

# Antimicrobial resistance, heavy metal resistance and integron content in bacteria isolated from a South African tilapia aquaculture system

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**ABSTRACT:** Antibacterial compounds and metals co-select for antimicrobial resistance when bacteria harbour resistance genes towards both types of compounds, facilitating the proliferation and evolution of antimicrobial and heavy metal resistance. Antimicrobial and heavy metal resistance indices of 42 Gram-negative bacteria from a tilapia aquaculture system were determined to identify possible correlations between these phenotypes. Agar dilution assays were carried out to determine susceptibility to cadmium, copper, lead, mercury, chromate and zinc, while susceptibility to 21 antimicrobial agents was investigated by disk diffusion assays. Presence of *merA*, the mercury resistance gene, was determined by dot-blot hybridizations and PCR. Association of mercury resistance with integrons and transposon Tn21 was also investigated by PCR. Isolates displayed a high frequency of antimicrobial (erythromycin: 100%; ampicillin: 85%; trimethoprim: 78%) and heavy metal (Zn<sup>2+</sup>: 95%; Cd<sup>2+</sup>: 91%) resistance. No correlation was established between heavy metal and multiple antibiotic resistance indices. Significant positive correlations were observed between heavy metal resistance profiles, indices, Cu<sup>2+</sup> and Cr<sup>3+</sup> resistance with erythromycin resistance. Significant positive correlations were observed between *merA* (24%)/Tn21 (24%) presence and heavy metal resistance profiles and indices; however, significant negative correlations were obtained between integron-associated *qacE 1* (43%) and *sulI* (26%) gene presence and heavy metal resistance indices. Heavy metal and antimicrobial agents co-select for resistance, with fish-associated, resistant bacteria demonstrating simultaneous heavy metal resistance. Thus, care should be taken when using anti-fouling heavy metals as feed additives in aquaculture facilities.

**KEY WORDS:** Heavy metal resistance · Antimicrobial resistance · Bacteria · Aquaculture · *Oreochromis mossambicus*

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## INTRODUCTION

Heavy metals have been implicated in the maintenance and proliferation of antimicrobial resistance, due to anthropogenic-derived heavy metal environmental contamination (Baker-Austin et al. 2006). Environmental co-selection of antimicrobial and heavy metal resistance (HMR) occurs in a diversity of metal-contaminated environments (Baker-Austin et al. 2006). In areas where metals are of no major risk to the

environment or to plant, animal or human use of environmental resources, metals play a role in retention of antimicrobial resistance, once genes are acquired (Ugur & Ceylan 2003).

The response of microbial communities to heavy metals depends on the concentration and availability of metals and is dependent on the actions of complex processes, involving multiple factors such as the type of metal, the nature of the environment and microbial species (Hassen et al. 1998). These

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mechanisms include reduced uptake, formation and sequestration of heavy metals in insoluble complexes, enzymatic oxidation or reduction to a less toxic species, efflux from the cell, metabolic by-pass and repair (Baker-Austin et al. 2006, Lemire et al. 2013).

HMR operons have been stably integrated into some bacterial chromosomes, but they may also be located on endogenous plasmids or transposons that often contain multiple, often redundant, resistance or detoxification operons (Busenlehner et al. 2003). Metal- and antimicrobial resistance genes are linked, particularly on plasmids, which carry determinants encoding heavy metal and antimicrobial resistance, flanked with insertion and transposase sequences and may have been acquired through multiple recombination events (Ugur & Ceylan 2003, Baker-Austin et al. 2006). The extent of this co-resistance is related to the abundance of transposons in clinically relevant and environmental bacteria. HMR genes may also be located adjacent to integrons or closely associated with them, often on the same transposon or plasmid (Bass et al. 1999, Baker-Austin et al. 2006). Given a proposed correlation between HMR and antimicrobial resistance (Baker-Austin et al. 2006), high heavy metal concentrations may increase the selective pressure for the maintenance of antimicrobial resistance genes (Spain 2003).

Heavy metals accumulate in aquatic sediment, debris and organic matter, where they may be consumed by bottom-dwelling fish, such as tilapia, which tend to accumulate it in the gills and intestines and therefore, associated microbial flora are continually exposed to not only heavy metals (Hassen et al. 1998), but also to antimicrobial compounds used as growth promoters or prophylactics (Seiler & Berendonk 2012). Resistant aquatic microorganisms arise due to exposure to contaminated aquatic environments, and sometimes due to coincidental co-selection of resistance determinants for either antimicrobial agents or heavy metals (Pathak & Gopal 2005, Seiler & Berendonk 2012). Heavy metals can be bioaccumulated by fish, and these contaminants gain entry into the aquaculture system through fish-based feeds that introduce fat-soluble contaminants such as heavy metals and polychlorinated biphenyls (Akinbowale et al. 2007). Bacterial isolates displaying multi-drug resistance (MDR) and HMR have been identified in fish tissue commonly consumed by humans, raising potential public health concerns (Pettibone et al. 1996, Sadhukhan et al. 1997, Pathak & Gopal 2005).

Gram-negative bacteria belonging to the genera *Aeromonas*, *Chryseobacterium*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Myroides*, *Salmonella*, *Serratia* and *Vibrio* are associated with diseases of farmed and wild fish (Austin & Austin 2016). Given the MDR phenotypes in their clinically isolated counterpart strains, fish-associated bacteria from these genera are of interest in understanding the presence and potential horizontal transfer of resistance genes in a cultured fish environment. This study is part of a broader investigation, which aimed to investigate the presence of plasmids and antimicrobial resistance determinants in Gram-negative fish-associated bacteria (*Aeromonadaceae* and *Flavobacteriaceae*; Jacobs & Chenia 2007, Chenia 2016), as well as investigate their biofilm-forming abilities (Basson et al. 2008, Jacobs & Chenia 2009). In the present study, HMR profiles of a selection of Gram-negative bacterial flora from an *Oreochromis mossambicus* (tilapia) aquaculture system were determined to identify a possible correlation between the HMR and MDR phenotypes. While not mobile, integrons tend to be associated with transposons and/or conjugative plasmids, which can serve as vehicles for their transmission (Jacobs & Chenia 2007). The association of HMR with the presence of mobile genetic elements (plasmids and *Tn21*) and integrons was also investigated, since a strong association between integron presence and MDR phenotypes has been observed (Leverstein-van Hall et al. 2002).

## MATERIALS AND METHODS

### Selection of isolates

Forty-two plasmid-containing, Gram-negative bacteria were selected for study following sampling from a South African *Oreochromis mossambicus* aquaculture system (fish: gill, skin, mouth and fin samples; tank water; tank sediment; and unmedicated feed samples). Over a 6 wk period, fish and tank water samples were collected fortnightly from the aquaculture facility. Samples of tank water were collected in sterile water-sampling bottles, sediment was collected in sterile glass flasks, and unmedicated food samples were collected in sterile plastic bags (Miranda & Rojas 2007). Five apparently healthy fish from each tank were randomly selected and placed into sterile plastic bags with some water from the same tank, oxygenated and sealed. All samples were placed on ice, transported immediately to the laboratory and processed within 3 h after collection.

Tank water, sediment and feed samples were processed as described by Miranda & Rojas (2007). Fish were euthanized by lethal thermal shock and externally washed with 0.85% sterile physiological saline to reduce potential contamination with skin bacteria (Miranda & Rojas 2007). Samples were taken from fish extremities (eyes, fins, gills, mouth and skin) as well as from internal organs (homogenised pooled kidney, liver and spleen samples).

Culturable and tetracycline-resistant counts of heterotrophic bacteria were performed by a spread plate method using tryptic soy agar (TSA; Biolab) and brain-heart infusion agar (BHIA; Biolab) with and without 25 µg ml<sup>-1</sup> of tetracycline (Sigma), respectively (Miranda & Rojas 2007). All agar plates were incubated at room temperature (21 ± 2°C) for 5 d. Bacteria were randomly selected from TSA/BHIA plates supplemented with 25 µg ml<sup>-1</sup> of tetracycline or 30 µg ml<sup>-1</sup> of chloramphenicol. Purified cultures were stored at -80°C in tryptic soy broth supplemented with 20% glycerol.

Gram-negative isolates were selected from 200 bacteria based on Gram staining characteristics and identified using API 20E or API 20NE kits (bio-Merieux) and/or 16S rRNA gene amplification and sequencing (Coram & Rawlings 2002).

### Antimicrobial susceptibility testing

Using the disk diffusion method on Mueller-Hinton (MH, Biolab) agar plates following Clinical and Laboratory Standards Institute (CLSI) protocols (CLSI 2008), we determined antimicrobial susceptibilities of the 42 Gram-negative bacteria to 21 antimicrobial agents representing 8 classes: amikacin (AK-30), amoxicillin (A-10), ampicillin (AP-10), amoxicillin-clavulanate (AUG-30), azithromycin (ATH-15), ceftriaxone (CRO-30), cefuroxime (CXM-30), chloramphenicol (C-30), ciprofloxacin (CIP-5), colistin sulphate (CO-25), cotrimoxazole (TS-25), erythromycin (E-15), gentamicin (GM-10), imipenem (IMI-10), nalidixic acid (NA-30), ofloxacin (OFX-5), sulphamethoxazole (SMX-25), sulphatriad (ST-200), streptomycin (S-10), tetracycline (T-30) and trimethoprim (TM-1.25) (Mast Diagnostics). Overnight BHI cultures were diluted to a turbidity equivalent to a 0.5 McFarland standard, and MH agar plates were inoculated and incubated for 24 h at 26°C. *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 25923 were used as antimicrobial susceptibility testing (AST) controls. Testing was done in

duplicate, and resistance profiles were assigned after measuring average zone diameters using CLSI breakpoints (CLSI 2008). Multiple antibiotic resistance (MAR) index values ( $a/b$ , where 'a' represents the number of antimicrobial agents the isolate was resistant to and 'b' represents the total number of antimicrobial agents the isolate was tested against) were calculated for all isolates (Krumperman 1983).

### HMR testing

Resistance to 6 heavy metals (all sourced from Merck), viz. cadmium acetate (Cd<sup>2+</sup>), copper sulphate (Cu<sup>2+</sup>), lead acetate (Pb<sup>2+</sup>), mercury chloride (Hg<sup>2+</sup>), potassium chromate (Cr<sup>3+</sup>) and zinc sulphate (Zn<sup>2+</sup>) was determined by agar dilution assays. Overnight BHI broth cultures of 42 study isolates and 3 AST controls diluted to a turbidity equivalent to a 0.5 McFarland standard were inoculated onto heavy metal-containing MH agar plates using a multipoint inoculator. There are no universally accepted metal concentrations to define bacterial resistance. Resistance to the 6 respective heavy metals was initially determined on MH agar plates with the following heavy metal concentrations: 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 mM. Thereafter, final plate concentrations ranged from 0.005–2.5 mM for Hg<sup>2+</sup>, 0.005–12 mM for Cd<sup>2+</sup>, 0.005–10 mM for Cu<sup>2+</sup>, 0.005–15 mM for Pb<sup>2+</sup>, 0.005–20 mM for Cr<sup>3+</sup>, and 0.005–17 for Zn<sup>2+</sup> (Table 1). Minimum inhibitory concentrations (MICs) were documented as the lowest metal concentration at which bacterial growth was not observed. Heavy metal concentration resistance breakpoints (Table 1) were adapted from Nies (1999) following preliminary results. Tests were carried out in duplicate on 2 separate occasions in order to ensure reproducibility. Bacterial growth was scored in comparison to growth of isolates on MH agar without addition of the respective metal being tested, after 24 and 48 h.

*E. coli* ATCC 25922, which is susceptible to Cu<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup> and Cd<sup>2+</sup>, was included as a control strain. Interpretation of resistance levels was initially done using criteria described by Nies (1999); however, these were amended using the CLSI AST bacterial reference strains as a basis for comparison (Table 1). The HMR index was determined using the equation  $a/b$ , where 'a' represents the number of heavy metals the isolate was resistant to and 'b' represents the total number of heavy metals the isolate was tested against.

Table 1. Percentage of heavy metal resistant isolates based on adapted heavy metal minimum inhibitory concentration (MICs). MICs are based on a study by Nies (1999); adapted MICs are based on MICs of *E. coli* ATCC 25922 (CLSI reference strain). Resistance was defined at concentrations  $\geq$  to those of adapted MIC concentrations

Heavy metal ions	Concentration range tested (mM)	MIC concentrations (mM)	Adapted MIC concentrations (mM)	% of resistant isolates
Mercury (Hg <sup>+</sup> )	0.005–2.5	0.01	$\geq 0.1$	26.2 (11/42)
Chromate (Cr <sup>3+</sup> )	0.005–20	0.2	$\geq 10$	21.4 (9/42)
Cadmium (Cd <sup>2+</sup> )	0.005–12	0.5	$\geq 0.5$	90.5 (38/42)
Copper (Cu <sup>2+</sup> )	0.005–10	1	$\geq 2.5$	50.0 (21/42)
Zinc (Zn <sup>2+</sup> )	0.005–17	1	$\geq 2.5$	95.2 (40/42)
Lead (Pb <sup>2+</sup> )	0.005–15	5	$\geq 5$	19.1 (8/42)

### Identification of *merA* and Tn21

Genomic DNA was extracted (Ausubel et al. 1989) following overnight culture in BHI broth. Presence of the 1.2 kb *merA* gene fragment was investigated by PCR using the primer pair MerA-F (5'-ACC ATC GGC GGC ACC TGC GT-3') and MerA-R (5'-ACC ATC GTC AGG TAG GGG AAC A-3') (Bass et al. 1999). Plasmid pTM314 containing a 4.6 kb *merACR* *SaII* fragment (Kusano et al. 1992) was used as a positive control. PCR reaction mixtures (25  $\mu$ l) included 100 ng template DNA, 200  $\mu$ M of each dNTP (Roche Diagnostics), 50 pmol of each primer, 1.5 mM MgCl<sub>2</sub> and 1 U Super-Therm *Taq* DNA polymerase (JMR Holdings), together with 1 $\times$  reaction buffer. PCR cycling parameters consisted of 35 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 1 min in a PCR Sprint thermal cycler (Hybaid). An initial denaturation step of 95°C for 3 min and a final elongation step of 72°C for 10 min were included in the reaction. Ten  $\mu$ l of PCR mixture was subjected to electrophoresis in 1% agarose gels, stained with ethidium bromide and viewed by UV trans-illumination.

Genomic DNA of all isolates was denatured to single-stranded DNA by exposure to 100°C for 10 min, blotted onto a nylon membrane using a multipoint inoculator and cross-linked to the membrane by 3 min UV light exposure. The 1.2 kb *merA* PCR fragment amplified from pTM314 was labeled using the DIG High Prime kit (Roche) for use as a probe. Dot-blot hybridization and washes were carried out at 42°C, as per the manufacturer's recommendations.

The presence of Tn21 in *merA*-positive isolates was investigated by amplification of a 348 bp gene fragment with *tnpM*-F (5'-TCA ACC TGA CGG CGG CGA-3') and *tnpM*-R (5'-GGA GGT GGT AGC CGA GG-3') primers (Pezzella et al. 2004). PCR amplification was carried out using cycling parameters described above.

### Identification of integrons and associated resistance genes

Presence of integrase 1 (*intI*), the conserved (CS) region, *qacE 1* and *sull* genes were determined by PCR using previously described primer sets (Jacobs & Chenia 2007). On positive amplification of the CS region, specific gene cassettes encoding resistance to gentamicin (*aac(6')Ia*), streptomycin (*ant(3'')1a*), chloramphenicol (*catB3*), trimethoprim (*dhfr1*),  $\beta$ -lactam (*oxa2a*) and carbapenem (*pse1*) were identified by PCR (Jacobs & Chenia 2007). PCR reaction mixtures (25  $\mu$ l) included 100 ng template DNA, 200  $\mu$ M of each dNTP (Roche), 50 pmol of each primer, 1.5 mM MgCl<sub>2</sub> and 1 U Thermopol *Taq* DNA polymerase (New England Biolabs), together with 1 $\times$  Thermopol reaction buffer (New England Biolabs). PCR cycling parameters for the *intI*, *qacE 1*, *sull* and the resistance gene cassettes consisted of 35 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min in a PCR Sprint thermal cycler (Hybaid). An initial denaturation step of 95°C for 5 min and a final elongation step of 72°C for 10 min were included in the reaction. For the CS PCR, cycling parameters were identical except for an annealing temperature of 57°C.

### Statistical analyses

Relationships between MAR indices, HMR profiles, HMR indices, individual heavy metal phenotypes, individual antimicrobial resistance phenotypes, *merA*/Tn21 presence, integron-associated genes *intI*, CS, *qacE 1* and *sull* genes were determined using Pearson product moment correlation analysis (p-values < 0.05 were considered significant), using SigmaStat 3.5 (Systat Software).

## RESULTS

### Bacterial isolates

Forty-two heterotrophic, Gram-negative bacteria belonging to 12 genera were identified and selected for further study, based on carriage of 1 or more plasmids (single to multiple ranging from 5 to 45 kb). Predominant genera identified (Table 2) included *Aeromonas* (26.2%), *Salmonella* (26.2%), *Shewanella* (9.5%) and *Citrobacter* (7.1%), followed by members of the *Flavobacteriaceae* (*Chryseobacterium* and *Myroides*).

### Antimicrobial susceptibility testing

Isolates displayed resistance to the penicillins, while resistance to the second (cefuroxime) and third (ceftriaxone) generation cephalosporins, as well as imipenem was lower (Fig. 1). Isolates were susceptible to the fluoroquinolones ciprofloxacin and of-

loxacin; however, 24.4% of isolates displayed resistance to nalidixic acid. The majority of isolates appeared susceptible to the aminoglycosides and macrolides (Fig. 1), although a high frequency of resistance to streptomycin and erythromycin was observed. Resistance to the sulphonamides sulphamethoxazole and sulphatriad was observed in <50% of isolates, and 78.1% of isolates were resistant to trimethoprim. Cotrimoxazole (sulphamethoxazole + trimethoprim) resistance was only observed for 24.4% of isolates (Fig. 1).

MAR indices ranged from 0.14–0.67, with 28.6% (12/42) of isolates having MAR indices  $\geq 0.5$  (Tables 2 & 3). A MAR index  $\geq 0.2$  was observed for 88.1% (37/42) of isolates (Table 3).

### HMR testing

Given the lack of standardized heavy metal concentrations and breakpoint criteria, an *E. coli* ATCC 25922 was used as a quality control for HMR testing

Table 2. Heavy metal resistance (HMR) indices, phenotypes and minimum inhibitory concentration (MIC) ranges displayed by 12 Gram-negative bacterial genera isolated from a tilapia aquaculture system; MAR: multiple antibiotic resistance

Genus (no. of isolates)	MAR index range	HMR index range	HMR phenotype (no. of isolates)	Heavy metal MIC range (mM)					
				Cd <sup>2+</sup>	Cr <sup>3+</sup>	Cu <sup>2+</sup>	Pb <sup>2+</sup>	Hg <sup>2+</sup>	Zn <sup>2+</sup>
<i>Aeromonas</i> (11)	0.24–0.57	0–0.67	Cd, Zn (7/11) Cd, Zn, Cu (2/11) Hg, Cd, Zn, Cu (1/11)	1–2.5	5–10	2.5–8.5	5	0.1–1	2.5–12
<i>Salmonella</i> (11)	0.19–0.62	0.33–0.83	Zn, Cr (1/11) Zn, Cu (1/11) Hg, Cd, Zn (1/11) Cd, Zn, Cu, Cr (4/11) Cd, Zn, Pb, Cu (3/11) Hg, Cd, Zn, Pb, Cu (1/11)	0.5–2.5	5–15	2.5–8.5	5–10	0.1–1	5–12
<i>Shewanella</i> (4)	0.19–0.57	0.33–0.5	Cd, Zn (3/4) Hg, Cd, Zn (1/4)	1	5–10	2.5	5	0.1–0.5	7.5–12
<i>Citrobacter</i> (3)	0.33–0.48	0.67–0.83	Cd, Zn, Pb, Cu (1/3) Cd, Zn, Cu, Cr (1/3) Hg, Cd, Zn, Cu, Cr (1/3)	1–5	10–15	8.5	5–7	0.1–1.75	12
<i>Myroides</i> (3)	0.62–0.67	0.5	Cd, Zn, Cu (1/3) Cd, Zn, Pb (1/3) Hg, Cd, Zn (1/3)	1–2.5	5–10	2.5–5.5	5–10	0.1–1	5–7.5
<i>Chryseobacterium</i> (2)	0.48–0.67	0.67–0.83	Cd, Zn, Pb, Cu (1/2) Hg, Cd, Zn, Pb, Cu (1/2)	2.5	10	5.5–7	7	0.1–1	2.5
<i>Enterobacter</i> (2)	0.33–0.38	0.5–0.67	Cd, Zn, Cu (1/2) Hg, Cd, Zn, Cu (1/2)	1–2.5	10	8.5	5	0.1–1	12
<i>Serratia</i> (2)	0.14–0.38	0.33–0.83	Hg, Zn (1/2) Hg, Cd, Zn, Cu, Cr (1/2)	1–2.5	10–15	1–8.5	5	1–1.75	5–12
<i>Bordetella</i> (1)	0.33	0	–	0.5	5	2.5	5	0.1	2.5
<i>Klebsiella</i> (1)	0.43	0.33	Cd, Zn (1/1)	2.5	10	1	5	0.1	5
<i>Pseudomonas</i> (1)	0.52	0.83	Hg, Cd, Zn, Pb, Cu (1/1)	7.5	5	6.5	7	2	12
<i>Vibrio</i> (1)	0.14	0.33	Cd, Zn (1/1)	2.5	5	2.5	5	0.1	5

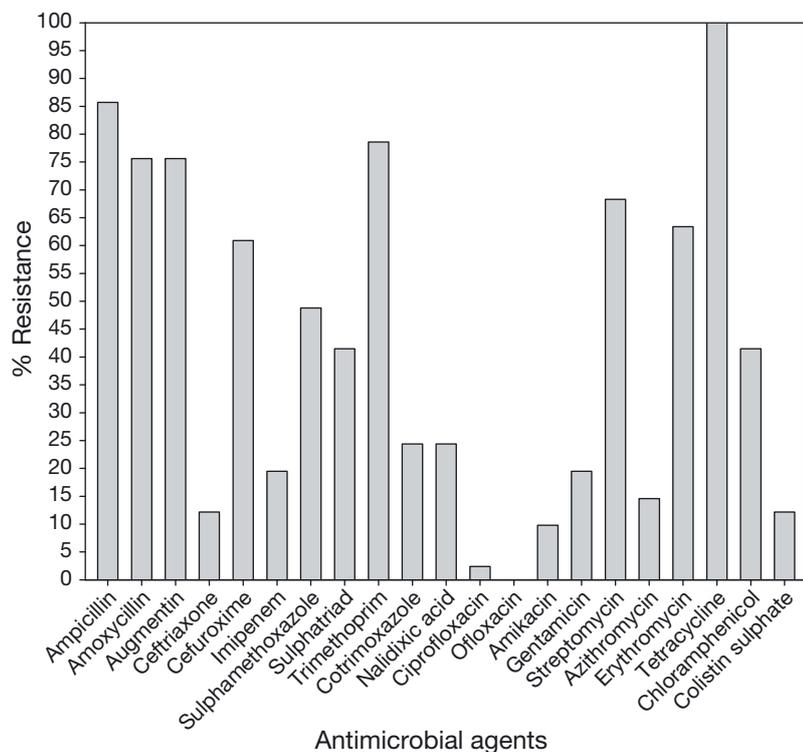


Fig. 1. Overall resistance profiles of bacterial isolates ( $n = 42$ ) from a tilapia aquaculture system to 21 antimicrobial agents representing 8 classes of antimicrobial agents

(Table 1). HMR indices and heavy metal MIC ranges for each of the 12 bacterial genera identified are listed in Table 2. HMR indices ranged from 0.33–0.83, with an HMR index  $\geq 0.5$  for 59.5% of isolates (Tables 2 & 3).

Mercury MICs ranged from 0.1–2 mM (Table 2), with 14.3, 7.1 and 2.4% of isolates tolerating Hg at 1, 1.75 and 2 mM, respectively. Cadmium MICs ranging between 0.5 and 7.5 mM, and MICs of 1 and 2.5 mM (Table 2) were observed for 47.6 and 38.1% of isolates, respectively. The majority of the isolates (95.2%) had Zn MICs  $> 2.5$  mM, of which 28.6, 14.3 and 52.4% had MICs of 5, 7.5 and 12 mM, respectively. Isolates with Cd MICs of 2.5 mM also displayed Zn MICs of 12 mM. A general trend of 4- to 5-fold higher Zn MICs to Cd MICs was observed. At Cd concentrations of  $\geq 2.5$  mM, *Pseudomonas aeruginosa* isolate Z11 also showed increased pyocyanin pigment production. Lead MICs ranged between 5 and 10 mM, with 80.9, 14.3 and 4.8% of isolates displaying MICs of 5, 7 and 10 mM, respectively (Table 2). At a Pb concentration  $\geq 2.5$  mM, resistant isolate colonies turned an intense brown colour. *P. aeruginosa* isolate Z11, however, did not change colour, but demonstrated increased pigment production. MICs for Cu ranged from 1–8.5 mM (Table 2), with 45.2

and 33.3% of isolates displaying MICs of 2.5 and 8.5 mM, respectively. The majority of isolates (92.5%) and all 3 control strains turned brown at  $\geq 1$  mM Cu, which intensified with increasing concentrations. MICs for Cr ranged between 5 and 15 mM (Table 2), with 35.7, 40.5 and 21.4% of isolates demonstrating MICs of 5, 10 and 15 mM, respectively.

HMR indices and HMR phenotypes of individual bacterial isolates are listed in Table 3. Heavy metal MICs appeared to be strain-specific rather than genus-specific. *Aeromonas* spp. isolates displayed low HMR indices, were sensitive to Cr, Cu, Pb and Hg and displayed simultaneous Cd and Zn resistance. *Salmonella enterica* isolates predominantly displayed Cd, Zn, Cu and Cr resistance (Table 2). Members of the family *Flavobacteriaceae* (*Chryseobacterium* and *Myroides* spp.) also displayed high levels of HMR, as did the *Citrobacter*, *Enterobacter* and *Serratia* spp. isolates (Table 2). Only 2 isolates, belonging to the genera *Bordetella* and *Aeromonas*, were 100% sensitive to all 6 heavy metals tested (Table 3).

MAR and HMR indices  $\geq 0.5$  were observed for 21.4% (9/42) of isolates (Table 3). However, high MAR indices were not necessarily accompanied by high HMR indices and vice versa (Table 3). Although no correlation was observed between the MAR and HMR indices, significant positive correlations were observed between erythromycin and HMR profiles ( $r = 0.31$ ,  $p = 0.04$ ) and HMR indices ( $r = 0.33$ ,  $p = 0.03$ ). Significant positive correlations were also observed between isolates demonstrating erythromycin resistance as well as Cu and Cr resistance ( $r = 0.38$ ,  $p = 0.01$ ;  $r = 0.31$ ,  $p = 0.04$ ), respectively.

Isolates displayed resistance to 2 to 5 metals simultaneously (Table 4). Since isolates appeared to be most sensitive to Pb, Cr and Hg (Tables 1 & 2) and resistant to Cu, Cd and  $Zn^{2+}$ , the order of toxicity was  $Pb > Cr > Hg > Cu > Cd > Zn^{2+}$  (Table 4). A significant positive correlation was observed between resistance to Cu and Cr and Pb sensitivity ( $r = 0.38$ ,  $p = 0.01$ ;  $r = 0.34$ ,  $p = 0.03$ ). Only 4 isolates (9.5%) displayed resistance to 5 metals simultaneously, although many of the isolates tested (35.7%) displayed resistance to differ-

Table 3. Multiple antimicrobial resistance (MAR) indices, heavy metal resistance (HMR) phenotype, HMR indices and antimicrobial resistance gene content of individual bacterial isolates (n = 42) from a tilapia aquaculture system. CS: conserved region. On positive amplification of the CS region, specific gene cassettes encoding resistance (R genes) were identified by PCR, where G: (*aac(6')*Ia) gentamicin gene cassette; S: (*ant(3'')*Ia) streptomycin gene cassette; C: (*catB3*) chloramphenicol gene cassette; D: (*dhfr1*) trimethoprim gene cassette; O: (*oxa2a*)  $\beta$ -lactam gene cassette; P: (*pse1*) carbapenem gene cassette (Jacobs & Chenia 2007)

Isolate identification	MAR index	HMR		<i>merA</i> / Tn21		Integron content			
		Phenotype	Index	<i>int1</i>	CS (bp)	<i>qacEΔ1</i>	<i>sulI</i>	R genes	
A14 – <i>Enterobacter</i> spp.	0.38	Cd, Zn, Cu	0.50	–	–	850	–	–	S
A18 – <i>Shewanella oneidensis</i>	0.59	Cd, Zn	0.33	–	–	–	–	–	
A19 – <i>Aeromonas veronii</i> bv sobria	0.43	Cd, Zn	0.33	+/+	+	850 + 946	+	+	S, G, P
A20 – <i>Aeromonas veronii</i> bv veronii	0.33	Cd, Zn	0.33	–	+	850 + 1000	+	+	S, G, P
A31 – <i>Aeromonas veronii</i>	0.38	Cd, Zn	0.33	–	–	850	+	+	S, G, P
A37 – <i>Aeromonas veronii</i> bv veronii	0.38	Cd, Zn	0.33	–	+	850	+	+	S, G, P
A41 – <i>Aeromonas veronii</i> bv sobria	0.43	Cd, Zn	0.33	–	+	850	+	+	G, P, O
B1 – <i>Bordetella</i> spp.	0.33	–	0	–	–	–	+	–	
B3 – <i>Shewanella oneidensis</i>	0.19	Hg, Cd, Zn	0.50	+/+	+	800 + 1350	+	–	S
B5 – <i>Shewanella oneidensis</i>	0.43	Cd, Zn	0.33	–	–	–	+	–	
B7 – <i>Shewanella oneidensis</i>	0.43	Cd, Zn	0.33	–	–	–	+	–	
B13 – <i>Aeromonas veronii</i>	0.38	Cd, Zn	0.33	–	–	650 + 600	+	+	S, G, P
B19 – <i>Aeromonas veronii</i> bv sobria	0.38	–	0	–	–	650 + 600	+	+	S, G, P
B28 – <i>Klebsiella pneumoniae</i> ssp ozaenae	0.43	Cd, Zn	0.33	–	–	–	–	–	S
C9 – <i>Serratia liquefaciens</i>	0.14	Hg, Zn	0.33	-/+	–	–	–	–	
D3 – <i>Vibrio fluvialis</i>	0.14	Cd, Zn	0.33	+/+	–	–	–	–	
D7 – <i>Aeromonas veronii</i>	0.24	Cd, Zn, Cu	0.50	–	+	550	+	–	S, G
D18 – <i>Salmonella enterica</i> ssp. arizonae	0.57	Cd, Zn, Cu, Cr	0.67	–	–	–	–	–	
D27 – <i>Salmonella enterica</i> ssp. arizonae	0.38	Cd, Zn, Pb, Cu	0.67	–	–	–	–	–	
D29 – <i>Citrobacter freundii</i>	0.33	Cd, Zn, Pb, Cu	0.67	–	–	–	–	–	
D40 – <i>Aeromonas veronii</i>	0.52	Hg, Cd, Zn, Cu	0.67	-/+	–	900	+	–	
D42 – <i>Chryseobacterium indologenes</i>	0.67	Hg, Cd, Zn, Pb, Cu	0.83	-/+	–	–	–	–	
D44 – <i>Salmonella enterica</i> ssp. arizonae	0.52	Hg, Cd, Zn	0.50	-/+	–	1000	+	+	S
Y1 – <i>Chryseobacterium</i> spp.	0.48	Cd, Zn, Pb, Cu	0.67	–	–	–	–	–	
Y10 – <i>Salmonella enterica</i> ssp. arizonae	0.19	Cd, Zn, Pb, Cu	0.67	+/+	+	–	–	–	
Y18 – <i>Serratia liquefaciens</i>	0.38	Hg, Cd, Zn, Cu, Cr	0.83	+/-	–	–	–	–	
Y22 – <i>Salmonella enterica</i> ssp. arizonae	0.33	Hg, Cd, Zn, Cu, Cr	0.83	+/-	–	–	–	–	
Y29 – <i>Citrobacter koseri</i>	0.33	Cd, Zn, Cu, Cr	0.67	–	–	–	–	–	
Y32 – <i>Myroides odoratus</i>	0.62	Cd, Zn, Cu	0.50	–	–	–	+	–	
Y43 – <i>Myroides odoratus</i>	0.67	Hg, Cd, Zn	0.50	+/-	–	550	–	–	S, G
Y53 – <i>Myroides odoratus</i>	0.62	Cd, Zn, Pb	0.50	–	–	–	–	–	
Y57 – <i>Citrobacter freundii</i>	0.48	Hg, Cd, Zn, Cu, Cr	0.83	–	–	–	–	+	
Y65 – <i>Salmonella enterica</i> ssp. arizonae	0.33	Cd, Zn, Pb, Cu	0.67	+/-	+	–	–	–	
Z11 – <i>Pseudomonas aeruginosa</i>	0.52	Hg, Cd, Zn, Pb, Cu	0.83	+/+	–	–	–	–	
Z24 – <i>Salmonella enterica</i> ssp. arizonae	0.62	Cd, Zn, Cu, Cr	0.67	–	+	1900	+	+	S
Z39 – <i>Salmonella enterica</i> ssp. arizonae	0.33	Cd, Zn, Cu, Cr	0.67	+/-	–	–	–	–	
Z45 – <i>Salmonella enterica</i> ssp. arizonae	0.52	Zn, Cr	0.33	–	–	1200	–	–	
Z49 – <i>Enterobacter</i> spp.	0.33	Hg, Cd, Zn, Cu	0.67	–	–	–	–	–	
Z51 – <i>Salmonella enterica</i> ssp. arizonae	0.33	Zn, Cu	0.33	–	–	–	–	–	
Z54 – <i>Aeromonas veronii</i>	0.48	Cd, Zn, Cu	0.50	–	–	600 + 1000/1100	+	–	S
Z58 – <i>Aeromonas veronii</i>	0.57	Cd, Zn	0.33	-/+	+	600 + 1300	+	+	S, P
Z64 – <i>Salmonella enterica</i> ssp. arizonae	0.19	Cd, Zn, Cu, Cr	0.67	–	+	–	–	–	

ent combinations of 2 heavy metals (Table 4). Cd and Zn resistance were observed simultaneously in 88.1% (r = 0.61, p = 0.00) of isolates (Table 4). Hg resistance was observed simultaneously with Cd and Zn resistance in 90.0% (9/10) of Hg<sup>2+</sup>-resistant isolates (Table 4). An HMR pattern of Cd = Zn > Cu > Hg > Cr<sup>3+</sup> > Pb was observed for *Aeromonas* spp. isolates, while that of *Salmonella* spp. was Zn > Cd = Cu > Cr = Pb > Hg.

#### Identification of *merA* and Tn21

Apart from the *merA* positive control, only the 2 isolates Z11 (*P. aeruginosa*) and B3 (*Shewanella oneidensis*) produced 1.2 kb *merA* amplicons, while 4 isolates (Y22, Y43, Y65 and Z39) produced truncated 0.9–1 kb amplicons. A positive *merA* hybridization result was obtained for 16.7% (7/42; A19, B3, D3, Y10, Y65, Z11, Z39) of isolates (Table 3). These 7 iso-

Table 4. Prevalence of combinations of heavy metal resistance displayed by bacterial isolates (n = 42) from a tilapia aquaculture system

No. of metals	Simultaneous resistance to combinations of heavy metal ions	% of isolates resistant (no. of isolates)
2		35.7 (15/42)
	Cd + Zn	80.0 (12/15)
	Zn + Cu	6.7 (1/15)
	Zn + Cr	6.7 (1/15)
	Hg + Zn	6.7 (1/15)
3		16.7 (7/42)
	Cd + Zn + Cu	42.9 (3/7)
	Hg + Cd + Zn	42.9 (3/7)
4		14.3 (1/7)
		30.9 (14/42)
	Cd + Zn + Cu + Cr	50.0 (7/14)
	Cd + Zn + Cu + Pb	35.7 (5/14)
5	Hg + Cd + Zn + Cu	14.3 (2/14)
		9.5 (4/42)
	Hg + Cd + Zn + Cu + Pb	75.0 (3/4)
	Hg + Cd + Zn + Cu + Cr	25.0 (1/4)

lates were primarily *Aeromonas* and *Salmonella* spp., and included the 2 isolates identified by PCR. The *merA* gene was thus identified in 23.8% of isolates overall (Table 3). Of the isolates positive for *merA*, 40% had Hg MICs of 0.1 mM (phenotypically susceptible), while the remaining MICs ranged from 0.5–2 mM.

The *tnpM* fragment was amplified from 23.8% (10/42) of isolates (A19, B3, C9, D3, D40, D42, D44, Y10, Z11, Z58; Table 3). However, Tn21 was only associated with *merA* in 50% of the cases (Table 3). Forty percent of isolates with Hg MICs  $\geq 1$  were positive only for Tn21, but not *merA*. Only a single Hg-resistant isolate (Z39, *Enterobacter* spp.) was negative for both Tn21 and *merA* (Table 3).

Statistically significant positive correlations were observed between *merA*/Tn21 presence and mercury resistance ( $r = 0.51$ ,  $p = 0.00$ ) as well as between *merA*/Tn21 presence and HMR indices ( $r = 0.36$ ,  $p = 0.02$ ).

#### Identification of integrons and associated resistance genes

Although the *int1* amplicon was obtained for 26.2% (11/42) of isolates (Table 3), only 4 of the 7 *merA*-positive isolates (isolates A19, B3, Y10 and Y65) yielded a positive integrase result. CS fragments ranging in size from 550 bp to 1.9 kb were

amplified from 40.5% (17/42) of isolates (Table 3). No strict association could be observed between CS fragment amplification and *merA* and/or Tn21. Only 11.8% (2/17) of isolates produced *merA*, Tn21, *int1* and CS amplification products, while Tn21, *int1* and CS fragments were amplified from 17.7% (3/17) of isolates (Table 3). The *qac E1* and *sull* fragments were amplified from 42.8% (18/42) and 26.2% (11/42) of isolates, respectively (Table 3). Significant negative correlations were observed between HMR profiles and *qac E1* and *sull*, respectively ( $r = -0.54$ ,  $p = 0.00$ ;  $r = -0.40$ ,  $p = 0.01$ ) as well as between HMR indices and *qac E1* and *sull*, respectively ( $r = -0.52$ ,  $p = 0.00$ ;  $r = -0.32$ ,  $p = 0.04$ ).

The streptomycin cassette was the most prevalent, being amplified from 88.2% (15/17) of CS-positive fragments. The *aac(6')Ia*, *pse1* and *oxa2a* fragments were amplified from 52.6% (9/17), 47.1% (8/17) and 5.9% (1/17) of isolates, respectively (Table 3).

## DISCUSSION

Antimicrobial resistance determinants selected for in aquaculture ecosystems may be transmitted to human pathogenic bacteria, and Rhodes et al. (2000) proposed that the aquaculture and hospital environments should be considered as a single interactive compartment. Antimicrobial-resistant bacteria present in an aquaculture setting may be transferred to fish-farm workers or processing-plant workers with open wounds following exposure to contaminated water or fish, or downstream handling of fish for food preparation or consumption of aquaculture fish, especially following disease outbreaks (Jacobs & Chenia 2007). Additionally, many reports have suggested that bacterial exposure to metal contamination in natural environments could play an important role in the maintenance and proliferation of antibiotic resistance (Baker-Austin et al. 2006, Seiler & Berendonk 2012). As part of a larger study exploring antimicrobial resistance and biofilm formation in fish-associated *Aeromonadaceae* and *Flavobacteriaceae*, Gram-negative bacteria from a tilapia aquaculture facility were screened for their HMR and antimicrobial resistance phenotypes.

Study isolates were resistant to different concentrations of heavy metals. Although toxicity was observed in the present study with low Hg concentrations (MIC: 0.1 mM), it occurred at a lower frequency than with Pb and Cr, whose toxicity occurred at much higher MICs (5 mM). Pelgrom et al. (1995) observed extremely high bacterial MICs for Cd (2 mM), Cu (6

mM) and Hg (1.2 mM). Tilapia fingerlings were observed to accumulate increased amounts of Hg and Cd with increased exposure time, although Zn concentrations were maintained, in relation to the nutritional status of the fish (Cuvin-Aralar 1994, Pelgrom et al. 1994). Isolates in the present study demonstrated similar or much higher heavy metal MICs compared to those documented in other studies (Nies 1999, Filali et al. 2000). This may be attributed to the bioconcentration of aquatic bacteria such as coliforms and aeromonads in the gut and liver and muscles of tilapia (Pathak & Gopal 2005), with the tilapia intestine wall serving as a storage organ and excretion route for heavy metals (Pelgrom et al. 1995).

A high proportion of Cd-resistant, Cr-susceptible isolates and a pattern of Cd > Cu > Hg > Cr resistance was observed for freshwater Chilean aeromonads displaying a multi-resistance phenotype (Miranda & Castillo 1998), while Australian fish aeromonads had a Cu = Pb > Mn > Cr > Zn > Co > Cd HMR pattern (Akinbowale et al. 2007). *Aeromonas* and *Salmonella* spp. from shrimp displayed a resistance pattern of Hg > Cr > Cd > Cu (Lee et al. 2009). *Aeromonas* spp. isolates in the present study displayed similar characteristics, with Cd = Zn > Cu > Hg > Cr > Pb (Tables 2 & 4), while Zn > Cd = Cu > Cr = Pb > Hg was observed for *Salmonella* spp. isolates. The HMR observed in this study could be the result of heavy metal contamination with fertilisers, since the aquaculture facility sampled was within an agricultural research area or could be due to the use of heavy metals such as copper, an anti-fouling agent (Seiler & Berendonk 2012).

Given the natural abundance of heavy metals in nature and the promiscuous nature of genes residing on resistance plasmids and transposons, HMR is increasingly being linked with antimicrobial resistance due to shared structural and functional characteristics of these resistance systems (Baker-Austin et al. 2006). Although HMR isolates in the present study displayed a high frequency of resistance to penicillin, tetracycline, streptomycin and trimethoprim, no statistically significant correlation was observed between MAR and HMR indices. A high frequency of resistance to ampicillin, streptomycin and tetracycline and maximum resistance to Cr was identified for aeromonads from Chilean salmon (Miranda & Zemelman 2002), while HMR aeromonads and pseudomonads from Australian rainbow trout displayed resistance to  $\beta$ -lactams, trimethoprim and florfenicol (Akinbowale et al. 2007).

The high frequency of Zn and Cd resistance might be attributed to the presence of the *czc* system, which

results in diminished cellular accumulation of the cations due to cation efflux (Hassan et al. 1999). Constitutive expression of the *P. aeruginosa* CzcCBA efflux system is associated with induced resistance to carbapenems (Perron et al. 2004). Although 17.9% and 12.8% of Zn<sup>2+</sup>-resistant isolates in the present study displayed resistance or moderate susceptibility to imipenem, no statistically significant association was observed between Zn<sup>2+</sup> resistance and imipenem resistance. Isolates Z58, B28 and D3 displayed Cd resistance but did not tolerate Zn, potentially indicating the presence of specific Cd resistance mechanisms similar to the *cad* operon of Gram-positive organisms (Oger et al. 2003). Although erythromycin resistance (*mphBM*) appeared to be linked to Cd resistance (*cadC* and *cadA*) in the Gram-negative bacterium *Stenotrophomonas maltophilia* (Alonso et al. 2000), and the majority of the erythromycin-resistant isolates in the present study were resistant to both Cd and Zn, no statistically significant relationship was observed. Instead, erythromycin resistance appeared to be linked to Cu and Cr resistance. The Cu resistance phenotype has been previously linked with macrolide resistance (Hasman & Aarestrup 2002).

Copper sulphate, used in large amounts as a feed additive in food animal production and as an anti-foulant, may select for resistant intestinal microorganisms (Hasman & Aarestrup 2002, Seiler & Berendonk 2012). Since Cu is an important co-factor for enzymatic reactions, it is constantly present in bacterial cells, and the 50% Cu resistance prevalence observed in the present study might be attributed to natural Cu homeostasis or acquired Cu resistance. A visual prediction of the periplasmic sequestration of Cu<sup>2+</sup> was made, due to colony colour change by some of the isolates at high concentrations. Isolates without the colour change might possess mechanisms similar to the *E. coli* Cu efflux mechanism where Cu<sup>2+</sup> is not accumulated in the periplasm but removed from the cell via efflux proteins encoded by plasmid-bound *pco* genes (Silver 1996, Spain 2003). Chromate-resistant isolates from tannery effluent showed resistance to Cu, Mn, Zn, As, Cd, Ni, Co and Hg, as well as MDR phenotypes (Verma et al. 2001). Although the mechanism has not been defined, many bacteria of the genera *Pseudomonas*, *Aeromonas*, *Enterobacter*, *Escherichia* and *Bacillus* can reduce Cr(VI) to Cr(III) (Upreti et al. 2004). Chromate resistance >10 mM was observed predominantly amongst *Salmonella enterica* isolates in the present study.

Pb appeared to be the most toxic to study isolates, with only 19.1% displaying resistance to >5 mM.

Although the specific mechanism of resistance could not be confirmed, the colour change at 2.5 mM indicated a periplasmic sequestration mechanism similar to that observed for Cu-resistant isolates. Pb-resistant *Staphylococcus aureus* and *Citrobacter freundii* have been observed to accumulate lead at the cell surface, and Cd resistance and ampicillin resistance accompanied the Pb resistance in *C. freundii* (Levinson & Mahler 1998). Isolates in the present study displaying resistance to Pb were found to be resistant to Zn and/or Cd, and resistant to a variety of  $\beta$ -lactams and cephalosporins.

Hg-resistant bacteria isolated from freshwater fish of Calcutta wetland fisheries displayed both antibiotic and heavy metal multi-resistance (Sadhukhan et al. 1997). Bottom-dwelling fish like koi, shol and tilapia contained higher numbers of Hg-resistant bacteria than surface or middle water-dwelling fish and this could be correlated with cross-resistance to other heavy metals and/or antimicrobials (Sadhukhan et al. 1997). Tn21 and other Tn21-like transposons carrying integron elements have been detected in *E. coli* from agricultural, veterinary and clinical sources (Bass et al. 1999, Pezzella et al. 2004). Tn21 encodes *mer* resistance and contains a class 1 integron encoding resistance to sulphonamides and streptomycin/spectinomycin (Liebert et al. 1999). In the present study, although a significant positive correlation was observed between Hg resistance and *merA*/Tn21 presence, as described for avian *E. coli* (Bass et al. 1999), integron presence was not similarly linked to Hg resistance and *merA*/Tn21 presence.

The MDR phenotypes demonstrated by study isolates were surprising given that the tilapia aquaculture system had not been exposed to antibiotic treatment. This might to some extent reflect transmission from microflora of the external environment, water and human handlers to the indigenous fish microflora. The high frequency of HMR might be attributed in part to the intrinsic mechanisms of HMR resulting from bacterial ultrastructure and physiology, and potential environmental exposure from water used in the aquaculture system. In the present study, isolates displayed both antimicrobial resistance and HMR despite not being deliberately exposed to either heavy metals and/or antimicrobial compounds, highlighting the need for surveillance of commensal and pathogenic microorganisms associated with cultured fish. A greater insight into the dynamics of co-selection due to linkage of HMR and antimicrobial resistance genes could be obtained by whole-genome and/or plasmid sequencing.

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