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Full Length Research Paper

Tetracycline resistance determinants of heterotrophic bacteria isolated from a South African tilapia aquaculture system

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Tetracycline-resistant bacteria are frequently isolated from aquaculture systems, where mobile resistance genes often transfer between bacteria associated with fish kept at high stocking densities. Bacterial isolates from an *Oreochromis mossambicus* (tilapia) aquaculture system (Stellenbosch, South Africa) were screened for their susceptibility to tetracycline. Genomic and plasmid DNA were used in PCR-RFLP assays employing six degenerate primer sets to identify the prevalence of nine tetracycline resistance genes. Isolates displaying a *tet*(A)-type tetracycline resistance gene were examined further for an association with transposon Tn*1721*. *tet*(A) was identified as the predominant tetracycline resistance determinant, followed by *tet*(B), -(E), and –(C) determinants. Isolates appeared to possess multiple *tet* genes simultaneously. Of the isolates presented with a *tet*(A) determinant, 73.9% appeared to be associated with Tn*1721*. No association between type of tetracycline resistance gene, presence on chromosome or plasmid, and MIC could be established. The Tn*1721* association may explain the high frequency of isolation of *tet*(A). The high levels of resistance displayed by isolates from the tilapia aquaculture system not previously exposed to antimicrobial agents is of concern and will have implications for future therapeutic interventions in disease outbreak situations.

Key words: Tetracycline resistance, aquaculture, tilapia, transposons.

INTRODUCTION

The widespread use and abuse of antimicrobial agents for both human and animal medicine has enormous implications in the generation of a diversity of continually evolving antimicrobial-resistant microorganisms. The broad-spectrum activity of tetracyclines has been exploited for human clinical therapy and prophylaxis as well as in animal husbandry for therapy, prophylaxis and as a growth promoter (Chopra and Roberts, 2001; Roberts, 2003; Seyfried et al., 2010).

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The tetracycline group of broad-spectrum antimicrobial agents inhibits protein synthesis in both Gram-negative and Gram-positive bacteria by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (Chopra and Roberts, 2001). Bacterial resistance to tetracycline is mediated mainly by two mechanisms, protection of ribosomes by large cytoplasmic proteins and energy-dependent efflux of tetracycline (Roberts, 2005). Forty-six different *tet* and *otr* genes have been identified, of which 30 encode energy-dependent efflux proteins, 12 ribosomal protection proteins, three inactivating enzymes, and one with an unknown resistance mechanism, respectively (Roberts, 2005). The efflux proteins export tetracycline out of the cell, reducing the intracellular

concentration and protecting the ribosomes *in vivo*, and is seen more frequently in Gram-negative bacteria (Roberts, 2003). Ribosomal protection proteins play a role when tetracycline binds to the ribosome, changing the conformational state and disrupting protein synthesis. The ribosomal protection proteins, via allosteric disruption of the primary tetracycline binding site, facilitate the separation of the tetracycline molecule from the ribosome (Roberts, 2005). The third mechanism involves enzymatic inactivation, whereby an NADPH-requiring oxidoreductase inactivates tetracycline in the presence of oxygen and NADPH (Roberts, 2005).

Acquired *tet* genes tend to be associated with mobile elements, that is, plasmids, transposons, conjugative transposons and/or integrons which carry other antimicrobial resistance genes or heavy metal tolerance genes. These mobile genetic elements facilitate the transfer of the *tet* genes between unrelated species and genera by conjugation (Roberts, 2003). The *tet* efflux genes of Gram-negative bacteria tend to be transposon- or integron-associated and inserted into a diversity of plasmids. The ribosomal protection proteins, on the other hand, tend to be chromosomally-located and are parts of conjugative or non-conjugative transposons (Roberts, 2003). The prevalence and host range of specific *tet* genes appears to be determined by linkage with specific types of mobile elements, with those on broad host range conjugative transposons more likely to be found in a large number of diverse bacteria than the *tet* genes on non-conjugative elements or plasmids with a narrow host range (Roberts, 2005).

Large amounts of antimicrobials, including tetracycline, are applied in intensive fish farming systems in order to treat disease or prevent potential disease outbreaks (Gao et al., 2012; Ryu et al., 2012). This has been accompanied by an increased frequency of tetracycline resistance among fish commensal and pathogenic bacteria (Akinbowale et al., 2007; Gao et al., 2012; Ryu et al., 2012). In aquaculture ecosystems, several *tet* determinants, *tet*(A) – *tet*(W), have been identified in fish-pathogenic bacteria from a number of geographical locations and fish species (DePaola et al., 1988; Adams et al., 1998; Rhodes et al., 2000; Schmidt et al., 2001; Miranda and Zemelman, 2002; Furushita et al., 2003; Miranda et al., 2003; Akinbowale et al., 2007; Seyfried et al., 2010; Gao et al., 2012) as well as amongst commensals (Ryu et al., 2012).

Isolates from the aquaculture setting, either fish- or water-associated, may be potential opportunistic human pathogens during the processing, packaging, or preparation of fish, especially following disease outbreaks. The aquaculture and hospital environments should be regarded as a single interactive compartment (Rhodes et al., 2000) and transmission of resistance determinants could potentially occur via the spread of mobile genetic elements such as plasmids from fish pathogens to human pathogens (Kruse and Sørum, 1994).

Since the products of aquaculture are destined for human consumption, and since many antimicrobial resistance determinants are encoded by transferable plasmids, cultured fish may serve as a vehicle for transmission of antimicrobial resistance to bacteria that are commensal or pathogenic to humans in different ecosystems (Rhodes et al., 2000). The study of antibacterial resistance of authocthonous bacteria permits an evaluation of its role in the maintenance and transfer to other bacteria, including those which are potentially pathogenic, in order to understand the gene flux encoding for bacterial resistance in fish culture and carriage to the human compartment of the environment (Miranda and Zemelman, 2002). The present study was thus undertaken to examine the natural prevalence of tetracycline resistance determinants among Gram-negative bacteria in an *Oreochromis mossambicus* (tilapia) aquaculture system which had not been previously exposed to tetracyclines or any other antimicrobial agents.

MATERIALS AND METHODS

Isolation of bacterial strains

Fish, tank water, tank sediment and unmedicated feed samples from an *O. mossambicus* (tilapia) aquaculture system (AquaStel, Stellenbosch, South Africa) were plated out onto Brain Heart Infusion (BHI) and Tryptone Soy (TS) agar plates containing 25 µg/ml tetracycline, as well as plates without tetracycline and incubated at 30 to 37°C for 24 h. Sampling was carried out over a month, with sampling every week. Forty-four heterotrophic, Gram-negative bacterial isolates belonging to diverse genera including *Acinetobacter, Aeromonas, Bordetella, Chryseobacterium, Enterobacter, Myroides, Pseudomonas, Salmonella* and *Shewanella,* were identified by standard biochemical and physiological tests and selected for further study based on their multiple antimicrobial resistance phenotypes and carriage of one or more plasmids. These isolates were maintained at room temperature (25°C) on BHI agar supplemented with 25 µg/ml tetracycline, while long-term stocks were stored at -70°C in 20% glycerol.

Antimicrobial susceptibility testing

Study isolates were grown overnight at 25°C in BHI broth and then diluted in phosphate-buffered saline (PBS) to achieve a turbidity equivalent to a 0.5 McFarland standard. Tetracycline resistance was determined by placing tetracycline discs (25 µg; Mast laboratories, UK) and tetracycline E-test strips (0.016 to 256 µg/ml; AB Biodisk, Sweden) onto inoculated Mueller-Hinton (MH) plates, to obtain tetracycline zone diameters and minimum inhibitory concentration (MIC) values, respectively. Testing was done in duplicate and resistance profiles (resistant, intermediate, or susceptible) were assigned after measuring average zone diameters following CLSI breakpoints (CLSI, 2006). Tetracycline E-test MIC values were determined according to manufacturer's criteria. Bacterial strains *Escherischia coli* ATCC 25922,

Table 1. Primer sets used to amplify *tet,* transposon and β-lactamase genes.

Resistance determinant	Primer name	Primer sequences	Expected PCR fragment size (bp)	Annealing temperature and time	Reference
tet(A), (B), (C)	TetABC-F TetABC-R	GCY RTV GGS ATH GGC YTK RTY ATG C ACM GCM CCW GTV GCB CCK GTG AT	293	55°C for 45 s	Schnabel and Jones (1999)
tet(D), (E), (H)	TetDEH-F TetDEH-R	GCB ATK GGD MTY GGB MTN ATY ATG C ACV GCD CCD GTB GCR CCN GTR AT	293	55°C for 45 s	Schnabel and Jones (1999)
tet(A), (C)	TetAC-F TetAC-R	CGC YTA TAT YGC CGA YAT CAC CCR AAW KCG GCW AGC GA	417	55°C for 1 min	Furushita et al. (2003)
tet(B), (D)	TetBD-F TetBD-R	GGD ATT GGB CTT ATY ATG CC ATM ACK CCC TGY AAT GCA	967 / 964	52°C for 1 min	Furushita et al. (2003)
tet(E), (H), (J)	TetBD-F TetEHJ-R	GGD ATT GGB CTT ATY ATG CC AWD GTG GCD GGA ATT TG	650	52°C for 1 min	Furushita et al. (2003)
tet(G), (Y)	TetGY-F TetGY-R	TAT GCR TTK ATG CAG GTC GAC RAK CCA AAC CCA ACC	917 / 911	52°C for 1 min	Furushita et al. (2003)
Tn*1721*	TetAR3 3'TAF	GGC ATA GGC CTA TCG TTT CCA GTA ATT CTG AGC ACT GTC GC	1200	55°C for 1 min	Pezzella et al. (2004)
TEM β-lactamase	Bla-TEM-F Bla-TEM-R	TTG GGT GCA CGA GTG GGT TAA TTG TTG CCG GGA AGC	503	55°C for 1 min	Guerra et al. (2001)

Enterococcus faecalis ATCC 29212, and *Staphylococcus aureus* ATCC 25923 were used as antimicrobial susceptibility controls according to CLSI recommendations (CLSI, 2006).

DNA extraction

Genomic DNA was extracted, following overnight culture, according to the CTAB/NaCl miniprep protocol (Ausubel et al., 1989). Plasmid DNA preparations were obtained using the modified alkaline lysis method of Birnboim and Doly (1979).

Identification of tetracycline resistance genes

Plasmid and genomic DNA were used as templates to amplify nine genes belonging to the *tet*(A) determinant family of *tet* genes, using two and four degenerate primer sets described by Schnabel and Jones (1999) and Furushita et al. (2003), respectively (Table 3). PCR amplification was carried in 25 µl reaction mixtures containing 50 ng plasmid DNA or 100 ng genomic DNA, 0.2 mM of each dNTP (Roche, Germany), 100 µM of each respective primer set, 1.5 mM $MgCl_2$, 1 U SuperTherm *Taq* DNA polymerase (JMR Holdings, UK), together with 1 × reaction buffer in a PCRSprint thermal cycler (Hybaid, UK). Amplification consisted of 35 cycles of denaturation at 94°C for 30 s, specific primer annealing temperatures and durations for the different primer sets are indicated in Table 1 and elongation at 72°C for 90 s. A previous denaturation step of 94°C for 3 min and a final elongation step of 10 min

Table 2. Restriction endonucleases and respective PCR-RFLP profiles of amplified *tet* genes.

PCR amplimer	Predicted amplified fragment size (bp)	Target gene	Restriction endonuclease	Restriction fragment sizes (bp)
TetABC	293	*tet*(A)	*Hae*III	75 +130
		tet(B)	*Hae*III	240 or 254
		tet(C)	*Hae*III	104 + 123
TetDEH	293	*tet*(D)	*Hae*III	293
		tet(E)	*Hae*III	153 + 140
		tet(H)	*Hae*III	293
TetAC	417	*tet*(A)	*Sma*I	267 + 150
		tet(C)	*Sal*I	216 + 201
TetBD	967 or 946	*tet*(B)	*Sph*I	492 + 302 + 173
		tet(D)	*Sph*I	640 + 324
TetEHJ	650	*tet*(E)	*Nde*II	425 + 148 + 77
		tet(H)	*Nde*II	383 + 267
		tet(J)	*Nde*II	297 + 236 + 117
TetGY	917 or 911	*tet*(G)	*Eco*RI	549 + 368
		tet(Y)	*Sph*I	714 + 197

at 72°C were included in the amplification cycle. Amplification products were visualized by UV transillumination following electrophoresis in 2% 1 × TAE agarose gels and staining with ethidium bromide. DNA molecular weight marker VI (Roche) was included in every electrophoretic run to provide a DNA size standard.

In order to identify the specific *tet* determinant, PCR fragments were subjected to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis using specific enzymes described by Schnabel and Jones (1999) and Furushita et al. (2003), respectively (Table 2). Respective PCR fragments were digested for 3 h with restriction enzymes (Fermentas, Canada), indicated in Table 2, at the required temperatures. Restriction fragment profiles were obtained following polyacrylamide gel electrophoresis in 8% polyacrylamide gels, staining with ethidium bromide, and UV visualization. The O'GeneRuler™ 100 bp DNA Ladder Plus (Fermentas) was included in every run for DNA size analysis of fragments. Molecular weights of fragments were determined using the UVIDOC Gel Documentation System (UVIDOC V.97, UVITec, UK).

Association of TetA determinants with transposons

Transposon Tn*1721* fragments were amplified using TetAR3 and 3'TAF primers (Table 1) described by Pezzella et al. (2004). The 1.2 kb PCR fragments were obtained following amplification at the following parameters: 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min and elongation for 1 min at 72°C in the PCR Sprint thermal cycler (Hybaid, UK). A previous denaturation step of 94°C for 3 min and a final elongation step of 10 min at 72°C were included in the amplification cycle.

Prevalence of TEM-1 resistance gene

The prevalence of the TEM-type β-lactamase gene was also investigated using primers (Table 1) described by Guerra et al. (2001), to amplify a 503 bp fragment, using previously described amplification conditions.

RESULTS

Antimicrobial susceptibility testing

All isolates (100%) displayed zone diameters indicative of tetracycline resistance, while tetracycline E-test MIC values ranged between 12 and 256 µg/ml.

Identification of tetracycline resistance genes

The Schnabel and Jones (1999) and Furushita et al. (2003) primer sets amplified *tet* genes from 75% (33/44) and 90.6% (40/44) of isolates, respectively (Figure 1). The *tet*(A) determinant was isolated from majority of the study isolates by both the Schnabel and Jones (1999) and Furushita et al. (2003) primer sets (Figures 2 and 3, Table 3). The Schnabel and Jones (1999) primer set appeared to be more effective in identifying the *tet*(B) and *tet*(C) determinants (Figure 2) while the Furushita et al.

Table 3. Comparison of *tet* determinant types identified using different degenerate primer sets.

tet determinant type	% of isolates presenting with specific *tet* determinant type (no. of isolates positive/overall no. of isolates tested)	
	Schnabel and Jones (1999)	Furushita et al. (2003)
tet(A)	34.1 (15/44)	40.9 (18/44)
tet(B)	29.6 (13/44)	22.7 (10/44)
tet(C)	25 (11/44)	13.6 (6/44)
tet(D)	2.3 (1/44)*	6.8 (3/44)
tet(E)	11.4 (5/44)	31.8 (14/44)
tet(GY)	n/a	15.9 (7/44)
tet(H)	*	6.8 (3/44)
tet(J)	n/a	0

*TetD/H determinant could not be differentiated by *Hae*IIIPCR-RFLP analysis.

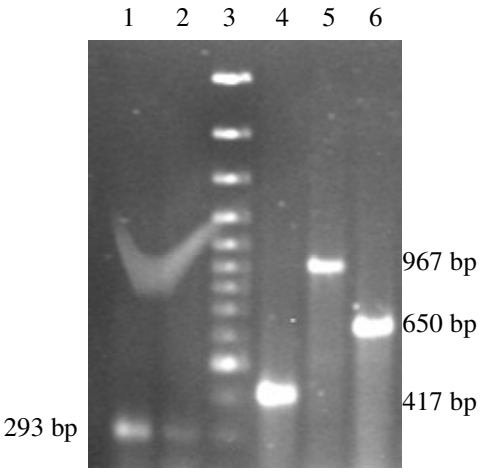

Figure 1. Agarose gel electrophoresis of amplification products with Schnabel and Jones (1999) and Furushita et al. (2003) *tet* primers. Lane 1: amplification product with TetABC primer; lane 2: amplification product with TetDEH primer; lane 3: O'GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Canada); lane 4: TetAC amplification product; lane 5: TetBD amplification product; and lane 6: TetEHJ amplification product.

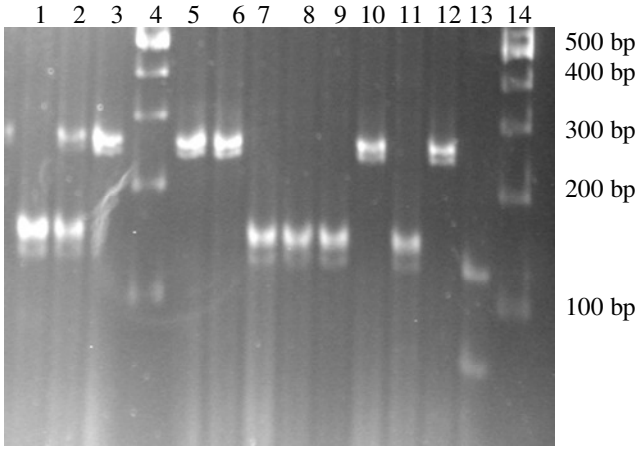

Figure 2. Polyacrylamide gel electrophoresis of *Hae*III-digested TetABC amplification products. Lanes 1, 7-9, and 11: isolates possessing *tet*(A) resistance gene; lane 2: isolate with multiple *tet*(A)+*tet*(B) resistance genes; lanes 3, 5, 6, 10 and 12: isolates presenting *tet*(B) resistance gene; lane 13: isolate with *tet*(C) resistance gene; lanes 4 and 14: O'GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Canada).

(2003) primer set was more effective at identifying the *tet*(E) determinant (Table 3). There was no significant correlation between the determinants amplified using the Schnabel and Jones (1999) and Furushita et al. (2003) primer sets for individual isolates.

Using the Schnabel and Jones (1999) primer sets, 36.4% (12/33) of the isolates appeared to carry two *tet* determinants simultaneously. These included combinations of *tet*(A) + *tet*(B) (6.1%), *tet*(B) + *tet*(C) (6.1%), *tet*(A) + *tet*(C) (6.1%), *tet*(B) + *tet*(D/H) (3%) and *tet*(C) + *tet*(E) (15.1%). The combination of *tet*(C) + *tet*(E) determinants appeared predominantly among *Aeromonas* spp. isolates.

A greater diversity of *tet* determinants was indentified with the Furushita et al. (2003) primer sets (Table 1). A number of isolates were found to be carrying multiple *tet* determinants, that is, 25% (11/44); 13.6% (6/44), and 2.3% (1/44) of isolates carried two, three, and four *tet* determinants, respectively.

It was not possible to correlate tetracycline MIC values with the presence of one or multiple *tet* determinants with both the Schnabel and Jones (1999) and Furushita et al. (2003) primer sets, since some isolates carrying a single

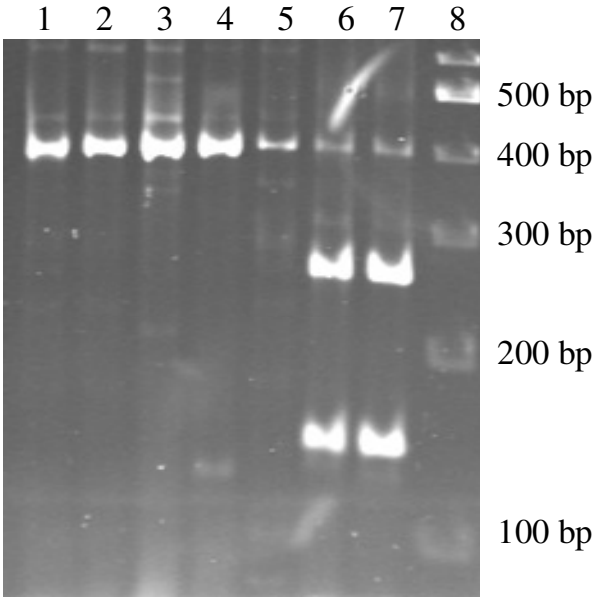

Figure 3. Polyacrylamide gel electrophoresis of *Sal*I- and *Sma*I-digested TetAC amplification products. Lanes 1 to 5: *Sal*I digestion of TetAC amplification product indicating a *tet*(A) resistance gene; lanes 6 and 7: *Sma*I digestion of TetAC amplification product indicating a *tet*(A) resistance gene; lane 8: O'GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas, Canada).

tet determinant had a higher MIC than those with multiple *tet* determinants. A greater diversity of *tet* determinants were identified when genomic DNA was used as template for amplification of *tet* gene fragments compared to plasmid DNA template.

Association of *tet*(A) determinants with transposons

Of the 52.3% (23/44) of isolates presented with the *tet*(A) gene, 73.9% (17/23) appeared to be associated with Tn*1721*, which was indicated by amplification of the 1.2 kb fragment of Tn*1721*. The majority of these isolates were *Aeromonas* spp. isolates, followed by *Salmonella enterica* serotype Arizonae isolates.

Prevalence of TEM-1 resistance gene

The TEM-1 β-lactamase fragment was amplified from 52.3% (23/44) of study isolates. Of these, 30.4% (7/23) were amplified using the plasmid DNA template, while the remaining 69.6% (16/23) were amplified using genomic DNA as the template. *Aeromonas* spp. isolates appeared to be the major genus harbouring the TEM-1 β-lactamase gene in this system.

DISCUSSION

Altogether, the prevalence of nine genes belonging to the *tet*(A) family of tetracycline determinants was investigated in the present study, using both plasmid and genomic DNA templates. Since more genes were targeted with the Furushita primer sets, more variation was observed for study isolates (Table 3). In the present study, there appeared to be a high prevalence of *tet*(A), followed by *tet*(E), *tet*(B) and *tet*(C) determinants, while the *tet*(D), *tet*(H) and *tet*(GY) determinants appeared infrequently.

The overall 54.5% prevalence of *tet*(A) among study isolates is similar to that identified in other fish farm studies (DePaola et al., 1988; Adams et al., 1998; Schmidt et al., 2001; Miranda et al., 2003; Jun et al., 2004; Seyfried et al., 2012). Furushita et al. (2003) reported a higher prevalence of *tet*(B) genes among Japanese fish farm bacteria, but did not detect the *tet*(A) and *tet*(C) genes. Ryu et al. (2012) identified *tet*(B) and *tet*(D) as the prevalent resistance genes in *E. coli* from Korean commercial fish and seafood. No *tet*(B) and *tet*(C) genes were detected by Schmidt et al. (2001) in the Danish fish farming environment. A higher prevalence of the *tet*(E) determinant, compared to *tet*(A), was not detected in the present study as observed on other fish farms worldwide (DePaola et al., 1988; Andersen and Sandaa, 1994; DePaola and Roberts, 1995; Akinbowale et al., 2007).

Screening of Gram-negative isolates revealed that < 10% carried multiple *tet* genes (Chopra and Roberts 2001; Miranda et al., 2003). However, this scenario is changing with evidence collated from farmed vs. wild animals and human studies. More than 10% of the tetracycline-resistant Gram-negative populations may possess multiple *tet* genes in some ecosystems and this affects characterization of *tet* genes (Roberts, 2005). Although the majority of isolates in the present study presented with a single *tet* determinant, a large number of isolates presented with multiple *tet* determinants. Schmidt et al. (2001) and Furushita et al. (2003) observed combinations of *tet*(A)+(E), *tet*(A)+(D), *tet*(E)+(D), *tet*(B)+(C) and *tet*(D)+(E). In the present study, a higher prevalence of *tet*(C)+(E), *tet*(B)+(E), *tet*(A)+(C) and *tet*(A)+(B) were observed. Akinbowale et al. (2007) observed that there was no correlation between the presence of multiple tetracycline resistance genes and the MIC. This lack of correlation was also observed in the present study.

Studies on *tet* determinants in the fish farm environment suggested that the *tet*(A)-(E) determinants were not uniformly distributed but appeared to be associated with specific genera and species (DePaola et al., 1988; Adams et al., 1998; Schmidt et al., 2001; Miranda et al., 2003). Among the *Aeromonas* spp. and *S. enterica* serotype Arizonae isolates assayed in the present study, the predominant *tet* determinant was

tet(A). Using both the Schnabel and Jones (1999) and Furushita et al. (2003) primers sets, the *tet*(C)+(E) combination was identified for isolates predominantly belonging to the genus *Aeromonas*. It is possible that *tet* determinant distribution patterns vary with the species or the sampling origin, implicating ecosystem-specific reservoirs for tetracycline resistance genes (Guillaume et al., 2000).

Tn*1721* and Tn*1721*-like elements are significant in the dissemination of the *tet*(A) determinant, playing an important role in the global dissemination of these genes and other plasmids (Rhodes et al., 2000). Among *Salmonella* spp., *tet*(A) is part of transposon Tn*1721* as well as a truncated Tn*1721*, which lacks a portion of the left arm (Pezzella et al., 2004). The 1.2 kb Tn*1721* fragment was amplified from 73.9% of study isolates including *Aeromonas* and *Salmonella* spp. isolates as well as *Enterobacter* spp. and *Pseudomonas aeruginosa* isolates containing *tet*(A), *tet*(A)+(C) and/or *tet*(C)+(E) determinants. The *tet*(A) determinant and associated Tn*1721* was frequently detected amongst *Aeromonas* spp. isolates as has been reported by other studies (DePaola et al., 1988; Adams et al., 1998; Schmidt et al., 2001; Miranda et al., 2003; Hatha et al., 2005). This was also observed for the *S. enterica* serotype Arizonae isolates, which is typical for salmonellae (Frech and Schwarz, 2000). The role of transposons associated with these determinants should not be underplayed since movement of these genes is facilitated via plasmid vectors or by direct transposition.

tet genes of classes A, B, D and H are associated with non-conjugative transposons or transposon-like elements, while those of classes C, E, and G are often found on plasmids (Butaye et al., 2003). The *tet*(B) and *tet*(G) determinants are regarded as being non-mobile (Jun et al., 2004) and this may explain their limited distribution amongst bacteria. *tet*(A) genes from *S. enterica* isolates from animals were found to be both plasmid- and chromosomally-located, while *tet*(B), *-(C),* and *–(D)* were chromosomally located (Pezzella et al., 2004). While it was attempted to localise the *tet* determinants to plasmid or chromosomal locations, the different primer sets, in the present study, were not useful in this regard. A greater diversity of *tet* determinants was identified using genomic DNA as a template.

Tetracyclines are used most frequently in veterinary medicine, while β-lactam use ranks third. Resistance to β-lactams and aminoglycosides was observed among tetracycline-resistant isolates. There might be co-selection of other resistance genes during tetracycline use. It is also possible that co-selection of *tet* genes occurred under selective pressure from use of other antimicrobials (Frech and Schwarz, 2000). The tetracycline resistant study isolates also appeared to carry other unrelated resistance determinants, for example the TEM-1 β-lactamase gene encoding β-lactam resistance. A number of these isolates also carry integrons containing resistance cassettes encoding aminoglycoside resistance as well as resistance to sulphonamides and quaternary ammonium compounds, and were resistant to heavy metals (data not shown). It is thus not unlikely, that the combination of different resistance genes, whether associated with mobile genetic elements or not, will facilitate selection of the resistant subpopulations of bacteria in the aquaculture environment when challenged with tetracycline (therapeutically or prophylactically) and vice versa – "hunting as a pack" phenomenon.

The high prevalence of resistance genes in the aquaculture system surveyed cannot be explained since the fish system had not encountered any disease outbreaks and thus has not been exposed to any type of antimicrobial agent. Seyfried et al. (2010) observed that many aquaculture facilities had a higher prevalence of tetracycline resistance genes irrespective of tetracycline use and dosage. Increased tetracycline resistance can be observed in any aquatic environment where there is an accumulation of fish feed, since fish farm influents and pelletised feed may serve as reservoirs of resistance genes even in the absence of antibacterial agents (Miranda and Zemelman, 2002; Seyfried et al., 2010). Dead commensal and pathogenic bacteria or bacteria in excreta may serve as vectors for the transmission of mobile resistance determinants. Additionally, a biofilm lifestyle (sides of aquaculture tanks, inlet and outlet pipe systems) may serve not only as potential reservoirs of resistance determinants and their associated mobile genes but also provide microniches where transfer events can occur at high frequencies sources (Cvitkovitch, 2004).

The diversity of *tet* resistance determinants isolated from an aquaculture system not previously treated with antimicrobial agents highlights the need to have continuous surveillance of resistance genes within this environment in order to ensure judicious use of antimicrobial agents for therapy and/or prophylaxis. Since the product of aquaculture is destined for human consumption, knowledge of genes circulating amongst zoonotic bacteria is crucial in trying to limit the movement of these genes through the food chain and decrease their impact on human health and therapy (Seyfried et al., 2010; Gao et al., 2012; Ryu et al., 2012).

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