampicillin (AMP), cefazolin (FAZ), cefoxitin (FOX), ceftazidime (TAZ), ceftriaxone (AXO), cefuroxime (FUR), cehpalothin (CEP), chloramphenicol (CHL), ciprofloxacin (CIP), clarithromycin (CLA), clinadamycin (CLI), erythromycin (ERY), gentamicin (GEN), lomefloxacin (LOM), mezlocillin (MEZ), nitrofuratoin (NIT), norfloxacin (NOR), ofloxacin (OFL), oxacillin + 2% NaCl (OXA+), penicillin (PEN), piperacillin (PIP), rifampin (RIF), sulfisoxazole (SUL), tetracycline (TET), ticarcillin/clavulanic acid (TIM), trimethorprim/sulfamethoxazole (SXT), vancomycin (VAN).)

Table 2b. (continued)

Genus	Isolate	Cell wall synthesis	Protein synthesis	Nucleic acid synthesis	Folate synthesis	$HgCl_2$ (μM)
Enterobacteriales						
Providencia sp.						
	BT2B	AMP, A/S, AUG, FAZ, CEP			SUL	500
	BT2Jt	AMP, A/S, AUG FAZ, CEP, FOX, FUR	TET, NIT		SUL	500
Serratia sp.						
	BT2F, BT2G, BT2Jw	AMP, A/S, TIM, AUG FAZ, CEP, FOX, FUR	NIT		SUL	500
	BT2M	AMP, A/S, AUG, FAZ, CEP, FOX, AXO, FUR				1000

Yersinia sp.						
	LTF, LTG	AMP, AUG FAZ, CEP, FOX				≥1000
Unidentified Enterobacteriaceae						
	BT2B1, BT2-B1c, BT2B1y, BT2B2					≥1000
	RTNov63	AMP, A/S, FAZ, CEP, FOX	NIT		SUL	250

Table 2b: Maximal antibiotic resistance of Gram negative bacteria as determined by Sensititre™ plate dilution MIC analysis.

Genus	Isolate	Cell wall synthesis	Protein synthesis	Nucleic acid synthesis	Folate synthesis	$HgCl_2$ (µM)
Bacillales						
Bacillus sp.						
	BT9B				SXT	500
	LTJ		CLI			≥1000
	RTJune4					250
Staphylococcus sp.						
	BT2A	AMP, PEN, OXA+, A/S, FAZ, CEP	CLI, VAN, CLA, ERY	RIF		500
	LTC2					250
	RTNov5					500
	RTNov8				SUL	1000
	RTNov33	PEN, OXA+, FAZ, CEP	CLI, CLA, ERY			≥1000
	RTNov68	AMP, PEN, OXA+, A/S, FAZ, CEP	CLI, VAN, CLA, ERY	RIF		250

Table 3a: Maximal antibiotic resistance of Gram positive bacteria as determined by Sensititre™ plate dilution MIC analysis.

Table 3b.

Genus	Isolate	Cell wall synthesis	Protein synthesis	Nucleic acid synthesis	Folate synthesis	$HgCl_2$ (µM)
Lactobacillales						
Carnobacterium sp.						
	BT5B, BT2K	OXA+	CLI, GEN			500
	BT2C, BT2P	OXA+	CLI, GEN			250
	LTE2, LTH, LTI	OXA+	CLI, GEN			250
	RTJune14, RTJune15B	OXA+	TET			250
Enterococcus sp.						
	BT2H	AMP, PEN, OXA+, A/S, FAZ, CEP	CLI, TET, NIT, CHL, VAN, CLA, ERY			500

Table 3b: Maximal antibiotic resistance of Gram positive bacteria as determined by Sensititre™ plate dilution MIC analysis.

Table 3c. (continued)

Genus	Isolate	Cell wall synthesis	Protein synthesis	Nucleic acid synthesis	Folate synthesis	$HgCl_2$ (µM)
Actinomycetales						
Dietzia sp.						
	BT7B	OXA+	NIT			1000
Dermacoccus sp.						
	BT3D	OXA+, FAZ, CEP	TET, CLA, ERY	RIF		250
	BT3E	AMP, PEN, OXA+, A/S, FAZ, CEP	CLI, NIT, CHL, VAN, CLA, ERY	RIF		250
Micrococcus sp.						
	BT6B, BT8B			LOM		1000

Table 3c: Maximal antibiotic resistance of Gram positive bacteria as determined by Sensititre™ plate dilution MIC analysis.

All of the isolates were originally selected based on their growth on tryptic soy agar plates supplemented with 50 µM $HgCl_2$. The MIC for inorganic mercury ($HgCl_2$) was tested by spotting 5 µL of a McFarland 0.5 dilution on TSA plates containing $HgCl_2$. Thirty-one of the bacterial isolates showed a $HgCl_2$ MIC greater than 250 µM $HgCl_2$ and some as high as 1000 µM $HgCl_2$ (Tables 2 and 3).

Antibiotic resistance was tested using Sensititre™ panels designed for either Gram negative or Gram positive bacteria. Only 13 isolates (Table 2) showed no maximal resistance to any of the more than 20 antibiotics tested – these include isolates identified as *Acinetobacter*, *Aeromonas*, *Enterobacter/Salmonella*, *Bacillus* and *Staphylococcus* (Tables 2, Table 3). Ten or more maximal antibiotic resistances were observed in twelve bacterial isolates, including nine Gram negative *Pseudomonas* isolates BT1B, BT4B1, BT4B2, and BT2I, *Aeromonas* isolate BT3H, *Providencia* isolate BT2Jt and *Serratia* isolates BT2F, BT2G, and BT2Jw (Table 2). One isolate each of *Staphylococcus* (BT2A), *Enterococcus* (BT2H), and *Micrococcus* (BT3E) also demonstrated resistance to at least ten antimicrobial compounds tested (Table 3). Twenty-four Gram negative bacteria demonstrated resistance to first generation cephalosporins (cephalothin and cefazolin-Table 2) and twelve of those demonstrated maximal resistance to at least one second generation cepholosporin (cefuroxime and/or cefoxitin-Table 2). Only one isolate, BT2M, demonstrated resistance to a third-generation cephalosporin ceftriazone (Table 2b). Seventeen Gram negative and three Gram positive isolates demonstrated maximal tested resistance to both ampicillin and ampicillin/sulbactam (Tables 2, Table 3) and sixteen Gram positive bacteria demonstrated resistance to oxacillin plus NaCl (Table 3).

Most of the antibiotic resistance demonstrated using the Sensititre™ MIC plates was based on cell wall synthesis inhibitors (Tables 2, Table 3), some of this resistance may be due to intrinsic resistance. Twelve Gram negative and sixteen Gram positive isolates demonstrated maximal resistance to at least one protein synthesis inhibitor (Tables 2, Table 3, respectively). Only seven isolates demonstrated maximal resistance to nucleic acid synthesis inhibitors (two Gram negative - Table 2 and five Gram positive - Table 3). Nineteen Gram negative isolates demonstrated maximal resistance to folate synthesis inhibitors (Table 2) while only two Gram positive isolates demonstrated resistance to the same inhibitors (Table 3).

There are most likely duplicate isolates within the individual fish samples tested, however, there are several examples of possibly identical bacteria isolated from different fish species based on differences in preliminary sequencing results and MIC patterns.

Discussion

A total of 44 distinct mercury-resistant bacterial isolates were collected and analyzed from three different hatchery-reared salmonid species' gastrointestinal tracts. These represent 14 different genera of bacteria based on partial 16S rDNA sequencing, including eight different Gram negative genera and six different Gram positive genera. Several of these genera are similar to ones identified Meredith et al. [10] isolated from feral brook trout. Sullam et al. [14] performed an extensive meta-analysis on fish intestinal bacteria from a number of different fish species. In agreement with their observations, we identified mercury-resistant bacterial isolates in the orders of Aeromonadales, Enterobacteriales, Pseudomonadales, Alteromonadales, Lactobacillales, Bacillales, and Actinomycetales.

Sixteen of the Gram-negative isolates demonstrated maximal tested resistance to at least some cephalosporins, while five of the Gram-positive bacteria demonstrated maximal resistance to cephalosporins. Interestingly, co-resistance to both cephalothin and cefazolin was observed in all of the resistant isolates. Nineteen of the Gram-negative isolates and eleven of the Gram-positive

isolates demonstrated resistance to penicillins. No Gram-negative bacteria demonstrated resistance to macrolides or aminoglycosides. Only six bacterial isolates demonstrated sensitivity to all antibiotics tested.

Shewanella isolates were identified only from the hatchery-reared rainbow trout in this study, but Meredith et al. [10] identified *Shewanella* species in feral brook trout. Several of these isolates demonstrated resistance to cephalosporins and sulfisoxazole. Meredith et al. [10] identified one *Pseudomonas* isolate that demonstrated resistance to multiple antibiotics, and the four isolates of *Pseudomonas* characterized here demonstrate resistance to similar antibiotics, including penicillins, cephalosporins, nitrofuratoin, and sulfisoxazole. *Pseudomonads* have been identified in salmonid species in other research [10,15,16]. Several different isolates of *Aeromonas* were identified in both Meredith et al. [10] and this research. McIntosh et al. [17] characterized an *A. salmonicida* subsp. *salmonicida* strain that harbored a large plasmid containing resistance to mercury (mer operon) and several different antibiotics.

This research demonstrates the co-occurrence of multiple antibiotic resistances with resistance to the heavy metal mercury. The hatchery environments used in this study receive water from natural sources as well as rainwater. None of these hatcheries reportedly use antibiotics in the normal course of rearing the fish and only the lake trout spend more than several months in the hatchery environment before ultimately being released into local bodies of water. There are two potential sources of mercury and/or other heavy metals in these environments - atmospheric precipitation/ground water or fish feed. It has previously been demonstrated that commercial fish feed is a source of dietary mercury in the hatchery environment [18,19].

While the fish in this study appeared to be healthy, they harbored potentially pathogenic (e.g., *Aeromonas*) bacteria. The selective force(s) for these mercury-and antibiotic-resistant bacteria within the fish gastrointestinal tract is unknown, but in the absence of known use of antibiotics, could be due to environmental exposure of the fish to heavy metals, such as mercury.

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References

1. Lu K, Mahbub R, Fox JG (2015) Xenobiotics: Interaction with the intestinal microflora. Institute for Laboratory Animal Research Journal 56: 218-227.

2. Clements KD, Angert ER, Montgomery WL, Choat JH (2014) Intestinal microbiota in fishes: what's known and what's not. Molecular Ecology 23: 1891-1898.

3. Wong S, Waldrop T, Summerfelt S, Davidson J, Barrows F, et al. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Applied and Environmental Microbiology 79: 4974-4984.

4. Lyons PP, Turnbull JF, Dawson KA, Crumlish M (2015) Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS). Aquaculture Research 48: 77-91.

5. Barkay T (2000) Mercury Cycle in Encyclopedia of Microbiology (San Diego, CA: Academic Press).

6. Hobman JL, Wilson JR, Brown NL (2000) Microbial mercury reduction in Environmental Microbe-Metal Interactions. (Washington, D.C.: ASM Press).

7. Schuster PF, Krabbenhoft DP, Naftz DL, Cecil LD, Olson ML, et al. (2002) Atmospheric mercury deposition during the last 270 years: glacial ice core record of natural and anthropogenic sources. Environmental Science and Technology 36: 2303-2310.

8. Poulain AJ, Aris-Brosou S, Blais JM, Brazeau M, Keller W, et al. (2015) Microbial DNA records historical delivery of anthropogenic mercury. The ISME Journal 9: 2541-2550.

9. Finley MLD, Kidd KA, Curry RA, Lescord GL, Clayden MG et al. (2016) A comparison of mercury biomagnification through lacustrine food webs supporting brook trout (*Salvelinus fontinalis*) and other salmonid fishes. Frontiers in Environmental Science 4: 1-13.

10. Meredith MM, Parry EM, Guay JA, Markham NO, Danner GR, et al. (2012) Concomitant antibiotic and mercury resistance among gastrointestinal microflora of feral brook trout, *Salvelinus fontinalis*. Current Microbiology 65: 575-582.

11. Johnson KA (2007) Colony lysis and PCR amplification for environmental bacterial identification. Focus on Microbial Education May. Electronic

12. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gel electrophoresis analysis for polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59: 695-700.

13. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

14. Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, et al. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Molecular Ecology 21: 3363-3378.

15. Trust TJ, Sparrow RAH (1974) The bacterial flora in the alimentary tract of freshwater salmonid fishes. Canadian Journal of Microbiology 20: 1219-1228.

16. Yoshimizu M, and Kimura T (1976) Study on the intestinal microflora of salmonids. Fish Pathology 10: 243-259.

17. McIntosh D, Cunningham M, Ji B, Fekete FA, Parry EM, et al. (2008) Transferable, multiple antibiotic and mercury resistance in Atlantic Ca-

nadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. Journal of Antimicrobial Chemotherapy 61: 1221-1228.

18. Choi MH, Cech JJ (1998) Unexpectedly high mercury level in pelleted commercial fish feed. Environmental Toxicology and Chemistry 17: 1979-1981.

19. Berntssen MHG, Hyllan K, Julshamn K, Lundebye AK, Waagbø R (2004) Maximum limits of organic and inorganic mercury in fish feed. Aquaculture Nutrition 10: 83-97.