Université de Montréal

Characterization of antimicrobial resistance in *Aeromonas* and *Vibrio* isolated in Canada from fish and seafood

par

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Characterization of antimicrobial resistance in *Aeromonas* and *Vibrio* isolated in Canada from fish and seafood

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Summary

Multiple studies have examined antimicrobial susceptibility in bacteria from aquacultured products microorganisms and their environment. However, no information is available concerning antimicrobial resistance in bacterial flora of fish and seafood available at the retail level in Canada. This is particularly true for the common aquatic commensals, *Aeromonas* and *Vibrio,* for which some species are known zoonotic pathogens. In the course of this study, the antimicrobial susceptibility among *Aeromonas* spp. and *Vibrio* spp. from domestic and imported fish and seafood was characterized. *Aeromonas* and *Vibrio* spp. isolates cultured from finfish and shrimp samples were evaluated for antimicrobial susceptibility by broth microdilution and/or disk diffusion techniques. Antimicrobial classes examined in detail included: tetracyclines (TET), folate pathway inhibitors (sulfadimethoxine-trimethoprim, SXT), florfenicol (FLO), and the quinolones (nalidixic acid / enrofloxacin, NA/ENO). Epidemiological cut-off values (ECV's) for *Aeromonas*/*Vibrio* were established using normalized resistance interpretation (NRI) of disk diffusion data. Isolates were further examined by PCR and microarray for genes associated with their antimicrobial resistance. Of 201 *Aeromonas* and 185 *Vibrio* isolates, those classified as resistant were as follows, respectively: TET (n=24 and 10), FLO (n=1 and 0), SXT (n=2 and 8), NA (n=7 and 5) and ENO (n=5 and 0). Various combinations of *tet*(A)*, tet*(B)*, tet*(E)*, floR, sul1, sul2* and *intI1* genes were detected with *tet*(E)*, intI1, sul2* and *tet*(B) being the most common. *Vibrio* and *Aeromonas* species isolated from retail fish and seafood sources can harbor a variety of resistance determinants, although their occurrence is not high. The risk represented by these resistances remains to be evaluated in view of the potential for bacterial infection and their role as a reservoir for antimicrobial resistance.

Key words: Aeromonas, Vibrio, antimicrobial resistance, normalised resistance interpretation (NRI), microarray, PCR, resistance genes

Résumé

Plusieurs études ont examiné la sensibilité aux antimicrobiens chez les bactéries d'organismes provenant de produits issus de l'aquaculture ou de leur environnement. Aucune information n'est cependant disponible concernant la résistance aux antimicrobiens dans les bactéries de la flore de poissons ou de fruits de mer vendus au détail au Canada. C'est particulièrement vrai en ce qui a trait aux bactéries des genres *Aeromonas* et *Vibrio,* dont certaines espèces sont des agents pathogènes zoonotiques connus. Au cours de cette étude, la sensibilité aux antimicrobiens d'isolats d'*Aeromonas spp*. et de *Vibrio spp*. provenant de poissons et de crevettes domestiques et importés a été mesurée à l'aide de techniques de micro dilution en bouillon et/ou de diffusion sur disque. Les classes d'antimicrobiens examinés comprenaient les tétracyclines (TET), les inhibiteurs de la voie des folates (sulfadiméthoxine-triméthoprime, SXT), le florfenicol (FLO), et les quinolones (acide nalidixique / enrofloxacine, NA/ENO). Des valeurs seuils épidémiologiques pour *Aeromonas* et *Vibrio* ont été établies en utilisant la méthode d'interprétation normalisée des données de résistance provenant de diffusion sur disque. La recherche de gènes de résistance associés au profil de résistance des isolats a été effectuée en utilisant des PCRs et des puces ADN. Le nombre d'isolats résistants aux divers antimicrobiens parmi les 201 isolats d'*Aeromonas* et les 185 isolats de *Vibrio* étaient respectivement les suivants: TET (n=24 et 10), FLO (n=1 et 0), SXT (n=2 et 8), NA (n=7 et 5) et ENO (n= 5 et 0). Diverses associations de gènes *tet*(A), *tet*(B), *tet*(E), *floR*, *sul1*, *sul2*, et *intI1* ont été détectées, les gènes *tet*(E), *intI1*, *sul2* et *tet(B)* étant les plus communs. Les espèces d'*Aeromonas* et de *Vibrio* isolées de poissons au détail et de fruits de mer peuvent héberger une variété de gènes de résistance, bien que peu fréquemment. Le risque que représente ces gènes de résistance reste à évaluer en considérant le potentiel infectieux des bactéries, l'utilisation des ces agents antimicrobiens pour le traitement des maladies en aquaculture et en médecine humaine et leur rôle en tant que réservoir de la résistance antimicrobienne.

Mots-clefs: ***Aeromonas*****,** ***Vibrio*****, résistance aux antimicrobiens, normalised resistance interpretation (NRI), puce d'ADN, PCR, gènes de résistance**

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List of abbreviations

AAC : *N*-acetyltransferases

ABC : ATP binding cassettes

ANT : *O*-nucleotidyltransferase

APH *: O*-phosphotransferases

AMR : Antimicrobial resistance

APW : Alcaline peptone water

ATCC : American type culture collection

BP : Base pair

Clav : Clavulanic acid

CAT : Chloramphenicol acetyl transferase

CIPARS : Canadian Integrated Program for Antimicrobial Resistance Surveillance

CLSI : Clinical and Laboratory Standards Institute

DHFR : Dihydrofolate reductase

DHPS : Dihydropteroic acid synthase enzyme

DNA : Deoxyribonucleic acid

EBP: Epidemiologic breakpoint

ECV : Epidemiological cut-off value

EDTA : Ethylenediamine-tetra-acetic acid

EPI : Efflux pump inhibitors

ENO : Enrofloxacin

FFC : Florfenicol

ESBL : Extended spectrum β-lactamase

GEI : Genomic islands

HGT : Horizontal gene transfer

ICE : Integrating conjugative element

IS : Insertion sequence element

KBP : Kilobase pair

NA : Nalidixic acid

NRI : Normalised resistance interpretation

MATE : Multidrug and toxic compound extrusion

MBL : Metallo-β-lactamase

MDF : Multi-drug resistant

MFS : Major faciliator superfamily

MIC : Minimum inhibitory concentration

MLSKO antimicrobials : Macrolides, lincosamides, streptogramins, ketolides and oxazolidinones

mRNA : Messenger ribonucleic acid

NADPH : Nicotinamide adenine dinucleotide phosphate

ORF : Open reading frame

PBP : Penicillin binding protein

PCR : Polymerase chain reaction

PMF : Protein motive force

QRDR : Quinolone resistance determining region

RFLP : Restriction fragment length polymorphism

RNA : Ribonucleic acid

rRNA : Ribosomal ribonucleic acid

RND : Resistance nodulation cell division

RPP : Ribosomal protection protein

SMR : Small multidrug resistance

SXT : Sulfadimethoxine-trimethoprim

SXT constin : C***on***jugative, ***s***elf-***t***ransmissible, and ***in***tegrating element

TET : Tetracycline

TSI : Triple sugar iron agar

0129 : 2,4- diamino-6,7-di-isopropyl- pteridine phosphate

XAT : Xenobiotic chloramphenicol acetyl transferase

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Introduction

Aquaculture is the fastest growing animal production industry in the world. It is predicted that in the year 2020, worldwide demand will surpass the wild fisheries supply by 15-20% [1]. This would indicate that more fish will be produced in the aquaculture setting if supply is to meet the demand. Not only will the demand for seafood surpass the wild fisheries capacity, but the annual personal consumption of seafood is also expected to increase from 16 kg today to 31 kg in 2030 [2]. This would predict an increased human exposure to fish, seafood and their bacterial flora during the production, marketing and consumption activities.

Aquaculture was in its infancy in Canada in the early 1980's and now salmon growing operations on the East and West coasts of Canada compare favourably with other modern agriculture industries. Disease control is maintained with strict biosecurity measures as well as preventative measures such as vaccination, site fallowing/rotation and in certain cases, the judicious use of antimicrobials. Although antimicrobial exposure in the aquaculture setting is generally considered low as compared to other types of animal production, with an increase in consumption of aquacultured products as opposed to wild caught, there is a concomitant relative increase in the exposure to fish, seafood and their bacterial flora which possibly have had exposure to antimicrobials in an aquaculture setting. There are equally important differences in the regulations governing antimicrobial usage in agriculture (including aquaculture) depending upon the country examined. The availability of antimicrobials for use in aquaculture is extremely limited in North America where only three classes are available; tetracyclines, potentiated sulfa's and phenicols. Although there are regulatory mechanisms to obtain antimicrobials without homologation, this is the exception rather than the rule. In other areas of the world such as in Asia, it is believed that antimicrobial usage is widespread and poorly regulated. Antimicrobial therapy in aquatic production is primarily administered orally whereas treatment by injection is reserved for highly valuable individuals such as broodstock. Therefore, in addition to the bacterial population causing disease, the bacterial flora on the fish and in the surrounding environment are equally exposed to the antimicrobials which evade consumption or their active metabolites secreted by the diseased organism.

Resistance to antimicrobials in bacteria derived from aquacultured animals and/or the aquatic environment have been reported in numerous publications in the scientific literature [3]. In addition, there have been studies that indicate transmission of resistance

determinant between terrestrial and aquatic environments does take place [4, 5]. Multiple laboratory studies have also demonstrated that resistance determinants can be transferred between aquatic bacteria which are low pathogen risks to humans to *Enterobacteriaceae* for example [6-8]. If this exchange occurs, at what frequency it occurs, and how it occurs in the environment or in association with seafood has yet to be elucidated.

It could reasonably be assumed that there is a certain level of risk of AMR exposure from aquatic bacteria pathogenic to humans, and from the transfer of resistance determinants from aquatic bacteria found on seafood and in the environment to bacteria pathogenic for humans, but the level of risk is unknown. The first step towards clarifying this question is examining and quantifying the presence of resistance elements in aquatic bacteria.

Aeromonas and *Vibrio* are among the most common bacterial genera found on fish, shellfish and the aquatic environment. They are recognized pathogens of aquatic animals, causing economically important aquaculture diseases, as well as zoonotic pathogens capable of causing severe disease in humans. As such, there exists the possibility of the exposure of these genera to antimicrobials, and perhaps the development of resistance. In addition, they are easily cultivated on usual bacteriological media which is important when categorizing bacteria via standardized phenotypic AMR susceptibility testing methods. In this study, the prevalence of *Aeromonas* and *Vibrio* in retail seafood will be examined, as well as the prevalence of AMR and the genetic basis for the observed AMR phenotypes.

Review of the literature

Vibrio and Aeromonas: pathogens of humans and animals

Aeromonas and *Vibrio* species are Gram-negative, mobile, facultative anaerobic bacteria that are present in aquatic systems worldwide. Until 1986, *Aeromonas* and *Vibrio* species were found in the family *Vibrionaceae*. Following analysis of molecular genetic evidence by Colwell *et al.* in 1986, *Aeromonas* species were subsequently transfered to a new family, the *Aeromonadaceae* [9]. There are currently 22 and 83 named species in the *Aeromonas* and *Vibrio* genera, respectively. Members of both genera are recognized as human and animal pathogens, and certain are zoonotic in nature. For details of the different species of this genera the following resources should be consulted [10, 11].

Vibrio species are predominantly halophilic and are therefore found more frequently in marine systems although certain species are reported in brackish and freshwater systems. They are among the most common bacteria isolated from marine molluscs and seafood [12, 13]. The *Vibrio* species *anguillarum*, *ordalli*, *salmonicida*, *alginolyticus*, and *vulnificus* are common pathogens of cultured marine fish causing septicaemia or focal chronic disease, the last two being zoonotic agents [14-17]. *Vibrio vulnificus*, *Vibrio mimicus*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, are the species most often associated with disease in humans, and often in relation with consumption of raw or undercooked seafood, or in the case of *V. cholerae*, with fecal contamination of foodstuffs including water. The symptom most commonly encountered with *Vibrio* infections is gastroenteric upset, however in certain cases systemic disease may result, especially with *V. vulnificus* [18].

Aeromonas salmonicida as well as *Aeromonas hydrophila* and other motile *Aeromonas* species are also frequently found in fish, shellfish and other seafoods [19, 20]. This genus is associated with severe acute septicemic and chronic disease in aquacultured animal species including salmonids and non-salmonids such as carp and frogs. *Aeromonas* in humans can cause serious disease including extra-intestinal infections such as bacteraemia, meningitis, pulmonary and wound infections although food poisoning and associated gastroenteritis is probably the most common sequel to exposure [16, 21, 22].

Importance of antimicrobial resistance

Antimicrobial resistance (AMR) can be defined as the ability of microorganisms to resist the effect of an antibiotic or antimicrobial agent. This inefficacy of antimicrobials can be associated with intrinsic bacterial resistance, a mutation of the bacterial genome or by the acquisition of genetic material or a combination of these factors which will be discussed further on.

The presence of antimicrobial resistance in a zoonotic species of either genera causing severe systemic disease may decrease the chances of successful therapy [23-27]. Although the spectre of outright therapeutic failure and mortality is the most feared outcome of antimicrobial resistance development, the impact of antimicrobial resistance on humans and animals is difficult to evaluate. In human populations, increased levels of antimicrobial resistance have been associated with higher morbidity, mortality and increased hospitalization rates in the literature [28, 29]. This in turn may be explained by antimicrobial treatment failure or increased virulence of resistant bacterial strains, although the presence of pre-existing disease conditions and inadequate or delayed therapy may equally contibute [28, 30, 31]. Not all researchers agree however. Cosgrove (2006), Suneshine (2007) and Maragakis (2008) demonstrated an association between AMR and increases in mortality, morbidity and increased treatment costs whereas, conversely, Devasia et al. (2005) found no differences in treatment outcomes comparing patients infected with multi-drug-resistant *ampC* (MDR-AmpC) and pansusceptible *Salmonella* Newport [32]. The relationship between disease outcome and AMR is not clear cut. In addition to the health concerns, AMR represents a financial burden including direct costs such as hospitalisation for community acquired disease, increased hospital stay duration, prolonged therapy or changes to more costly medications, and repeat consulations [33]. Estimates of the monetary cost of AMR in the United States have been pegged from 1.3 to 5 billion dollars in the 1990's [33, 34].

Antimicrobials are an important part of disease control in most animal production systems and are vital for cost effective production in treatment of episodic disease. They are used for infectious disease control/treatment, prevention of disease in high risk situations and for growth promotion [35]. Aquaculture differs in that the use of antimicrobials for growth promotion is not a current production practice. The use of antimicrobials in animal production, including aquaculture, has been fingered as an

important source of both resistant pathogenic and commensal bacteria [36, 37]. In aquaculture, there are multiple contributors to the development of resistance in aquatic bacteria which are likely similar to other agriculture production systems. Important factors include poor husbandry, lack of an accurate diagnosis followed by indiscriminate antimicrobial usage, repeated use of the same antibiotic, undiagnosed underlying disease processes, and inappropriate record keeping, [3].

The most direct impact of non-susceptible bacterial pathogens in aquatic production is treatment failure. The availability of a limited number of antimicrobials approved for aquatic species; florfenicol (Aquaflor®), potentiated sulfas (Romet-30® and Tribrissen®) and tetracyclines (Terramycin-Aqua®), at least in North America, exacerbates this situtation. Strains of *Aeromonas salmonicida* resistant to all of the aforementionned medications have been reported in fresh water aquaculture in Canada [38, 39]. The costs incurred by the presence of AMR in aquatic animal production are difficult to estimate although attempts have been made to model costs of disease in other species [40, 41]. In addition to the financial burden directly incurred through loss of stock via inefficacious treatments, the impact of medication costs, increased manhours needed for treatment activities (medication preparation and administration, removal of dead animals etc.), and fees charged by health professionals are also to be considered.

Likely the most hotly debated issue of importance concerning AMR in agriculture/aquaculture is the impact on humans where the presence of antimicrobial resistance in a zoonotic species causing severe systemic disease may decrease the chances of successful therapy [23-27]. The danger that AMR development in animal production presents to humans is twofold. The first is the direct transmission of resistant human pathogens (eg. *E. coli*, *Aeromonas* sp. etc.) from animal to human, and the second is the contamination/infection by resistant commensal bacteria during manipulation or consumption somewhere along the food chain with a subsequent resistance gene transmission to human pathogens. Recent surveillance data generated by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) program has furthered evidence of antimicrobial use and transmission of resistant bacteria by demonstrating trend associations between cephalosporin usage and resistant *Salmonella* in retail chicken [42]. Multiple laboratory studies have demonstrated that resistance determinants can be transferred between aquatic bacteria, which are low pathogen risks to humans (ex. *Aeromonas salmonicida* to *Enterobacteriaceae*) [6, 7, 43]. If this exchange

occurs, at what frequency it occurs, and how it occurs in the environment or in association with seafood has yet to be elucidated. Studies by Rhodes (2000) and Furushita (2003) make the argument that transmission of resistance determinants between terrestrial and aquatic environments does take place [4, 5]. Further, Rhodes (2000) suggests that aquatic and terrestrial environments should be considered as one interactive unit [4]. If this is true, aquatic bacteria susceptibility will not only be affected by selective antimicrobial pressure on farm but from the availability of resistance determinants acquired from aquatic and terrestrial sources.

Mechanisms of antimicrobial resistance

To evade the effects of antimicrobials, bacteria have developed multiple strategies to neutralize their effects including avoidance, target modification and protection, inactivation, and active elimination of the offending molecules from the bacterial cytoplasm.

Intrinsic resistance

Bacteria which exhibit intrinsic or innate resistance would include those which lack or restrict access to targets for the antimicrobial in question. For example, anaerobic bacteria are insensitive to aminoglycosides because of they're inability to successfully carry out oxygen dependent antimicrobial transport across the cytopolasmic membrane and into the bacterial cell [44]. Additionally, certain resistance determinants are permanent fixtures in the bacterial genome such as described in species of *Aeromonas* with chromosomally located β-lactamases [45].

Enzymatic degradation/inactivation

Modification or destruction of antimicrobials to render them inactive is a strategy used by many bacteria for several classes of antimicrobial drugs including the aminoglycosides, macrolides, β-lactams and phenicols. Acetyltransferases for example are inactivating enzymes which are common to aminoglyocosides and phenicol resistance. Transfer of an acetyl group from an acetyl co-enzyme A donor to the antibiotic affects amino acid interaction and inhibits binding at strategic sites in the ribosome [46, 47]. Another example is the three constitutive chromosomal β-lactamases groups which can be present in some *Aeromonas* species including a penicillinase/carbapenemase, a cephalosporinase and an oxacillinase [45, 48]. They have different substrate preferences,

but their mode of action is similar, where enzymatic hydrolysis of amide bond in the β-lactam ring is responsible for inactivation [49].

Reduced accumulation (efflux pumps)

Antimicrobial avoidance by decreasing cytoplasmic concentrations of offending chemicals is common among many bacterial species. Bacterial efflux pumps serve as efficient gate keepers allowing entry of ions and nutrients, permitting communication between bacteria and their environment and limiting accumulation of unwanted metabolites or other toxic products [50]. In fact, it is quite likely that the affected antimicrobials are not the intended pump substrate. The first antimicrobial resistance efflux pumps were discovered on plasmids coding for resistance to tetracycline in *E. coli*, and have now been identified in many bacterial genera [51, 52]. Some efflux pumps may be substrate specific as is seen in bacteria which are producers of antimicrobial compounds such as the actinomycetes [53]. They may equally act upon many different substrates such as the multidrug resistance pump (MDR) AheABC of *A. hydrophila* described by Henrould (2008), and more than one efflux pump may be present in the same bacterium [54, 55]. Five major efflux pump classes have been identified including ATP binding cassettes (ABC), resistance nodulation cell division (RND), major faciliator superfamily (MFS), small multidrug resistance (SMR) and multidrug and toxic compound extrusion (MATE) [50, 56]. The ABC efflux pumps use ATP as the energy source to drive antimicrobial export, whereas MFS, SMR and RND efflux systems use the proton motive force (PMF) of the transmembrane electrochemical proton gradient to effectuate this action. MATE export pumps in contrast are considered H+ or Na+ coupled drug transporters [57]. Although differing in action, it remains that all functionally are considered to be capable of transporting a wide variety of substances including antimicrobials [56]. MFS, RND and SMR efflux pumps have been identified in *Aeromonas* species, whereas RND, MFS, SMR and MATE classes have been described in *Vibrio* sp. [50, 58].

Target modification and protection

Bacteria can evade antimicrobial action by modifiying or shielding antimicrobial targets to render them refractory to antimicrobial effects. This can arise from a mutational event or from horizontal gene transfer. Perhaps one of the best characterized is the resistance to sulfonamides via their interaction with folic acid metatoblism. Folic acid is an

important intermediate in many vital metabolic pathways in bacteria including the production of DNA and NADPH among others. Sulfonamides act as structural analogs of p-amino benzoic acid which competes for the dihydropteroic acid synthase enzyme (DHPS), thereby inhibiting the production of folic acid [59]. The acquisition of genes coding for DHPS enzymes with greater affinity for p-amino benzoic acid than sulfonamides renders the bacteria resistant [59]. This type of resistance has been reported in both *Aeromonas* and *Vibrio* genera.

Resistance acquisition

Acquired resistance refers to a modification or acquisition of genetic material which confers antimicrobial resistance to a bacterium. This may be associated with mutation of the bacterial genome conferring resistance or transfer and incorporation of resistance determinants via exchange of naked DNA, bacteriophage infection or mobile genetic structures such as plasmids, transposons and integrative conjugative elements (ICE's) [60]. Collectively, these non-mutational mechanisms are referred to as horizontal gene transfer or HGT. Further, the maintenance of acquired genes is generally facilitated by environmental selective pressure where survival of the bacteria with the genetic modifications is favoured, as with chronic antibacterial use in hospitals or animal production for example.

Mutation

Spontaneous mutation of the bacterial genome occurs during normal bacterial growth due to copy errors in DNA replication. Mutation rates are variable, dependent upon the bacteria under consideration but are generally found to be 10^{-10} – 10^{-9} per base pair replicated [61]. Those bacteria harbouring a mutation which is beneficial to survival in a given environment are favoured. Therefore in a situation where a bacterium is exposed to an "antimicrobial environment", those with an adaptive resistant mutation will survive, followed by clonal expansion of the bacterium within the bacterial population. Resistance to quinolones (ex. naladixic acid) is an example where point mutations principally in the gyrase (*gyrA)* or topoisomerase IV (*parC)* genes confer increased resistance to 1st and 2nd generation quinolones [48, 62]

Transformation

Transformation refers to the bacterial uptake of naked DNA from the environment

resulting in a different genotype [63]. Experimental or *in-vitro* transformation is used to artificially introduce DNA into bacteria following competence induction by chemical, physical or enzymatic treatments. Natural transformation is a function encoded in the bacterial genome and implies the survival of naked DNA in the environment, localisation and internalisation by a competent host followed by incorporation into the host genome. This type of acquisition occurs in both Gram-positive and Gram-negative species although they differ principally because of the differences in cellular barriers [64]. Natural transformation of plasmid DNA has been reported for *Vibrio* sp. with frequencies ranging from 0.3 to 3.1 x 10^{-8} transformants/recipient in sediment microcosms [65]. AMR transfer by transformation has not been described in *Vibrio* or *Aeromonas*, but is a well known mechanism first recognized in the Gram-positive species *Streptococcus pneumonia* [66, 67]. More recently, Neilsen (2010), describes transfer of *erm* (B), *mef* (E), *mef* (I), *tet* (M) and *catQ* genes between streptococcal isolates [68]. Although it has been argued that this form of genetic exchange is less important than conjugative events as they relate to HGT and AMR, transformation does not require conjugative elements, physical contact, or even a live DNA donor [69]. Therefore, its importance in complex bacterial communities such as biofilms may be underestimated.

Transduction

Bacterial infection with viral particles or bacteriophages may also occasion the transfer of genetic material between bacteria resulting in the incorporation of novel genetic material into the bacterial genome [70]. Bacteriophages are ubiquitous microorganisms endowed with a simple structure including the viral coat or capsid, an injection apparatus and the genetic material, variably DNA or RNA being simple or double stranded [71]. The viral infection of bacteria may have two general results: bacterial death (lytic phages) or incorporation of viral DNA into the bacterial genome (lysogenic phages). Cellular targets of bacteriophages are not clearly understood, however it is generally considered that they are specific to receptor types and likely to bacterial species and are not a probable genetic transfer mechanism between distantly related bacterial genera. However, it has been shown recently that transfer of virulence determinants between different bacterial genera via bacteriophages can occur with as high a frequency as intrageneric exchange [72]. Bacteriophage DNA capacity may effectively limit the efficacity of resistant determinant transfer. Bacteriophages are known to be important for the transmission of virulence

factors such as the case with the CTX prophage coding for cholera toxin in *Vibrio cholerae*. Lan (2009) describes prophages in *Vibrio parahemolyticus* containing putative bacterial DNA. They suggest that this bacteriophage may be involved in horizontal genetic exchange and may contribute to bacterial genetic diversity in this species [70]. Large bacteriophage capacity is estimated to be approximately 200 kb wherein resistance determinants such as *tet*(A) (1250 bp) and *cat* (1348 bp) would fit comfortably [71, 73-75]. Additionally, bacteriophages in the aquatic environment are considered the most abundant biological entity (up tot 2.5×10^8 per ml) and presumably an important element for genetic diversification [76, 77]. Bacteriophages have been shown to transfer various AMR determinants in *Pseudomonas aeruginosa [78]* . Although there are numerous references to bacteriophages reported in the literature concerning *Aeromonas* and *Vibrio* species, there is no specific mention of AMR containing viral particles [79, 80].

Conjugation

Conjugation refers to the exhange of DNA between bacteria via pili, and is often erroneously referred to as "sexual reproduction". Requirements for successful conjugation include the intimate (direct) contact of a gene donor and recipient, the gene targeted for transfer and the presence of the "machinery" necessary for the transfer of the genetic material. The most commonly recognized conjugative mechanism is plasmid mediated genetic exchange, first described in *E. coli* by Tatum and Lederberg in 1947 [81]. Plasmids are autonomously replicating, extrachromosomal circular or linear double stranded DNA fragments, with sizes ranging form 300 bp to 2400 kpb [82]. Minimally, a conjugative plasmid must contain transfer genes (*tra*), which code for the pilus assembly and associated transfer related proteins as well as a replicative starting point or origin of transfer (*oriT*) which is distinct from *oriR* which is necessary for intrabacterial replication [83]. This is followed by a transfer of one DNA strand to the recipient cell and the synthesis of the complementary strands in the donor and recipient cells. The transmission of resistance determinants associated with plasmids has been reported by many authors and transfer frequency in experiments between *A. salmonicida* and *E. coli* in the laboratory have been found to range between $10^{-1} - 10^{-9}$ [7, 39, 84, 85]. Upon examining plasmid transfer in a natural microenvironment involving salmon contaminated with resistant *A. salmonicida* and *E. coli* on a cutting board, a transfer frequency of 3×10^{-6} to 8×10^{-3} was noted [6]. Broad host range plasmids such as the IncU class possess shorter rigid pili which

lend to greater transfer efficiency, as much as 2,000 to 300,000 times faster on solid surfaces [43, 86, 87]. This is an important distinction when considering consumer risk originating from aquatic animals or their environment. Coexistence of different plasmids within the same bacterium is possible, however, two plasmids which share the same or similar types of plasmid transfer genes (considered as being of the same incompatibility group) are inhibitory to each other [83]. Certain types of plasmid incompatibility groups may be more commonly associated with specific bacterial genera. The plasmids of the incompatibility groups U and C are the most common in the aermonads, whereas group C plasmids are found principally in *Vibrio* species [88-90]. Plasmids from these incompatibility groups have a wide host range and have been associated with phenotypic resistance in these species.

Not all plasmids are capable of conjugative transfer. Smaller plasmids which do not contain the necessary 35 kb of transfer genes may piggyback on other mobile plasmids if they simply contain the appropriate origin of replication (*oriT*) recognized by the transfer machinery of the co-residing plasmid. Most early studies examining the genetic causes of antimicrobial resistance phenotypes concentrated on the absence or presence of mobile genetic elements which could confer AMR to sensitive bacterial strains. *Aeromonas* and *Vibrio* species are known to harbour a plethora of plasmids, ranging in size from 11 – 200 kbp, coding for resistance to various antibiotics [8].

Transposition

DNA elements which can "hop" or transpose from one location to another in bacteria are called transposons. They consist minimally of an open reading frame coding for a transposase enzyme (which controls their movement among different DNA locations) bounded by inverted repeats. These most simple transposons are called insertion sequence elements (IS) and may be only 1000 bp in length. IS's may "hop" close enough together on chromosomes or plasmids to mobilize the intervening DNA forming "composite transposons" [83]. This interaction of related transposons may mobilize larger DNA elements, including AMR genes, within the bacterium. Non-composite transposons may also contain AMR genes as a part of the minimal transposable unit. They may also have the capacity to integrate AMR genes or "cassettes" due to the presence of integrons in their structure (see below). Transposons in association with plasmids and integrons appear to be involved in long-range AMR distribution. The truncated or complete transposon Tn*1721*

containing *tet*(A) for example, has been found on pRAS1 or pRAS1-like plasmids in *A. salmonicida* from different regions including Scotland, Norway and Japan [4, 43, 91].

Integrative elements

Integrons are genetic elements capable of capturing and incorporating genetic material into their DNA structure. They are not capable of self replication or transmission, but are rendered "mobile" by other mobile genetic platforms such as transposons and plasmids. The integrons have been categorized into classes depending on the type of integrase that is incorporated in their structure (*intI1*, *intI2*, *intI3* and *intI4*). Classes 1-4 have been identified in *Aeromonas* and *Vibrio* sp. Class 1 integrons contain minimally a gene coding for an integrase enzyme (*intI*), a promoter and a recombination site (*attI*) in the 5' conserved segment of the integron [92]. The components *intI* and *attI* act on gene cassettes which are comprised of a promoterless gene, often coding for antimicrobial resistance, and a recombination site (*attC*) recognizing the complementary site (*attI*) of the integron [93]. Integrated genes become a part of the integron structure and many genes cassettes may become associated with the integron. PCR amplification of the variable region of the integron permits the identification of the residing cassettes. Class 1 integrons, thought to be degenerate transposons, are the most common in Gram-negative bacteria. They usually contain *sul1* and *qacE*Δ genes, coding for resistance to sulfonamides and quaternary ammonium compounds respectively, and an open reading frame (orf) of unknown function in their conserved 3' segment, downstream of the *attcI* site. The presence of *sul1*, along with *qacE*Δ is often used as markers for class 1 integrons though the presence of these genes or truncated relatives is variable [94, 95]. The functional capacity of the integron to accumulate resistance gene cassettes as well as the inclusion of resistant genes in their basic structure (ex. *sul1* in class 1 integrons) can lead to variable AMR phenotypes often exhibiting multiple drug resistance (MDR) in addition to diaminopyramidine resistance. Class 2 integrons have a similar structure and are found within transposons of the Tn*7* family, commonly code for trimethoprim resistance (*dhf*) [96, 97]. Class 2 integrons appear to be more limited in resistance cassette arrangements than Class 1, likely due to a non-functional integrase gene [96]. The recently described Class 3 integrons have a similar organization to Class 1 and Class 2 integrons including the capacity to carry resistance determinants [98]. Their presence was signalled in *Aeromonas* sp. from the african aquaculture environment [95]. Finally, Class 4 integrons have been

identified on the small chromosome of *Vibrio cholerae*, containing 100's of genes of mostly unknown function [99, 100]. Although the origin of integrons is unclear, these "Super-integrons" identified in *Vibrio* and also in *Pseudomonas* species are thought to be involved in their presence [101, 102]. Tapping into this vast depot of genetic material in the super-integrons may partly explain the seeming ease with which bacteria adjust to new antimicrobial molecules [103]. The presence of integrons is commonly reported in aquatic bacteria and clinical *Aeromonas* and *Vibrio* isolates [95, 104-112]. Class 1 integron carriage has been reported as varying from 3.6 – 73% [94, 95, 106, 107, 113]. These studies indicate that not only are *Aeromonas* and *Vibrio* species present in diverse environments, both as disease causing agents and commensals, but also that the various integron types are equally present which may contribute to resistance determinant mobility. This supports the argument that antimicrobial pressure in the environment is important for the maintenance and spread of AMR determinants in the bacterial population.

Integrating conjugative elements (ICE's) or Genomic islands (GIs)

In the early 1980's transposons were identified which not only had the capacity to integrate into the bacterial genome but also a conjugative capacity. ICE's tend to be large DNA structures due to the accompanying *tra* genes necessary for conjugative transfer however they are incapable of autonomous replication like plasmids [114]. Their DNA excision and integration functions (*xis* and *int* genes) resemble more phages than true transposons, hence their name [115]. Recently, it has been argued they should be included in a larger overarching family of syntenic blocks of transferred DNA called Genomic islands or GI's [83, 115]. The interbacterial transfer mechanism however, is similar to plasmids, where following excision from the bacterial DNA, a circular DNA intermediate where an *oriT* sequence is formed. (Hinerfield and Senghas in Snyder)[116]. ICEs can code for a variety of functions including virulence factors, antimicrobial resistance, and various metabolic functions [115]. The first ICE's, were identified in *Streptococcus faecalis* and *Bacteroides fragilis* in the early 1980's and were followed by several others [117, 118]. In 1996 Waldor *et al.* identified the first ICE in the *Vibrionaceae*, named SXT, in *Vibrio cholerae* [119]. Beaber described the SXT sequence as a melting pot of composite genes including those from bacteriophages, plasmids and other diverse sources [120]. The SXT element or "constin" (conjugative, self-transmissible, and integrating) has been found to be capable of antimicrobial resistance mediation, often in association with

integrons and transposons, and may also play a role in mobilizing plasmids carrying resistance determinants [121-123]. The resistance genes *floR*, *strA*, *strB*, *sul2*, *dfrA18*, and *dfrA1* have been associated with presence of *V. cholerae* SXT constin [124].

Antimicrobial resistance in Aeromonas and Vibrio

β-lactams

The β-lactam class of antibiotics includes the penicillins and the cephalosporins, carbapenems and monobactams, all characterized by a central β-lactam ring. This class of antibiotics are structural analogs of the terminal acyl-D-alanyl-D-alanine of the bacterial cell wall peptidoglycan subunit. Penicillin bindings proteins (PBP's) preferentially bind the β-lactams leading to an inhibition of cell wall synthesis and cell death [125]. Resistance to β-lactams is derived from multiple mechanisms including antimicrobial efflux, mutation of PBP's (reducing penicillin binding affinity), β-lactamase activity, overexpression of intrinsic β-lactamase activity and decreased permeability [49].

PBP's and their mutant variants are normally associated with resistance to β-lactams in Gram-positive bacteria and have not been reported to be a mechanism of importance for *Aeromonas, Vibrio* spp. or other Gram-negative bacteria associated with the presence of a plethora of β-lactamases in these species. Decreased outer membrane permeability in Gram-negative bacteria can be associated with low level resistance to the β-lactams as well as other antimicrobials. Oliver (2002) noted that alterations in porin expression in *E. coli* were linked to differences in β-lactam susceptibility, and furthermore Nikaido (1987) demonstrated a synergism between β-lactamase presence and decreased membrane permeability resulting in increased AMR in *E. coli* [126, 127]. Similar results have been shown for *A. salmonicida* where mutants with changes in outer membrane proteins demonstrated higher levels of AMR, including the β-lactams [128-130].

β-lactamase production by Gram-negative bacteria is the most important element when considering innate (chromosomal) and acquired resistance to β-lactams. Several classification schemes have been proposed for these enzymes based on molecular or functional characteristics, those of Ambler (1980) and Bush (1989, 1995) among the most commonly cited in the published literature [131-133]. The Ambler scheme proposes four classes (A-D) based on amino acid sequences where classes A, C and D are serine proteases and class B is a metallo-β-lactamase. The Bush scheme classifies β-lactamases as

to their preferred substrate and inhibiting molecules resulting in a more complex separation of enzymes [131, 133, 134]. These classifications schemes are contrasted in Annex 1.0, with mention of enzymes and substrates particular to each class.

Several enzymes classified loosely as pencillinases have been identified in *Aeromonas* and *Vibrio*. Aeromonads are generally considered resistant to penicillins due to the commonly identified chromosomally located β-lactamase enzymes, CeP-S, AMP-S and CPH-S. *Vibrio* species are not known to possess similar chromosomal enzymes. The presence of chromosomal β-lactamases in *Aeromonas* can be quite variable, where a penicillinase, a cephalosporinase and/or a metallo-β-lactamase may be present in different combinations depending on the species and strain examined [45, 135]. Walsh (1997) evaluated the prevalence of the three chromosomal β-lactamase genes in different *Aeromonas* species and found the $bla_{Amp\text{-}S}$ gene presence varied from 25 - 45% depending on the species, with *A. veronii* having the highest prevalence at 45% [136]. The $bla_{\mathrm{Cep\text{-}S}}$ gene was almost uniformly present in the *A. caviae*, *A. veronii* and *A. hydrophila* strains examined and its presence confers resistance to 1st generation cephalosporins [133, 136].

Although the chromosomal β-lactamases are considered immobile, others have been identified on mobile genetic structures making them more important when considering HGT of resistance elements. They are commonly found present as gene cassettes in class 1 integrons, with either a plasmidic or chromosomal location. In most cases they were associated with resistance determinants for other antimicrobials within the same integron or plasmid [106, 137].

The extended spectrum β-lactamases (ESBL's) have become extremely important when considering β-lactam therapy due to their large spectrum of activity, and their genetic mobility. ESBL's are generally recognized as being capable of hydrolyzing penicillins, 1st, 2nd, and 3rd generation cephalosporins (with the exception of cephamycins and carbapenems) and monobactams [138]. To date three ESBL's have been identified in both the *Aeromonas* and *Vibrio* genera (see Table I below).

The metallo-β-lactamases (MBL) to date have only been recognized in *Aeromonas* species. They are categorized as Bush class 3/Ambler class B and can hydrolyze most classes of β-lactams. [134]. Wild chromosomal MBL + strains may be differentiated form acquired MBL's due to their susceptibility to carbapenem [139]. Acquired MBL's are commonly found as gene cassettes within class1 integrons and as such may be associated with other AMR determinants. European MBL's strains often exhibit a truncated gene

cassette fused with the gene *aacA4*, resulting in resistance cross-selection between aminoglycosides and β-lactam [139]. The following table denotes the various determinants responsible for β-lactam resistance described for *Aeromonas* and *Vibrio* sp. Additional information concerning the β-lactamase genes described above as well as available associated MIC data can be found in Annex 1 and 2.

Table I *Aeromonas* and *Vibrio* β-lactam resistance genes

Gene	Species/genus	Bush-Ambler class[a]	Associated genetic element	Ref.
***Aeromonas*/Penicillinases**				
bla_{SHV}	*A. media*	2b-A	-	[140]
bla_{TEM}	*Aeromonas sp.*	2b-A	-	[140]
bla_{OXA-2}	*Aeromonas* sp.	2d-D	Class 1 integron	[95, 106, 140]
bla_{OXA-21}	*Aeromonas* sp.	2d-D	Class 1 integron	[107]
bla_{PSE-1}	*Aeromonas* sp.	2d-D	Class 1 integron	[95]
***Vibrio*/Penicillinases**				
bla_{SAR-1}	*V. cholerae*	2b-A	Plasmid	[141]
bla_{TEM-1}	*V. cholerae*	2c-A	Plasmid	[142, 143]
bla_{CARB2}	*V. cholerae*	2c-A	Class 1 integron	[104, 112, 144]
$bla_{CARB6,7,9}$	*V. cholerae*	2c-A	Chromosome	[145-147]
***Aeromonas*/Extended spectrum β-lactamases, cephalosporinases**				
bla_{CMY-2}	*A. salmonicida*	1-C	IncA/C plasmid	[39]
bla_{Cep-S}	*Aeromonas sp.*	1-C	Chromosome	[136]
bla_{TEM-24}	*A. caviae*, *A. hydrophila*	2be-A	Plasmid	[135, 148]
bla_{PER-1}	*V. cholerae*	2be-A	Plasmid	[142]
***Vibrio*/Extended spectrum β-lactamases, cephalosporinases**				
$bla_{CTX-M-2like}$	*V. cholerae*	2be-A	Plasmid	[142]
bla_{PER-2}	*V. cholerae*	2be-A	Plasmid	[142]
$bla_{OXA-142}$	*V. fluvialis*	2be-A	Class 1 integron	[109]
bla_{Amp-C}	*V. fischeri*	1-C	Chromosome	[149]
***Aeromonas*/Metallo-β-lactamases**				
bla_{Cph-A}	*Aeromonas sp.*	3-B	Chromosome	[150, 151]
bla_{IMP-19}	*A. caviae*	3-B	Class 1 integron	[152]
bla_{VIM-4}	*A. hydrophila*	3-B	Class 1 integron	[153]

[a] Additional information concerning β-lactamases is found in Annex 1

Tetracyclines

Tetracyclines are bacteriostatic antimicrobial compounds derived from *Streptomyces* spp. Their effects are mediated by the interaction with the 30S subunit of the ribosome, thereby preventing association with aminoacyltRNA and thus protein synthesis [154]. Resistance to the tetracycline family was recognized in *Aeromonas* species as early as 1959 by Snieszko and later attributed to a transferable R factor by Aoki in 1971 [155]. At least 41 tetracycline resistance genes have been characterized to date. They are divided into four classes including efflux proteins (26), ribosomal protection proteins (11), enzymatic modifying proteins (3), and one with an unknown mechanism [156, 157].

The first category, the efflux proteins, which are members of the major facilitator superfamily (MFS), reduce intrabacterial tetracycline concentrations via an energy dependent protonic exchange [158]. The efflux proteins associated with tetracycline resistance in Gram-negative bacteria are doted with two components, a repressor and an efflux protein, where in the presence of tetracycline, the efflux protein coding gene is derepressed allowing transcription [154]. The *tet* efflux genes are not found as gene cassettes within class 1 integrons, however they are commonly found in other mobile genetic structures such as on mobile plasmids and within transposons [73, 84, 157, 159]. The *tet*(A) gene is an example of a *tet* gene which has been associated with full or truncated *Tn1721* transposon in *A. salmonicida* and other *Aeromonas* species [43, 85]. Early studies localized certain *tet* efflux genes on nonmobile plasmids or chromosomes due to apparent low experimental transfer frequencies [160, 161]. Subsequently, other authors have described these genes as highly prevalent on transferable plasmids, perhaps due to different or improved experimental methods [161-164]. Due to the plasmidic location of *tet* genes co-resistance to other antimicrobials is often reported. Co-resistance to sulfonamides ± trimethoprim as well as streptomycin is common, likely due to presence of integrons on *tet* determinant containing plasmids [7, 85, 95]. The *tet* efflux genes are commonly found singly, but they may also cohabit in the same bacteria either chromosomally or on the same mobile genetic element with other *tet* genes or resistance determinants to other antibiotic classes [154]. Determinant combinations in *Aeromonas spp.* have been reported in various studies, including *tet*(A)-*tet*(E), *tet*(B)-(D), *tet*(A)-*tet*(C), and *tet*(E)-*tet*(D) and even *tet*(A), *tet*(B), *tet*(D)/*tet*(H), and *tet*(E) [95, 159-161, 163, 164]. The presence of more than one *tet* determinant has also been signalled in *Vibrio* spp. including *tet*(A)/*tet*(B),

tet(A)/*tet*(B)/*tet*(D), *tet*(D)/*tet*(E) combinations [165, 166]. Therefore, one or combinations of *tet* determinants may be responsible for observed phenotypes in these genera.

Seven efflux *tet* genes have been identified in *Aeromonas* species to date including *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(Y), *tet*(31). The *tet*(A) and *tet*(E) determinants are often quoted as being the most common although few studies have examined the prevalence of all *tet* determinants within a bacterial population. There have also been differences ascribed to variations in molecular techniques such as multiplex and single PCR protocols [163]. Prevalence for *tet*(A) in tetracycline resistant strains varies between 3 and 88% [4, 7, 84, 85, 91, 95, 160, 161, 163, 167], whereas prevalence of *tet*(E) genes among resistant isolates has been reported anywhere from 42% to 90% *[95, 159-161, 163, 168-170]*. The *tet* genes B, C and D appear to be present in lower numbers, ranging from as little as 1% to 28% in resistant bacteria [169, 170]. The lack of identifiable *tet* determinants in the literature is common [5, 7, 160, 161, 170]. In a study by Schmidt (2001) for example, only 30% (66/216) resistant isolates could be assigned a known *tet* determinant [7].

Several efflux pumps have also been identified in *Vibrio* species including *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), and *tet*(35). The determinants *tet*(D) and *tet*(B) seem to be identified with a greater frequency in marine environments. The prevalence of *tet*(B) in *Vibrio* was 43% and 100% in studies conducted by Furushita (2003) and Kim (2007) respectively [5, 171]. The *tet35* gene was identified in a tetracycline resistant strain of *V. harveyi* isolated from a prawn. Unlike previous tetracycline export pumps identified, *tet35* appears to have a primary physiological role in Na+/H+ transport rather than antimicrobial export, is chromosomally located and results in inferior MIC's in transconjugants as compared to *tet*(A) for example [172].

The second class of *tet* resistance genes are the ribosomal protection proteins (RPP's). These act by permitting continual protein synthesis in the presence of tetracyclines. Although traditionally considered as Gram-positive tetracycline resistance genes these are now being identified in other cases including Gram-negative aquatic bacteria [171]. The determinant *tet*(M) is the only RPP identified among *Vibrio* and *Aeromonas* species [157]. It can be found in co-residence with other *tet* determinants such as *tet*(B), *tet*(D) and/or *tet*(E) [164, 165, 173]. The *tet*(M) determinant was the most common among tetracycline resistant *Aeromonas* isolates in an Australian study, where 7 out of 10 (70%) harboured the gene [164]. In Korean *Vibrio* isolates, the *tet*(M) and *tet*(B)

duo were found in 23 of 24 isolates and both *tet* determinants were located within a Tn*10* tranposon. Similarly, Kim (2004) examined the *tet* resistance genes from 151 tetracycline resistant marine bacteria originating from Japan and Korea. The majority of the *tet*(M) positive *Vibrio* isolates were found to be associated with a transposon of the Tn*1545*-Tn*1916* family and interestingly the Gram-positive bacteria, *Lactococcus gerviae* examined in the same study, carried a similar gene/transposon combination. The association of *tet*(M) resistance elements with transposable elements may be responsible for this ever-enlarging host range [154].

The last class of *tet* resistance elements are the enzymatic proteins which inhibit tetracycline activity by inactivation of the antimicrobial or by an acceleration of protein transcription bypassing the *tet* resistance mechanism and includes *tet*(34), t*et*(X) and *tet*(37) (refer to Annex 5) [174, 175]. They act via an NADPH-requiring oxidoreductase (*tet*(37) *and tet*(X)), or an enzyme similar to xanthine–guanine phosphoribosyl transferase from *Vibrio cholerae* (*tet*(34)). The *tet*(34) gene was identified in a *Vibrio* isolate grown from marine fish intestinal contents, and was associated with the relatively high MIC of 500µg/ml to oxytetracycline [174]. Additional information concerning the tetracycline resistance genes described above as well as available associated MIC data can be found in Tables II and III below and in Annex 3 and 4.

Table II Tetracycline resistance genes identified in *Aeromonas* sp.

Genus/species	Associated genetic elements	Genes identified[a]	Ref.
A. salmonicida	pRAS1/pAr-32, Class 1 integron, transposon	*dfrA16, qac, sul1* ***tet*(A)**, *aadA2,qac,sul1, catAII*	[43]
A. salmonicida	pRAS2, Tn5393	***tet*(31)**, *sulII, strA, strB*	[176]
A. salmonicida	pRAS3	***tet*(C)**	[177]
A.salmonicida, atypical	pRAS-1 like, Tn1721, IS6100	*dfrA16, sul1*, ***tet*(A), *tet*(B)**	[85]
A. salmonicida	pASOT	***tet*(A)**	[84]
Aeromonas sp.	pFBAOT, Tn1721	***tet*(A)**	[4]
A. caviae(punctata)	pFBAOT6,Class 1 integron, transposon	***tet*(A)**, *sul1,qacEd1, aadA2*	[178]
Aeromonas sp.	pSS2, chromosome	***tet*(A), *tet(E)***	[160]
A .hydrophila	-	***tet*(A),*tet*(E)**	[161]
Aeromonas sp.	-	***tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E)**	[167]
A. bestiarum	pAB5S9, ICE	*floR*, ***tet*(Y)**, *strA, strB, sul2*	[179]
Aeromonas spp.	Class 1 integron, plasmid	*ant(3")Ia, aac(6')Ia, dhfr1,* bla_{OXA2a}, bla_{PSE1} ***tet*(A),*tet*(B),*tet*(D),*tet*(E), *tet*(H)**	[95]
A.hydrophila and A.salmonicida	R-plasmid	***tet*(E), *tet*(A), *tet*(D)**	[163]
A. hydrophila	pJA5017, pES15, pES41, pTW537	***tet*(D)**	[180]
A. veronii	Plasmid	***tet*(A)-*tet*(E)**	[169]
A. salmonicida	Class 1 integron, plasmid	*ant(3")1a, dhfr1, dhfrIIc, dhfrXVI,* ***tet*(A), *tet*(C)**, *sul1*	[159]
A. salmonicida	Class 1 integron, plasmid (pSN254-like)	*aadA7, florR,* ***tet*(A)**, *sul2, strA, strB, sul1,* bla_{CMY-2}, *sugE, Hg*	[39]
Aeromonas sp.	Plasmid/chromosome?	***tet*(A), *tet*(E), *tet*(M), *tet*(D)**	[164]
Aeromonas sp.	Class 1 integron, plasmid	***tet*(A),*tet*(E),*tet*(D)**, *dhfr1, dhfr2a,ant(3")1a, catB2*	[7]
A .salmonicida	Class 1 integron, plasmid	***tet*(A), *tet*(E)**, *sul2,aadA1,aadA2, dfr16, dfrfIIc,*	[91]
A. hydrophila	-	***tet*(E), *tet*(D)**	[170]

[a]Tetracycline resistance genes are highlighted in bold faced type

Table III Tetracycline resistance genes identified in *Vibrio* sp.

Genus/species	Genetic element	Genes identified[a]	Ref.
Vibrio spp.	-	*florR, catII, catIV,* ***tet*(B), *tet*(D), *tet*(E), *tet*(M)**	[181]
Vibrio spp.	-	***tet*(A), *tet*(B), *tet*(D)**, *catII, catIV*	[166]
Vibrio spp.	-	***tet(A)*, *tet*(B), *tet*(D), *tet*(M)**, *catII, floR,*	[165]
Vibrio spp.	-	***tet*(A), *tet*(B),*tet*(G)**	[182]
Vibrio spp.	-	*catIV, catII,* ***tet*(A), *tet*(D), *tet*(B)**	[183]
Vibrio spp.	Transposon Tn*1545*-Tn*916* family	***tet*(M)**	[171]
Vibrio sp.	Chromosomal (or low copy plasmid)	***tet(34)***	[174]
V. anguillarum	Plasmid	***tet*(E)**	[184]
V. salmonicida	pRVS1	***tet*(E)**	[162]
V. harveyi	Chromosome (tet35), plasmid?(tetA), Tn*10*	***tet*(A), *tet*(35)**	[172]
V. fluvialis		***tet*(E)**	[167]
Vibrio sp.	Tn*10*, plasmid?	***tet*(M), *tet*(B)**	[173]
V. anguillum	pJA4320, pJA7601	***tet*(G)**	[74, 184]
Vibrio sp.	Plasmid, chromosome?	***tet*(A), *tet*(E), *tet*(M)**	[164]
Vibrio/Photobacterium	Plasmid? (conjugaison)	***tet*(B), *tet*(Y)**	[5]
V. cholerae	Class 1 integron, plasmid, SXT	*aadA2, sul1,* ***tet*(A)**	[185]
V. cholerae, V. parahaemolyticus	Class 1 integron, plasmid	*dfrA15 , bla_{P1}, qacH, aadA8,* ***tet*(G)**, *aph, cat1, sul2*	[104]
V. cholera	Class 1 integron, SXT	*aadA1, floR, strA, strB, sul2,* ***tet*(A)**	[124]

[a]Tetracycline resistance genes are highlighted in bold faced type

Phenicols

Phenicols are bacteriostatic compounds which were originally derived from *Streptomycetes*. These compounds inhibit protein production via reversible binding with the 50S subunit of the ribosome of prokaryotes. Resistance to the phenicols has been attributed principally to enyzmatic modification of the antimicrobial and efflux proteins.

The most common mechanism of enzymatic modification is via acetylation by a choramphenicol acetyltransferase (CATs), 24–26 kDa homotrimer proteins, which effectively inactivates the drug via deprotonation and transfer of an acetyl group from acetyl-CoA to the C3 alcohol of the choramphenciol molecule [186]. Schwarz (2004) classified *cat* genes into types A and B, the former being the classical CAT genes and the latter being xenobiotic CATs, also referred to as XATs [186, 187]. Within these CAT types, genes are further grouped according to an 80% similarity sequence identity ex. CAT1, 2 etc., and there are presently at least 16 type A groups and 5 of type B. The type A *catI* and *catII* genes appear to be the most common variants in Gram-negative bacteria coding for high level resistance to choramphenicol. Conversely, most Type B variants, associated primarily with Gram-negative bacteria impart low level resistance even with sur-expression, which may indicate that the intended acetylation substrate may be something other than chloramphenicols [186, 187]. The substitution of the C3 alcohol of the chloramphenicol (CM) molecule by a fluor group, as is found in florfenicol (FFC), an antimicrobial commonly used in aquaculture, renders the antimicrobial resistant to the CAT enzymes [187]. The *cat* genes have been described as chromosomal elements, but are often associated with mobile genetic structures such as plasmids, transposons and integrons [187]. The genes *cat1* and *catB2* for example were first described in *E.coli* as being part of the transposons Tn*9* and Tn*2424* respectively [188-190]. More than one *cat* gene may be present in chloramphenicol resistant bacteria [186]. The second of the main mechanisms for chloramphenicol resistance are export proteins including specific and multidrug exporters. There are eight described classes with only classes 3 and 4 having activity against both chloramphenciol and florfenicol [187].

The florfenicol resistance gene, *floR*, first described in florfenicol resistant strains of *Photobacterium damselae subsp. damselae* in 1996, is the only representative of the CM/FFC export proteins found in *Aeromonas* and *Vibrio* species, and confers resistance to both chloramphenicol and florfenicol [179]. This gene has been associated with the SXT

constins (***con***jugative, ***s***elf-***t***ransmissible, and ***in***tegrating) which are chromosomal conjugative elements located downstream of the *prfC* (protein release factor) in *Vibrio cholerae* [120, 121]. In *Vibrio* it has been associated with resistance gene clusters within the SXT element [121, 124]. Partial sequences of the SXT element had also been found associated with *floR* in *A. bestiarum*, perhaps belying the role played by SXT in interspecies transmission of genetic material [179]. Tables IV and V summarise the identified phenicol resistance genes identified during this review.

Table IV Phenicol resistance genes identified in *Aeromonas* sp.

Genus/species	Associated genetic elements	Genes identified[a]	Ref.
A. salmonicida	pRAS1/pAr-32, Class 1 integron, transposon	*dfrA16, qac, sul1 tet*(A), *aadA2, qac, sul2,* ***catAII***	[43]
A. bestiarum	pAB5S9, ICE, transposon	***floR****, tet(Y), strA, strB,sul2*	[179]
Aeromonas spp.	Class 1 integron	*dfr12, dfr2d, aadA1, aadA2,* $bla_{oxa\text{-}2}$*,* ***catB3, catB8****,qacE2*	[106]
Aeromonas sp.	Class 1 integron, chromosome	$bla_{OXA\text{-}2}$*,*bla_{TEM}*,*bla_{SHV}*,cphA, dfrA12, aadA1, aadA2,* ***catB8***	[140]
Aeromonas spp.	Class 1 integron	*aadA1, aadA2,aac-a4,aac(6')-II, aac(6')Ib,arr-2, arr-3, dfrA1, dfrA12, dfrA5, dfra17,dfra2d,dfrV,* bla_{oxa21}*,* ***catB3, catB8, cmlA1****,ereA2*	[107]
A. allosaccharophila	p34, integron	*qnrS2,aac(6')-Ib-cr,* $bla_{oxa\text{-}1}$*,* ***catB3****, arr-3*	[137]
A. salmonicida	Plasmid (pSN254-like), Class 1 integron	*aadA7,* ***florR****, tet*(A)*, sulII, strA/strB, sul1,* $bla_{cmy\text{-}2}$*, sugE, Hg*	[39]
Aeromonas sp.	Class 1 integron, plasmid	*tetA,tetE,tetD, dfr1, dhfr2a,ant(3")1a,* ***catB2***	[7]
A. hydrophila	-	***catII***	[191]
Aeromonas sp.	Class 1 integron	*dfr1, ant(3")1a,* ***catB2****, tet(*A)*, tet*(E)	[7]

[a]Phenicol resistance genes are highlighted in bold faced type

Table V Phenicol resistance genes identified in *Vibrio* sp.

Genus/species	Associated genetic elements	Genes identified[a]	Ref.
Vibrio spp.	-	***florR, catII, catIV***, *tet*(B), *tet*(D), *tet*(E), *tet*(M)	[181]
Vibrio spp.	-	*tet*(A), *tet*(B), *tet*(D), ***catII, catIV***	[166]
Vibrio spp.	-	*tet*(A), *tet*(B), *tet*(D), *tet*(M), ***catII***, ***floR***,	[165]
Vibrio spp.	-	***catIV, catII***, *tet*(A), *tet*(D), *tet*(B),	[183]
Vibrio-like bacteria	Class 1 Integron	*dfrIIc, dfrXII,aadA1a,* bla_{oxa2}, ***catB3, catB5***	[94]
Vibrio sp.	-	***catIV***	[191]
V. anguillarum	Plasmid (Rms418)	***Cat***	[192]
V. cholerae	Transposon-like structure, SXT	*dfr18*, ***floR***,*sul2, strA/strB*	[121]
V. cholerae, V. parahaemolyticus	Class 1 integron, plasmid	*dfrA15* , bla_{P1}, *qacH, aadA8,* *tet*(G), *aph*, ***cat1***, *sul2*	[104]
V. cholerae	Class 1 integron, SXT	*aadA1*, ***floR***, *strA, strB, sul2,* *tet*(A)	[124]
V. cholerae	Chromosome (superintegron)	***catB9***, *dfr2B*	[193]

[a]Phenicol resistance genes are highlighted in bold faced type

Sulfonamides and diaminopyrimidines

The sulfonamides are the oldest family of antimicrobial compounds dating back to the early nineteen hundreds with the development of protonsil, the first commercially available antimicrobial, by Gerhard Domagk, whereas the diaminopyrimidines are among the most recently developed compounds becoming available in the 1960's [194]. As previously described, sulfonamides and diaminopyrimidines are structural analogs of p-amino benzoic acid and folic acid respectively which compete for essential enzymes vital for cell function where they exert a bacteriostatic action on sensitive bacteria [59].

Resistance to these compounds is principally associated with modification of the target enzymes dihydropteroic acid synthase (DHPS) and dihydrofolate reductase (DHFR), and to a lesser extent on the reduction of antimicrobial entry into the bacteria due to

multidrug efflux pumps. The modification of DHPS and DHFR enzymes can arise from mutations which render bacteria non-susceptible, however acquisition of resistant enzymes carried by mobile genetic elements is much more common. There are currently over 30 *dfr* genes identified in a variety of different bacterial species. Some confusion exists in the literature pertaining to gene nomenclature and several names have been used for the same genes over the years. For example, the gene *dfrA1* has also been known as *dfr*, *dhfr1* and the the type I DHFR gene depending on the publication consulted. Current accepted nomenclature has divided genes into two groups *dfrA* and *dfrB*, followed by an Arabic number designating the gene variant [195-197]. They are often encountered as gene cassettes within integrons, although they may be found independently on plasmids [196]. These integrons have in turn been associated with transposons such as Tn*7* and Tn*21* where integration into chromosomal or plasmidic structures is important for AMR dissemination [198, 199]. A trimethoprim/sulfonamide resistance phenotype may be considered as an initial marker for their presence [7].

Only three mobile variants of the sulfonamide resistance genes have been described to date including *sul1*, *sul2* and *sul3* [59, 200, 201]. These genes are generally plasmid associated and *sul1* has been associated with the Tn*21* transposon family [200, 202]. The gene *sul1*is frequently found in the 3’ conserved end of the class 1 integron along with *orf5* and Δ*qacE* and is commonly found associated with other resistance cassettes although truncated forms or its absence is possible [94]. It is thought to originate from a transposon with a subsequent loss of mobility, as it resembles portions of Tn*1721* and Tn*1696* [43].

The *sul2* gene is less commonly encountered in *Aeromonas* than in *Vibrio* where it has been associated with the SXT constin in *V. cholerae*. In two studies, one examining a multiresistant plasmid identified in *A. bestiarum*, and the other in *A. salmonicida*, *sul2* was identified in a DNA segment identical to, or with similarities to, the SXT fragment of *V. cholerae* belying its possible genetic origin [39, 179].

The SXT constin in *V.cholerae* can mediate resistance via the presence of a transposed antimicrobial resistance gene cluster commonly including *floR*, *sul2*, *strA/str*B coding for chloramphenicol, sulfonamide and streptomycin resistance respectively [121]. In recently described *Vibrio cholerae* isolates, the *dfr* genes *dfrA1* (ElTor) and *dfrA18* (0139) have been identified conferring resistance to trimethoprim [121]. Conversely in Iwanaga’s (2004) study no *dfr* genes were found. The insertion and removal of transposons and integrons containing the *dfrA18* and *dfrA1* genes and further recombination within the

constin is probably responsible for this variability.

The prevalence of *dfr* genes among *Aeromonas* sp. has been reported to vary from 25 to 52% [91, 106, 107, 140, 159]. Certain authors attribute the elevated prevalence of these genes in the fish farming environment to the use of potentiated sulfonamides in treating fish diseases. Among human clinical isolates of *V. cholerae*, *dfr* gene prevalence varying from 13 - 32% [105, 144]. In a retrospective study of *V. cholerae* O1 epidemic isolates from Guinea-Bissau, all 6, 1997 isolates carried *dhfr18* on class 1 integrons whereas this gene was absent from those of previous years. Table VI and VII list the various *dfr* and *sul* genes responsible for sulfonamide/potentiated sulfonamide resistance described for *Aeromonas* and *Vibrio* sp. in the literature.

Table VI Sulfonamide resistance genes identified in *Aeromonas* sp.

Genus/species	Associated genetic elements	Genes identified[a]	Ref.
A. salmonicida	pRAS1/pAr-32, Class 1 integron, transposon	***dfrA16***, *qac*, ***sul1,*** *tet*(A), *aadA2,qac, catAII*	[43]
A. salmonicida	pRAS2, transposon	*tet*(31), ***sul2***, *strA,strB*	[176]
A. salmonicida, atypical	pRAS-1 like, Tn1721, IS6100	***dfra16***, ***sul1***, *tet*(A), *tet*(B)	[85]
A. caviae(punctata)	pFBAOT6, Class 1 integron, transposon	*tet*(A), ***sul1***, *qacEd1, aadA2*	[178]
A. bestiarum	pAB5S9, ICE, transposon	*floR, tet*(Y), *strA, strB,* ***sul2***	[179]
Aeromonas spp.	Class 1 integron, plasmid	*ant(3")Ia, aac(6')Ia,* ***dhfr1***, *bla_{oxa2a}, pse1 tet*(A), *tet*(B), *tet*(D), *tet*(E), *tet*(H)	[95]
Aeromonas spp.	Class 1 integron	***dfr12, dfr2d***, *aadA1, aadA2, bla_{oxa-2}, catB3, catB8, qacE2*	[106]
A. salmonicida	Class 1 integron, plasmid	*ant(3")1a,* ***dhfr1, dhfrIIc, dhfrXVI***, *tet*(A), *tet*(C), ***sul1***	[159]
Aeromonas sp.	Class 1 integron, plasmid, chromosome	*bla_{OXA-2}, bla_{TEM}, bla_{SHV}, cphA,* ***dfrA12***, *aadA1, aadA2, catB8*	[140]
Aeromonas spp.	Class 1 integron,plasmid	*aadA1, aadA2, aac a4, aac(6')-II, aac(6')Ib, arr-2, arr-3,* ***dfrA1, dfrA12, dfrA5, dfra17, dfra2d, dfrV,*** *bla_{oxa21}, catB3, catB8, cmlA1, ereA2*	[107]
A. salmonicida	Plasmid (pSN254-like), Class 1 integron	*aadA7, florR, tet*(A), ***sul2***, *strA, strB, sul1, bla_{cmy-2}, sugE, Hg*	[39]
Aeromonas sp.	Class 1 integron, plasmid	*tet*(A), *tet*(E), *tet*(D), ***dhfr1***, ***dhfr2a***, *ant(3")1a, catB2*	[7]
A. salmonicida	Class 1 integron, plasmid, Tn1721	*Tet*(A), *tet*(E), ***sul2***,*aadA1,aadA2,* ***drf16, drfIIc***	[91]

[a]Sulfonamide resistance genes are highlighted in bold faced type

Table VII Sulfonamide resistance genes identified in *Vibrio* sp.

Genus/species	Associated genetic elements	Genes identified[a]	Ref.
V. fluvialis	pBD146, Class 1 integron, SXT	***dfrV***, *arr3*, $bla_{OXA-142}$, *aadA1*	[109]
V. cholerae (non01,non0139)	Class 1 integron, plasmid, chromosome	*aac(6')-Ib*, *dfrA1*, *aadA1*, ***dfrA12***, *aadA2*, ***dfrA15***, ***dfrA5***, *ereA2*, ***sul1***, *qac*,	[203]
Vibrio-like bacteria	Class 1 Integron	***dfrIIc***, ***dfrXII***, *aadA1a*, bla_{oxa2}, *catB3*, *catB5*	[94]
V. cholerae	Class 1 integron, plasmid	***dfrA12***, *ant(3")-1a*	[144]
V. cholerae	Class 1 integron, plasmid	***dfrA15***, ***dfrA1***, *ant(3")-1a (aadA2)*, bla_{P1}(bla_{CARB-2}), *aadB*	[204]
V. cholerae	SXT, Transposon-like structure	***dfr18***, *floR*,***sul2***, *strA*, *strB*	[121]
V. cholerae 01	Class 1 integron	*ant(3")-1a*,***sul***	[111]
Vibrio spp.	Class 1 integron, SXT	***dfrA15***,bla_{P1}, *aadA2*	[112]
V. cholerae	Class 1 integron, plasmid, SXT	*aadA2*, ***sul1***, *tet*(A)	[185]
V. cholerae	Class 1 integron, SXT	*aadA1*, ***dfrA15***	[105]
V. cholerae	Plasmid	***dfr***	[205]
V. cholerae	Class 1 integron, plasmid, chromosome	***dfrA1***, *aad2*	[206]
V. cholerae, *V. parahaemolyticus*	Class 1 integron, plasmid	***dfrA15*** , *blaP1*, *qacH*, *aadA8*, *tetG*, *aph*, *cat1*, ***sul2***	[104]
V. cholerae	Class 1 integron, SXT	*aadA1*, *floR*, *strA*, *strB*, ***sul2***, *tet*(A)	[124]
V. cholerae	Chromosome,(superintegron)	*catB9*, ***dfr2B***	[193]

[a]Sulfonamide resistance genes are highlighted in bold faced type

Aminoglycosides and aminocyclitols

The aminoglycosides and aminocylitols are a mix of natural occurring and synthetically derived compounds which are polar organic bases consisting of aminated sugars joined to a dibasic cylcitol via glycosidic linkages [46]. Their antibacterial activity depends upon active oxidative transport across the bacterial envelope whereupon attaining the cytosol the antimicrobial can interact with the bacterial ribosome. The binding of the aminoglycosides to the 30S ribosomal subunit causes subsequent misreading of the mRNA and aberrant protein synthesis resulting in death of susceptible species. Multiple resistance mechanisms to the aminoglycosides have been identified, including decreased

uptake/increased efflux, modification of the 16S rRNA via mutation or methylation and most commonly, enzymatic inactivation.

Decrease uptake of aminoglycosides as a result of diminished permeability and/or increased export have been identified in *Pseudomonads* and *E. coli* respectively, which can lead to low level or intermediate resistance [46, 207].

Mutations which alter the aminoglycoside binding site in the ribosome may effectively increase resistance to this class of compounds. Meier (1994) for example, describes how a single point mutation in the 16S rRNA confers streptomycin resistance to *Mycobacterium tuberculosis* [208]. Methylation within the ribosomal aminoacyl site (A-site) of the 16S rRNA also interferes with aminoglycoside binding and has been recognized as one of the means of self protection utilised by bacteria which produce aminoglocysides such as the *Streptomyces* [209]. Acquired ribosomal methylases have a varied spectrum of activity and may confer resistance to some or all aminoglycosides containing the deoxystreptamine ring, including gentamicin, tobramycin, amikacin and paromomycin [209, 210]. They are however, as yet unreported in *Aeromonas* or *Vibrio* species [211].

Enzymatic inactivation of the aminoglycosides is achieved by modification of either the amino groups via the acetyl-CoA dependent activity of the *N*-acetyltransferases (AAC) or the ATP dependent activity of the *O*-nucleotidyltransferases (ANT) and *O*-phosphotransferases (APH) enzymes on hydroxyl groups [46]. Although there are only three mechamisms of inactivation, at least 50 aminoglycoside modifying enzymes have been described, therefore, within each class there are multiple variants. Moreover, the use of two different nomenclature systems, and the publication different names for the same aminoglycoside modifying enzyme, makes nomenclature confusing [212, 213]. Shaw provides an excellent review of the nomenculature and spectrum of activity of the various aminoglycoside inactivating enzymes [214].

These enzymes are commonly but not uniquely found, on mobile genetic elements, and are among the most commonly encountered in aquatic bacteria. Although the assumption that antimicrobial exposure is necessary to develop and maintain resistance in a bacterial population, members of the aminoglycoside family are not used in aquaculture. Aminoglycoside resistance gene cassettes appear to be more stable in integron structures which may help explain the facility of their transmission and occurrence and stability [7]. Numerous publications report the presence of aminoglycoside resistance genes in

Aeromonas and *Vibrio* species. Tables VIII and IX summarise those identified during this review.

Table VIII Aminoglycoside resistance genes identified in *Aeromonas* sp.

Genus/species	Associated genetic elements	Genes identified[a]	Ref.
A. salmonicida	pRAS1/pAr-32, Class 1 integron, transposon	*dfrA16, qac, sul1 tet*(A)/***aadA2***, *qac, sulI, catAII*	[43]
A. salmonicida	pRAS2, transposon	*tet*(31), *sul2*, ***strA, strB***	[176]
A. caviae(punctata)	pFBAOT6, Class 1 integron, transposon	*tet*(A), *sul1,qacEd1*, ***aadA2***	[178]
A. bestiarum	pAB5S9, ICE, transposon	*floR, tet*(Y), ***strA, strB***, *sul2*	[179]
Aeromonas spp.	Class 1 integron, plasmid	***ant(3")Ia***, ***aac(6')Ia***, *dhfr1*, bla_{oxa2a}, *pse1*, *tet*(A), *tet*(B), *tet*(D), *tet*(E), *tet*(H)	[95]
Aeromonas spp.	Class 1 integron	*dfr12, dfr2d*, ***aadA1***, ***aadA2***, bla_{oxa-2}, *catB3, catB8,qacE2*	[106]
A. salmonicida	Class 1 integron/plasmid	***ant(3")1a***, *dhfr1, dhfrIIc, dhfrXVI, tet(A), tet*(C), *sul1*	[159]
Aeromonas sp.	Class 1 integron, chromosome	bla_{OXA-2}, bla_{TEM}, bla_{SHV}, *cphA, dfrA12*, ***aadA1, aadA2***, *catB8*	[140]
Aeromonas spp.	Class 1 integron	***aadA1, aadA2, aac a4, aac(6')-II, aac(6')Ib***, *arr-2, arr-3, dfrA1, dfrA12, dfrA5, dfra17,dfra2d, dfrV*, bla_{oxa21}, *catB3, catB8, cmlA1, ereA2*	[107]
A. media	Plasmid	bla_{PER-1}, ***aphA6***, ***strA***	[215]
A. hydrophila	Class 1integron	bla_{vim-4}, ***aacA4***	[216]
A. caviae	pJDB2	bla_{imp-19}, ***aacA4***	[152]
A. allosaccharophila	p34 (IncU), integron	*qnrS2*, ***aac(6')-Ib-cr***, bla_{oxa-1}, *catB3, arr-3*	[137]
A. salmonicida	Plasmid (pSN254-like), Class 1 integron	***aadA7***, *florR, tet*(A), *sul2*, ***strA/strB***, *sul1*, bla_{cmy-2}, *sugE, Hg*	[39]
Aeromonas sp.	Class 1 integron, plasmid	*tet*(A), *tet*(E), *tet*(D), *dhfr1, dhfr2a*, ***ant(3")1a***, *catB2*	[7]
A. salmonicida	Class 1 integron, plasmid, Tn*1721*	*tet*(A), *tet*(E), *sul2*, ***aadA1, aadA2***, *drf16, drfIIc,*	[91]

[a]Aminoglycoside resistance genes are highlighted in bold faced type

Table IX Aminoglycoside resistance genes identified in *Vibrio* sp.

Genus/Species	Associated genetic elements	Genes identified[a]	Ref.
V. fluvialis	pBD146, Class 1 integron, SXT	*dfrV arr3, bla$_{OXA-142}$,* ***aadA1***	[109]
V. cholerae (non01,non0139)	Class 1 integron, plasmid, chromosome,	*aac(6_)-Ib, dfrA1,* ***aadA1****, dfrA12,* ***aadA,*** *dfrA15, dfrA5, ereA2, sul1, qac,*	[203]
Vibrio-like bacteria	Class 1integron	*dfrIIc, dfrXII,* ***aadA1a****, blaOXA$_{OXA2}$, catB3, catB5*	[94]
V. cholera	Class 1 integron, plasmid	*dfrA12,* ***ant(3")-1a***	[144]
V. cholera	Class 1 integron, plasmid	*dfrA15, dfrA1,* ***ant(3")-1a (aadA2),*** *bla$_{P1}$(bla$_{CARB-2}$),* ***aadB***	[204]
V. cholera	Transposon-like structure, SXT	*dfr18, floR, sul2,* ***strA/strB***	[121]
V. fluvialis	Class1 integron	***aac(3)-Id, aadA7***	[110]
Vibrio cholerae 01	Class 1 integron	***ant(3")-1a****, sul1,*	[111]
Vibrio spp.	Class 1 integron, SXT	*dfrA15, bla$_{P1}$,* ***aadA2***	[112]
V. cholera	Class 1 integron, SXT, plasmid	***aadA2****, sul1, tet*(A)	[185]
V. cholera	Class 1 integron, SXT	***aadA1****, dfrA15*	[105]
V. cholera	Class 1 integron, plasmid , chromosome	*dfrA1,* ***aad2***	[206]
V. cholerae, V. parahaemolyticus	Class 1 integron, plasmid	*dfrA15 , blaP1, qacH,* ***aadA8****, tet*(G)*, aph, cat1, sul2*	[104]
V. cholera	Class 1 integron, SXT	***aadA1****, floR,* ***strA/B****, sul2, tet*(A)	[124]

[a]Aminoglycoside resistance genes are highlighted in bold faced type

Quinolones

The quinolones are broad spectrum antimicrobials possessing a two ringed quinolone nucleus (4-oxo-1,8-naphthyridin-3-carboxylic acid), with a carboxylic acid side chain and oxygen at carbons 3 and 4 respectively being important for antibacterial activity [217]. These first generation molecules (oxalinic acid, nalidixic acid as examples) were later modified with the addition of a fluorine at carbon 6 and various substitutions at other ring sites to give us the modern fluoroquinolones. The quinolones owe their antibacterial activity to a non-covalent binding of the DNA gyrase and/or topoisomerase IV, stabilising breaks in the DNA, inhibiting replication [218].

Decreased permeability of Gram-negative bacteria to antimicrobials due to alterations in porins and/or the phospholipid bilayer may be a factor in resistance to

antimicrobials [219]. A decrease in the expression of the porin OmpF and outer membrane proteins of *E. coli* for example, can decrease the susceptibility to quinolones as well as other antimicrobials [220]. This type of change had been noted for *A. salmonicida* mutants which displayed low-level resistance to multiple antibiotics, including the quinolones, due to changes in outer membrane protein profiles [129].

The earliest type of resistance identified against the quinolones was associated with single or multiple point mutations in the QRDR (**q**uinolone **r**esistant **d**etermining **r**egion) of the gyrase gene (*gyrA/gyrB*) and the topoisomerase IV gene (*parC/parE*). These mutations affect quinolone binding sufficiently to permit DNA replication, and an accumulation of mutational events in the QRDR can lead to higher levels of quinolone resistance [221, 222]. Point mutations in the *gyrA* gene are the most frequently reported, occurring most commonly at the codons 83 and 87 [62, 218, 222-225]. The most common point mutations in *parC* are found at codons 80 and 84 and have been reported only to be identified in the presence of *gyrA* mutations [218, 226]. Mutations in *gyrB* and *parE* are less commonly identified, and are cited as causing low-level resistance to quinolones when present. However, in a recent study by Bansal (2011) in *E. coli*, a high frequency of *parE* mutations outside of the QRDR, was associated with high-level ciprofloxacin resistance in association with multiple *gyrA* and *parC* mutations [227].

In *Aeromonas* isolates, mutations in *gyrA* have been identified at codons 83 (Se83→Ile or Se83→Arg) and 87 (Asp87→Asn) [222, 228, 229]. *parC* mutations do not seem to occur in the absence of *gyrA* mutations and have been identified at codon 80 (Ser80→Ile). Arginine and isoleucine point mutation of the *gyrA* codon 83 seems to be the most important for high level quinolone resistance however multiple mutations in this gene coupled with mutations in *parC* may have additive effects resulting in highly resistance bacterial strains [222]. Giraud (2004) demonstrated however, that strains with identical *gyrA/parC* point mutations presented different levels of quinolone resistance indicating there are multiple factors involved [62]. Mutations in *gyrB* and *parE* in *Aeromonas*, associated with quinolone resistance, have not as yet been reported.

Vibrio species have demonstrated the same type of resistance mechanism with (Se83→Ile) *gyrA* mutations being by far the most common [230-234]. Roig identified an additional *gyrA* mutation (Se83→Arg) in *V. Vulnificus* [232]. *parC* mutations are habitually located at the 85th amino acid with a serine to leucine substitution, however a change at amino acid 113 (Ala113→Val) has also been identified resulting in elevated

MIC's [232, 233]. This not-withstanding Roig (2009) reported double mutations of *gyrB* in two strains of *V. vulnificus* (Ala386→Thr, Gln412→His) and (Glu425→Gly; Asn438→Lys) which were associated with increased resistance in combination with *gyrA* and/or *parC* mutations. *parE* mutations have not been identified to date in *Vibrio* species.

Until fairly recently, it was thought that mutational resistance along with impermeability to the quinolones were the only mechanisms of quinolone resistance. This type of resistance is less disconcerting because transmission from one bacterium to another is possible only by clonal expansion. Discoveries of quinolone resistance determinants such as efflux pumps, antimicrobial modifying enzymes, and proteins which protect intrabacterial quinolone targets, as well as plasmid mediated resistance phenotype that had been relatively recently reported for the quinolone family, have complicated the picture and make the rapid development and spread of quinolone resistance a real possiblity.

Efflux pumps which reduce intrabacterial concentrations of antimicrobials have been shown to make an important contribution to quinolone resistance in Gram-negative bacteria and several chromosomally located efflux systems have been identified [235]. More recently, a plasmid mediated efflux pump, QepA, coded for by the *qepA* gene, has been characterised in a clinical isolate of *E. coli* by Yamane (2007) and subsequently in other *Enterobacteriaceae* [236-238]. Giraud (2004) demonstrated indirectly the importance of efflux pumps in *Aeromonas* by comparing MIC's of bacteria with or without exposure to efflux pump inhibitors (EPI's). The EPI's reduced the MIC's by a factor of 2 – 75,000 times, with the effect being the most pronounced for the third generation quinolone ciprofloxacin [62].

Although as yet unreported in *Aeromonas* species, a member of the MATE family of efflux proteins with activity for the several antibiotics including the quinolones has been reported in *Vibrio parahemolyticus* [239]. This MDR efflux protein, NorM, demonstrated a predilection for hydrophilic quinolones such as ciprofloxacin and norfloxacin resulting in increased MIC's to these compounds, whereas MIC's to hydrophobic quinolones such as nalidixic acid and spafloxacin remained unchanged [239]

Robiscek (2006) noted a difference in MIC's to fluorquinolones between different populations of *E.coli* both containing the same plasmid-borne *qnrA* determinant. Upon further investigation they identified an aminoglycoside acetyltransferase (Aac(6')-Ib-cr) which possessed the capacity to modify and inactivate ciprofloxacin [240]. Two mutations in the gene led to its capacity to acetylate quinolones and thus contribute to

fluoroquinolone resistance. This same gene has been reported in *Aeromonas* by Picao where *aac(6')-Ib-cr* was located on a Class 1 plasmid borne integron along with a *qnrS* determinant [137].

In 1998 a gene localized on a plasmid coding for quinolone resistance was identified in *Klebsiella pneumoniae* [241]. The *qnr* gene, codes for a protein which protects the topoisomerases from quinolone binding, and five resistance determinant groups have been identified to date including *qnrA*(1-6), *qnrB*(1-10), *qnrC*, *qnrD* and *qnrS*(1&2) [242-246].

This class of plasmid borne resistance determinant was first reported in *Aeromonadaceae* by Cattoir in 2008 where a *qnrS2* gene was identified in a mobile Aeromonad isolated from water of the Seine River in Paris [247]. This was followed closely with a publication by Sanchez (2008) who found the same determinant in a clinical *A.veronii* isolate, and Picao (2008) whom identified the gene in *A. allosaccharophila* isolated from a lake in Switzerland [137, 248]. Cattoir and Picao both identified the *qnrS2* gene as being part of an insertion cassette structure bounded by inverted repeats [137, 247]. A vibrionic origin of the *qnr* determinants was suggested by Poirel (2005) and later by Cattoir (2007) [249, 250]. Following an "*in silico*" analysis of the bacterial genome of *Vibrio splendidus,* prospective *qnr* genes were cloned and found to increase MIC's to quinolones in recipient bacteria [249]. MIC's obtained in the various works cited here in relation to mutations or genes are presented in Annexs 5 and 6 for *Aeromonas* and *Vibrio*.

Macrolides, lincosamides, streptogramins, ketolides and oxazolidinones (MLSKO antimicrobials)

The MLSKO antimicrobials are bacteriostatic antimicrobials, which include the macrolides, lincosamides, streptogramins, ketolides and oxazolidinones, and are composed of 14 – 16 membered lactone rings with variable attached amino or neutral sugars [251]. The lincosamides are an exception, as they contain no lactone ring, but are considered in this group because of their mode of action. The MLSKO's inhibit bacterial protein synthesis via a reversible interaction with the 50S subunit of the 70S ribosome, preventing translocation of the tRNA.

There are multiple resistance mechanisms reported for MLSKO's in Gram-negative bacteria. Some Gram-negative bacteria, such as the *Enterobacteriaceae*, exhibit innate resistance due to impermeability of the bacterium [252]. This does not seem to be

universally true for *Aeromonas* and *Vibrio*, at least phenotypically, where resistance to erythromycin has been reported as ranging from 29.4 – 98% for *Aeromonas* and 34 – 100% for *Vibrio* species [251, 253-256]. Chromosomally located efflux pumps coding genes in Gram-negative and Gram-positive bacteria, belonging to the RND and the ABC or MFS families respectively are responsible for efflux macrolide resistance [251, 252]. The *vexAB* genes of *V.cholerae* code for an RND-family efflux pump protecting the bacteria from bile. Bina (2006) noted that *vexB* mutants were more susceptible to erythromycin indicating its non-specific implication in innate macrolide resistance [58]. The inactivation of MLSKO antimicrobials has been reported in many bacterial families implicating lyase, transferase, phosphorylase and esterase enzymes, this last being recognized in *Aeromonas* and *Vibrio* [107, 203, 257]. In Gram-negative bacteria, erythromycin esterases which hydrolyze the lactone ring of the 14-membered macrolides are responsible for resistance. In *Aeromonas* and *Vibrio*, erythromycin resistance determinants are rarely reported, however the erythromycin esterase gene *ere-A2* has been identified as a resistance cassette within class 1 integrons in both genera [107, 203].

Rifamycins

Rifamycins are natural or semisynthetic drugs derived from an actinomycete, *Amycolatopsis mediterranei* and their bactericidal activity is due to the inhibition of RNA synthesis due to binding with the DNA-dependant RNA polymerase [258]. This class of drugs has greater activity against Gram-positive bacteria and is used primarily for the treatment of mycobacterial infections [258]. Gram-negative bacteria are generally considered resistant due to impermeability to the drug. Resistance the rifamycins arises via two principal mechanisms. The first is by point mutations of the RNA polymerase rendering it insensitive to the rifamycins, and the second is enzymatic inactivation of the antibiotic with an ADP-ribosylating transferase via the *arr* gene [259]. In bacteria such as *M. tuberculosis*, the former is by far the most important, but for other bacteria additional resistance mechanisms include glycosylation, phosphorylation and ribosylation enzymes which inactivate the antimicrobial [258]. As was seen previously for the *ere* genes, those coding for rifamipicin resistance are not frequently encountered. It is likely that research has not been directed towards the resistance mechanisms for these classes of drugs because they are seldom recommended or used with the genera in question here. Although the

presence of these resistance elements has little consequence upon therapy options for the genera discussed here, their presence on transmissible genetic elements may serve as a reservoir for other human pathogens. In studies by Lee, Picao and Rajpara, either the genes *arr-2, arr-3* or both were identified as being present in the variable region of class 1 integrons [107, 109, 137].

The usage of antimicrobials in aquaculture may represent an exposure risk, to bacteria resistant to antimicrobials, especially in imported products. Although significant information concerning AMR and causal resistant determinants in *Aeromonas* and *Vibrio* is available in the literature, there is little data available concerning that found in fish and seafood at the retail level in Canada or elsewhere. The following manuscript describes and discusses the methods and results concerning 1) the identification of the targeted bacteria (*Aeromonas* and *Vibrio*); 2) the determination of the occurrence of phenotypic resistance in *Aeromonas* and *Vibrio* species found in fish and seafood; 3) and the characterization of the causal resistance genes.

Scientific article #1

Antimicrobial susceptibility in *Vibrio* sp. isolated from seafood using normalised resistance interpretation

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Abstract

Antimicrobial use in the aquaculture setting is generally considered low as compared to other types of animal production. However, where endemic disease requires frequent therapeutic intervention, or in countries where antimicrobial usage is poorly regulated, the quantities used may be considerably higher. This may lead to increased occurrence of antimicrobial resistance (AMR) in aquaculture products and increased the risk of human exposure. Little information is available concerning the occurrence of antimicrobial resistance in finfish and seafood available at the retail level in Canada. In this study, 175 *Vibrio* isolates were cultured from 323 finfish and shrimp sampled within the framework of the Canadian Integrated Program for Antimicrobial Resistance Surveillance. Epidemiological cut-off values were estimated for the tetracyclines, folic acid inhibitors, quinolones and florfenicol using Normalised resistance interpretation (NRI-ECV) of the disk diffusion data and then compared to corresponding MIC distributions and AMR gene presence. Gene presence associated with resistance phenotypes was evaluated using PCR and microarray analysis. *Vibrio* prevalence in finfish and shrimp was 16% and 49% respectively, where *V. parahaemolyticus* was the species most often identified. The NRI-ECV for tetracycline separated the population into distinct susceptible or wild-type (WT) and non-susceptible, non-wild-type (NWT) populations and correctly classified all isolates with identified *tet* resistance genes as NWT. Classification was less clear for SXT, where among 8 isolates classified as NWT, six contained genes for resistance to folic acid inhibitors (*sul2*, *sul2/dfrA7*) and two contained none of the resistance genes, whereas three isolates identified as WT by the NRI-ECV harboured *sul2*, *sul2/dfrA7* or *drfA5*. Fully susceptible populations were noted for florfenicol and enrofloxacin, whereas 4 isolates were classified as NWT for nalidixic acid and a *gyrA* mutation was identified in only one of these isolates. Reasons for misclassification could include: abnormal inhibition zone distributions, suboptimal PCR primers or non-specific probe design, and intermediate inhibition zones caused by stepwise decreases of susceptibility due to the accumulation of mutation or resistance determinants such as with the quinolones and potentiated sulfonamides. NRI analysis was useful in establishing ECVs for four antimicrobial classes for *Vibrio* species using laboratory specific isolates. The results obtained indicated a high correlation between isolates classification as WT/NWT and gene presence.

1.0 Introduction

Aquaculture is the fastest growing agri-industry in the world. It currently accounts for 46% of the world fish supply and is poised to overtake wild fisheries as the primary fish source [260]. Antimicrobial exposure in the aquaculture setting is generally considered low as compared to other types of animal production, however, in countries where endemic disease requires frequent therapeutic intervention, or in countries where antimicrobial usage is poorly regulated, the quantities used may be considerably higher [3]. Antimicrobial resistance has been reported in many species of aquatic bacteria in freshwater and marine environments including *Vibrio*. *Vibrio* species are Gram-negative, mobile, predominantly halophilic bacteria that are frequently found in marine systems. Some species are common pathogens of cultured marine fish causing septicemia or focal chronic disease and certain species can cause severe disease in humans [14-17].

In both aquacultured animal and human illnesses, antimicrobial therapy may be necessary. However, the presence of antimicrobial resistance may decrease the chances of successful treatment [23-27].

Disk diffusion and broth dilution are the most common methods used for susceptibility evaluation by clinical laboratories. Approved guidelines have been published by Clinical and Laboratory Standards Institute (CLSI) for both methods concerning bacteria isolated from aquatic animals [261, 262]. However, the current CLSI breakpoint recommendations used to interpret susceptibility of *Vibrio*, published in CLSI M45-A are adapted from the *Enterobacteriaceae* and may not be appropriate for *Vibrio* [263]. For example, *Vibrio* may require the addition of sodium chloride for adequate growth, which may affect test performance and interpretation. In addition, drug pharmacodynamic and pharmacokinetic considerations and clinical effectiveness are elements necessary to the traditional approach of ascertaining antimicrobial susceptibility. At this time, there is a paucity of information concerning these aspects for *Vibrio* species, making susceptibility testing and results interpretation a challenge.

Epidemiologic cut-off values (ECV's) have traditionally been determined using frequency distributions of disk diffusion and minimal inhibitory concentration (MIC) data [264]. This technique is based on rigorous adhesion to standard methods, and an inclusion of large numbers of isolates (> 300) is recommended. Using recommended susceptibility testing

methods, inter-laboratory variability susceptibility determination should be minimal for the same isolates. However, some studies have suggested otherwise [265, 266]. Normalised Resistance Interpretation (NRI) has been proposed to identify wild-type (WT) and non-wild type (NWT) bacterial populations [267, 268]. NRI-ECVs are defined using the normal distribution of disk inhibition zones of the population of susceptible isolates to define the wild type distribution. The advantages of this technique include generation of lab-specific epidemiological cut-off values, autocalibration, reliance on standardized methods independent of disc test standards, and therefore independent of interlaboratory variability [267].

In this study, 175 *Vibrio* isolates cultured from retail seafood were tested for susceptibility to antimicrobials using disk diffusion and broth dilution techniques. Epidemiological cut-off values (ECV's) were estimated using NRI of the disk diffusion data and then compared to corresponding MIC distributions and antimicrobial resistance gene presence.

2.0 Materials and Methods

2.1 Sample collection and bacterial isolation

Three hundred and twenty three retail seafood samples (164 salmon, 149 shrimp, 6 trout and 4 tilapia) were obtained between the 20th of October, 2008 and the 20th of July 2009, within the sampling framework of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) [42]. They originated from Québec, Ontario, Saskatchewan, British Columbia and the Maritimes. Finfish samples taken included filets or steaks (skin-on or skin-off) whereas raw shrimp samples were submitted whole deveined. The original retail samples were purchased as fresh, thawed or frozen. Approximately 100 grams of each sample were placed individually in 7x12inch sterile filtered bags (VWR International, Mississauga, ON) with 225ml of alkaline peptone water (APW), (Oxoid, Cambridge, UK) and homogenized by hand for two minutes with subsequent incubation at 28°C for 18-24 hours. *Vibrio* species were cultivated and selected by inoculating Thiosulfate Citrate Bile Sucrose agar plates (TCBS), (Oxoid, Cambridge, UK) with a loopful of the APW enrichment. Two yellow and two blue-green colonies were selected and plated onto half TSA + 5% sheep blood (TSA-Blood) + 2% NaCl agar plates for further testing.

2.2 Bacterial identification

Putative *Vibrio* isolates were subjected to an initial panel of tests including: Gram stain, motility, oxidase, triple sugar iron + 2% NaCl (TSI), (Oxoid, Cambridge, UK) agar slant and 0129 susceptibility (2,4-diamino- 6,7-diisopropylpteridine phosphate, 150mg) (BD-BBL, Mississauga, Ontario). A putative *Vibrio* sp. identification was given to those isolates found to be Gram-negative, motile, oxidase positive, sensitive to 0129 and demonstrated an acid/acid or alkaline/acid reaction without the presence of gas or H_2S in TSI agar slants. Up to two isolates per positive sample were preserved in tryptic soy broth (TSB) + 50% glycerol at -82°C for further testing.

One hundred and eighty five putative *Vibrio* sp. isolates were then identified using the Vitek2® identification system following manufacturer's protocols (bioMérieux, Marcy l'Etoile, France). This identification was confirmed via amplification of the ribosomal polymerase subunit gene *rpoB* as previously described, with some modifications [269]. PCR amplification was performed in 25 µl containing; 2ul 10X PCR buffer, 0.08mM dNTPs, 0.2mM $MgCl_2$, 10 pmol of opposing primers, 1.25U Taq polymerase (New England Biolabs, Pickering, Ontario) and from 50-100ng of DNA template. A temperature of 56°C was used during the hybridization step. *V. alginolyticus* ATCC17749, *V. fluvialis* ATCC33812, *V. parahaemolyticus* ATCC17802 and *V. vulnificus* ATCC27562 were used as controls. The same primers were used for subsequent sequencing reactions of the approximately 540bp PCR products. Sequencing was performed by the Plate-forme d'analyses biomoléculaires (PAB), Université Laval. Identification was made using the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) [270]. Identification to the species level was defined as an *rpoB* sequence 'Maximum identity score' of ≥99% with that of strain sequences in GenBank whereas identification to the genus level was defined as those isolates having a 'Maximum identity score' of ≥97%. A score of lower than 97% was considered as a failure of identification [271].

2.3 Antimicrobial susceptibility testing

All 185 isolates were tested for antimicrobial susceptibility by broth microdilution (MIC) and by disc diffusion using methods for aquatic organisms published by CLSI (M42-A, M49-A). Media supplemented with additional salt was not used. For MIC determination,

the ARIS automated system of Sensititre™ (Trek™ Diagnostic System Ltd) with a custom aquatic plate was used, containing (MIC range in µg/ml in parenthesis): enrofloxacin (0.002-1), florfenicol (0.03-16), oxalinic acid (0.004-2), oxytetracycline (0.015-8) and sulfamethoxazole/trimethoprim (0.015/0.3-1/19). Antimicrobials evaluated by disk diffusion included (antimicrobial disc content in µg in parenthesis): enrofloxacin (5), florfenicol (30), nalidixic acid (30), sulfamethoxazole/trimethoprim (23.75/1.25), and tetracycline (30) (BD-BBL, Mississauga, Ontario). Incubation temperature for all testing was 28°C. Quality control for both testing methods was performed using *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658.

2.4 Detection of antimicrobial resistance genes

Detection of individual antimicrobial resistance determinants by PCR (*tet* (A), (B), (C), (D) & (E)), *florR*, *sul1*, *sul2*, *qnr A*, *B*, and *S* and *gyrA/parC* mutations was performed to validate the NRI-ECV's. The primers utilised are presented as supplementary information in Table S1. Identical amplification reactions were used for *floR, sul1* and *sul2* with 2.0 µl of 10X PCR Buffer, 0.08mM dNTPs, 0.25mM $MgCl_2$, 10 pmol of each primer, 1 U of *Taq* DNA polymerase (NEB) and 50-100 ng of template DNA in a total volume of 20 µl. Briefly, the temperature cycles for PCR used were: *floR*: initial denaturation (94°C, 5 min), 35 polymerization cycles (94°C for 30s, 60°C for 30s and 72°C for 30s), final elongation at 72°C for 7 min; *sul1*: initial denaturation (95°C, 5 min), 25 polymerization cycles (95°C for 30s, 55°C for 30s and 72°C for 40s) final elongation at 72°C for 7 min; *sul2*: initial denaturation (95°C, 5 min), 35 polymerization cycles (95°C for 30s, 62°C for 30s and 72°C for 45s) and final elongation at 72°C for 7 min. All *tet* genes were amplified using identical amplification reactions and PCR conditions. The PCRs consisted of 2.0 µl of 10X PCR Buffer, 0.08 mM dNTPs, 0.14 mM $MgCl_2$ 10 pmol of opposing primers, 1 U of *Taq* DNA polymerase (NEB) and 50-100 ng of template DNA, in a total volume of 20 µl. The PCR conditions included an initial denaturation step (95°C, 5 min), followed by 35 polymerization cycles (95°C for 30s, 62°C for 30s and 72°C for 45s) and final elongation at 72°C for 7 min. Amplification of *qnrA*, *qnrB* and *qnrS* genes was undertaken utilizing a multiplex PCR as previously described [272]. The *gyrA* and *parC* genes were amplified using conditions described previously using the *gyrm*1-2, *parm*1-2 primer pairs [231]. The same primers were used for subsequent sequencing reactions of the approximately 500bp PCR products. Comparison to phenotypically susceptible isolates of the same genera was

performed to characterize mutations in the quinolone resistance determining region (QRDR).

Eleven isolates were further examined for the presence of resistance genes using microarray analysis as described by Bonnet (2009) [273]. Isolates were selected to confirm results, and to examine non-susceptible phenotypes for which no resistance genes were identified by PCR. The majority of the probes were designed based on gene sequences found in *E. coli*. Twelve probes were added to identify AMR genes not already present on the microarray. Information concerning the probes used on the microarray is available as supplemental information (Table S2).

2.5 Statistical analysis

The calculation of the Epidemiological Cut-Off Value (ECV) for the interpretation of susceptibility of isolates was accomplished using NRI of disk diffusion data as previously described [267, 274]. For the NRI calculations, peak values were established using four points rolling averages, and a plot of seven probit values versus zone size was used to identify the means and standard distribution of the susceptible population. ECV's were set at 2.5 standard deviations from the mean. Those isolates demonstrating inhibition zone sizes smaller than the the calculated ECV are considered Non-wild type (NWT) and those above wild-type (WT). All statistical analyses and graphics were generated in Microsoft Excel (2007) and the R program for statistical computing and graphics (open source software version 2.11.0).

3.0 Results

3.1 Bacterial isolation and identification

Of the 323 seafood samples examined, 185 putative *Vibrio* sp. isolates were recovered from 45 (15.6%) and 113 (48.9%) of the finfish and shrimp samples, respectively. Ninety-five percent of these (175/185) were confirmed as *Vibrio* species with the *rpoB* sequencing: *V. parahaemolyticus* (*n* = 86), *Listonella (Vibrio) anguillarum* (*n* = 45), *Vibrio* sp. (*n* = 27); *V. alginolyticus* (*n* = 7), *V. metschnikovii* (*n* = 5), *V.cholerae*, (*n* = 2); *V. harveyii*, (*n* = 2), *V. vulnificus*, (*n* = 1).

3.2 Tetracycline susceptibility

The zone size distribution for 175 *Vibrio* isolates to tetracycline (TET) is shown in Fig.1a. The mean zone size for the susceptible populations as estimated using NRI analysis was

27.9 mm with a standard deviation of 2.8 mm and an ECV of ≥20 mm. The use of this ECV enabled the classification of 10/175 (5.7%) isolates as NWT for TET with inhibition zones measuring from 6 – 17 mm. The disc diffusion zone size results of 174 isolates for tetracycline were compared with MIC results in Fig.2. The bimodality of the population is evident, with 4 dilutions (from 1-8µg/ml) separating the two populations. The genes *tet*(B), *tet*(A) and *tet*(E) were identified in 4, 2 and 1 isolates, among ten NWT isolates, respectively, corresponding to zone sizes of 6 - 17mm and MIC's of >8 µg/ml. The MIC for all 3 isolates which were negative for *tet* genes was >8 µg/ml.

3.3 Florfenicol susceptibility

The zone size distribution for 175 *Vibrio* isolates to florfenicol (FFC) is presented in Fig.1b. The mean zone size for the susceptible populations as estimated using NRI analysis was 31.8 mm with a standard deviation of 3.0 mm and an ECV of ≥24 mm. Only one isolate (1/175, 0.6%), with a zone size measuring 21mm was considered as NWT. When comparing disk diffusion zone sizes with corresponding MIC's in Fig. 2, an unimodal susceptible population is suggested. The single isolate which was classified as NWT by NRI, demonstrated an MIC of 0.5µg/ml. The *flo-R* gene was not found in this unique isolate identified by the NRI-ECV.

3.4 Trimethoprim-sulfamethoxazole susceptibility

The zone size distribution of *Vibrio* isolates for trimethoprim-sulfamethoxazole (SXT) is presented in Fig.1c. The mean zone size for the susceptible populations as estimated using NRI analysis was 29.9 mm with a standard deviation of 3.5 mm and an ECV of ≥21 mm. Using this ECV, 8 of 175(4.6%) isolates were classified as NWT for SXT with inhibition zones measuring from 12-18 mm. However, three isolates containing resistance genes (*sul2*, *dfrA5*, *dfrA7*/*sul2*) were classified as WT with regards to SXT by the NRI-ECV. When disc diffusion zone size results are compared with MIC's (Fig.2.), a bimodal distribution is present with a separation of the two populations at the 0.25µg/ml dilution. Six NWT isolates harbouring the gene *sul2* or the combination *dfrA7*/*sul2* had MIC's from 0.5-2 µg/ml, whereas the 3 WT isolates carrying *dfrA5*, *dfrA7*/*sul2 and sul2* were found at MIC's of 0.03, 0.125 and 0.5 µg/ml respectively.

3.5 Quinolones susceptibility

The zone size distribution of *Vibrio* isolates for nalidixic acid (NA) and enrofloxacin

(ENO) are presented in Figs.1d and 1e. The mean zone size for the susceptible populations as estimated using NRI analysis was 32.9 mm with a standard deviation of 5.4 mm for NA and 34.7 mm with a standard deviation of 5.6 for ENO, resulting in ECV's of ≥19 and ≥20 mm, respectively. The use of these ECVs enabled the classification of 4/175 (2.3%) isolates as being NWT for NA and 0/175 (0%) isolates as WT with respect to ENO. The disc diffusion zone size results of 174 isolates are compared with MIC results in Fig.2. The ENO distribution is unimodal, as compared to the comparison of the disc diffusion zone sizes of NA and MIC's of oxolinic acid where a bimodal distribution is evident with two dilutions (1-2 µg/ml) dividing the two populations. The point mutation *gyrA*$_{\text{Se83-Ile}}$ was identified in one of the five isolates at an MIC of 4 µg/ml, and was identified as NWT by the NRI-ECV.

3.6 Epidemiologic cut-off values for different species subgroups

Seven species of *Vibrio* were identified in this study with *V. parahaemolyticus* (86) being the most prevalent. When the NRI-ECV for all *Vibrio* sp. was compared to the NRI-ECV calculated for the inhibition zone diameters of *V. parahaemolyticus* the ECV's are similar for both groups with the exception of SXT (see Table 1) where 5 NWT isolates were reclassified as WT. Among the reclassified isolates, one harboured *sul2* and another, a *sul2/dfrA7* gene combination. Only those isolates with small inhibition zone diameters (12-13 mm) remained classified as NWT, all positive for *sul2*.

4.0 Discussion

The Epidemiologic cut-off values (ECVs) derived from NRI analysis, in general, agree well with the distribution of the zone diameter and MIC data for the antimicrobials and resistance genes. The NRI-ECV for TET separated the population into distinct WT and NWT populations, and classified all isolates with identified resistance genes as NWT. The presence of TET resistance genes has been associated with radically decreased susceptibility in several publications, which can result in widely separated bimodal distribution as seen here [161, 177, 275]. This could facilitate agreement of distribution data with NRI-ECV, and a similar ECV may have been derived from observation of the distribution data.

Among eight isolates classified as NWT by the NRI-ECV for SXT, six contained genes for resistance to folic acid inhibitors, but in two no resistance genes were identified. This could

indicate poor sensitivity of PCR (primer or probe design for the targeted gene) or misclassification by the NRI-ECV. The presence of *dfr* variants not yet described is also a possibility. Additionally, the microarray used in this study was designed for *E. coli*, it is also possible that the genes used for probe design were different enough from those found in *Vibrio* to make detection difficult. Three isolates harbouring genes coding for resistance to folic acid were classified as WT. It is generally considered that resistance to SXT requires the presence of genes coding for both sulfonamide and trimethoprim resistance. If this is the case, in those isolates where only a *sul* or *dfr* gene were identified, it would suggest that an additional gene remains unidentified and in the case where both *sul* and *dfr* genes were identified, there may have been a laboratory error. If isolates contain either *sul1* or *drfA* but not both, the inhibition zone size data may be between fully susceptible and fully resistant thus making interpretation difficult using an ECV based on inhibition zone size. As suggested by others [276], the use of single agents rather than drug combinations for susceptibility testing may help resolve some of the discrepancies observed here. The zone diameter distribution for SXT was not normal with our dataset, and this may also affect the NRI-ECV calculation.

Laboratory error or variability could also be responsible for the misclassification, and has been reported by other authors [265, 277, 278]. The inhibition zone size measurements are made to the nearest mm (+/- 1mm), which could change the susceptibility interpretation for isolates on or near the ECV as is the case with two of the isolates in the SXT distribution. This was echoed by Barry (1974) discussing the role of standardisation in disk diffusion techniques [279]. Trailing endpoints and fuzzy zones are also associated with susceptibility measurements of potentiated sulfonamides, and this may contribute to their imprecision [280].

The classification of quinolones using NRI-ECV's demonstrated a relatively low frequency of resistance, where only four isolates were found resistant to NA and a *gyrA* mutation was identified in only one of these isolates. When inhibition zones and MIC's were compared, a bimodal distribution was present with the NA/OXO data, however, the same was not true for ENO where only a susceptible population was identified (Fig.1&2). Outliers were noted for NA/OXO at dilutions of 0.015 and 4µg/ml (NA/OXO), and for FFC at 0.5µg/ml (Fig.2). Misclassification was likely due to the location of the isolate in the population distribution and the absence of resistance genes or mutations. An ECV was established for ENO in spite

of the absence of low or non-susceptible isolates, one of the advantages of using NRI. However, the setting of the ENO NRI-ECV was ambiguous with the *Vibrio* dataset. When analyzing the four points rolling averages of ENO, there were three peaks which could have been used to position the normalised susceptible population lending an element of subjectivity. This subjectivity may lead to the misclassification of isolates as falsely susceptible to ENO using the NRI-ECV, as has been previously reported [266]. In studies by Ruane et al. (2007) and Rodiguez-Avial (2005), a high level of correlation between zone sizes for oxalinic acid and ENO was noted, indicating probable cross-resistance [266, 281]. When the inhibition zone size data derived from our data are compared for NA and ENO, a similar linear relationship is noted, also indicating probable cross-resistance (Fig.3). The previously mentioned authors further suggest a first generation quinolone could be used as a reporter for all quinolone resistance [266, 281]. Although using a reporter quinolone such as NA may classify isolates as falsely non-susceptible to ENO, this would have less of an impact on treatment outcome than falsely classifying isolates as susceptible. The use of a reporter first generation quinolone for resistance evaluation may be useful until a wider sample of isolates with more variable and higher resistance to ENO can be evaluated. However, the recent discovery of mobile quinolone resistance elements which have a greater activity for fluoroquinolones than for quinolones, such as the *aac(6')-Ib-cr* gene, may make this extrapolation questionable [282].

Species-specific breakpoints have been developed for many important bacteria causing diseases in human and animals permitting improved discrimination of susceptible and non-susceptible populations [283, 284]. *V. parahaemolyticus* is the species most often associated with human illness in the United States and it was the most predominant species identified in this study [285]. A species specific NRI-ECV was calculated for *V. parahaemolyticus* to see if there were differences in WT/NWT classification and gene presence, particularly for SXT. Using the species-specific ECV, five additional isolates, two containing resistance genes were reclassified as WT, which indicates that the ECV specific to *V. parahaemolyticus* may be erroneous. It has been shown that precision of NRI-ECVs increases and standard deviations decrease with larger sample sizes numbers [286]. It could be argued that the sample size was not sufficiently large to allow for accurate NRI-ECV calculation. The NRI calculation used the high-zone side of the susceptible population to reconstruct the susceptible peak and set the ECV and assumes a normal distribution of

WT isolates. The SXT distribution of inhibition zone diameters for *V. parahaemolyticus* in this study was irregular, and likely the cause of the differences. Using larger samples sizes with improved coverage of the whole range of susceptibility resulted in improved interpretation.

The majority of current publications concerning antimicrobial resistance in *Vibrio* use CLSI breakpoints to evaluate susceptibility. When CLSI breakpoints for tetracycline and the fluoroquinolones were compared with NRI-ECV's, all isolates were classified identically. For SXT, however, three isolates were re-classified as susceptible by CLSI, containing *dfrA7/sul2*, *dfrA5*, and *sul2*. According to CLSI M45-A, for SXT, an MIC of ≤ 2µg/ml would indicate a susceptible isolate. However, Fig. 2 would suggest that when using the NRI-ECV, isolates with SXT MIC's of 0.5 - 2 µg/ml should be considered non-susceptible. And in fact, five of the isolates in this MIC range contained either *sul2* or a *dfrA7/sul2* gene combination. Using the CLSI MIC or disk diffusion breakpoints could result in the misclassification of some isolates carrying resistance genes.

To conclude normalised resistance interpretation analysis was useful in establishing ECVs for *Vibrio* species using laboratory specific isolates. It permitted the establishment of ECVs for four antimicrobial classes, resulting in an excellent classification of isolates as WT or NWT with associated gene presence. Among the quinolones tested, the interpretation with NRI-ECVs was least ambiguous with NA. Due to the linear correlation of zone diameters between the two quinolones, these results suggest that NA could be considered as a reporter for quinolone non-susceptibility.

6.0 Supporting Information

Table S1: Primers for simple PCRs used in this study.

Table S2: AMR probes present on the microarray and reference genes, used in this study.

7.0 Acknowledgements

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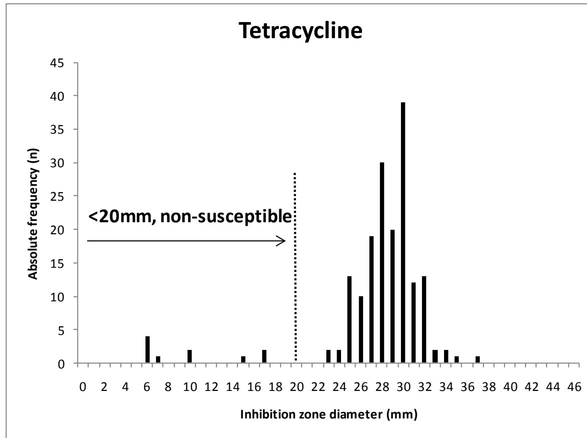
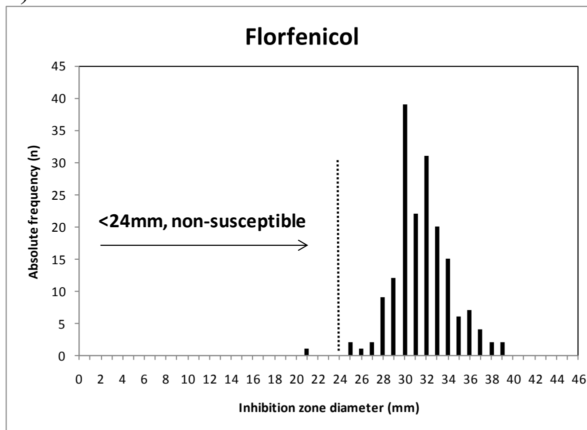
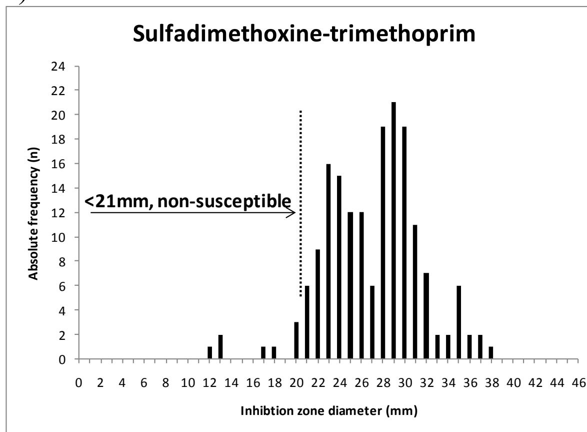
a).



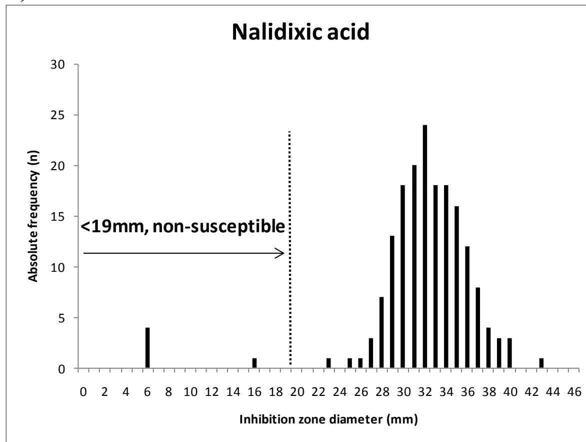
b).



c).



d).



e).

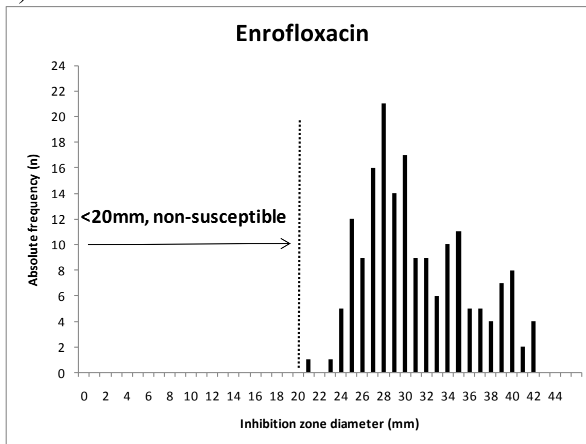


Figure 1: Histogram of inhibition zone diameters of *Vibrio* sp. population

Note: The calculated NRI-ECV value for each antimicrobial is indicated by the vertical dotted black line.

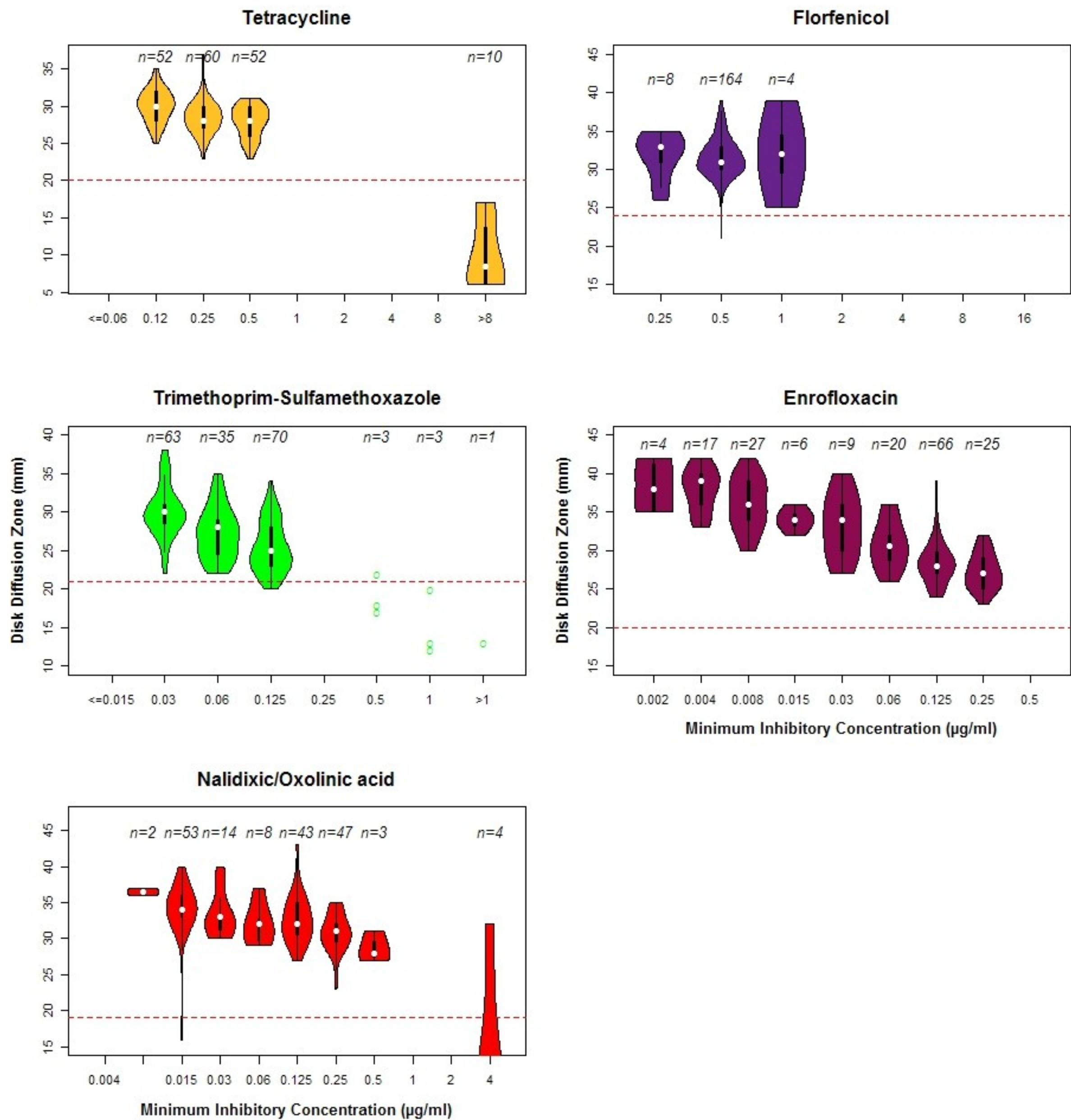


Figure 2: Comparison of MIC and disc diffusion zones for *Vibrio* sp. isolates

MIC and inhibition zone values for each antimicrobial are found on the x-axis and y-axis respectively. The width of the plot represents sample density and the extremities, the minimum and maximum values. The center point indicates the median and the quartile values are found at the extremities of the internal black bar. The calculated NRI-ECV value for each antimicrobial is indicated by the horizontal red-dashed line.

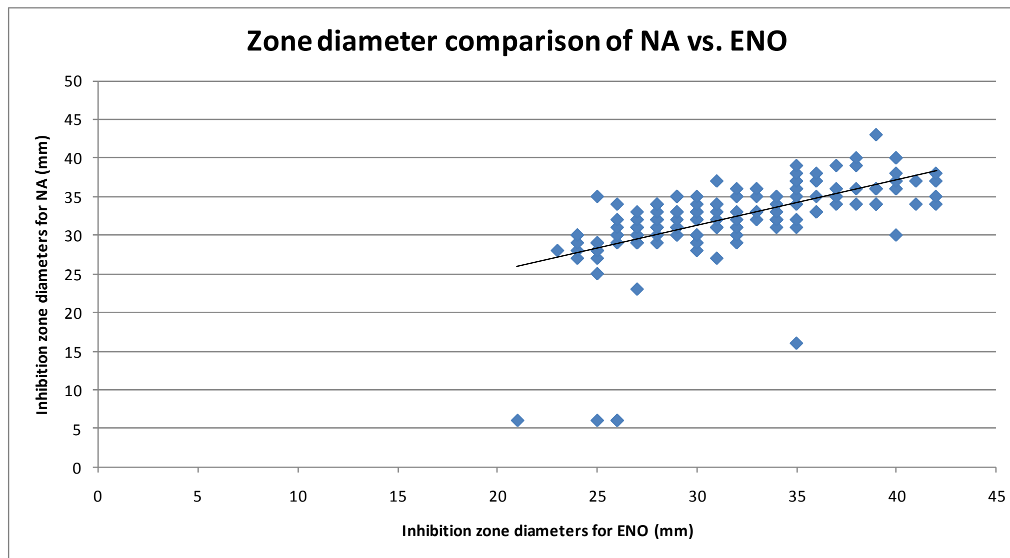


Figure 3: Comparison of the zone diameter distributions of Nalidixic acid (NA) and Enrofloxacin (ENO).

Table I: NRI-ECVs calculated for all *Vibrio sp.* and the *V. parahaemolyticus* subpopulation

Antimicrobial[a]	All *Vibrio* (*n*=175)[b]	*V. parahaemolyticus* (*n*=86)[b]
TET	≥ 20	≥ 21
FFC	≥ 24	≥ 22
SXT	≥ 21	≥ 14
NA	≥ 19	≥ 22
ENO	≥ 20	≥ 17

[a]Antimicrobial abbreviations: TET; tetracycline, FFC; florfenicol, SXT; sulfamethoxazole-trimethoprim, NA; nalidixic acid, ENO; enrofloxacin

[b]Isolates with a zone diameter less than the ECV indicated are considered non-susceptible.

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General discussion

The principle objective of this project was to generate information concerning AMR in *Aeromonas* and *Vibrio* species derived from fish and seafood while evaluating the methods used at various steps, from bacterial identification to antimicrobial susceptibility testing, to evaluate the necessity of the inclusion of those genera into a national antimicrobial resistance surveillance program in Canada. *Aeromonas* and *Vibrio* are ubiquitous in the aquatic environment, have the capacity to cause disease in animals and humans, and possess a genetic basis for antimicrobial resistance which is shared by important human pathogens such as *E. coli*. Multiple authors have also described their capacity for inter and intra-genic transfer of resistance determinants [4, 6, 39, 84]. Several publications describe the presence of *E. coli* and another human pathogen, *Salmonella,* in seafood [287-290]. Bacteriological isolation performed in parallel to this study indicated an overall prevalence in seafood of 27.2% and 2.5% for *E. coli* and *Salmonella,* respectively [287-290]. However, these genera are not considered as being commensals in the aquatic environment as are *Aeromonas* and *Vibrio*, their presence being rather a consequence of fecal contamination of either the culture methods or more likely the processing and retail chain [291, 292]. Therefore, the choice of examining *Vibrio* and *Aeromonas* in this study was made not only based on the genetic promiscuity of AMR genes and direct impact on human health, but also due to its presence in the aquaculture environment where AMR may be selected for by aquaculture practices, particularly in those countries where antimicrobial usage is poorly regulated [3].

Identification of Aeromonas and Vibrio spp.

Aeromonas and *Vibrio* were members of the same family until 1986 when they were separated by Colwell following analysis of ribosomal DNA sequencing and DNA/DNA hybridization results [9]. Both genera of bacteria, therefore, share many qualities including morphological, growth and habitat characteristics. Detailed biochemical identification keys for *Aeromonas* and *Vibrio* species have been published by Alsina (1994) and Abbott (2003) and various commercial systems have been used for definitive identification. [293, 294]. The advantages of biochemical characterisation is its relative simplicity, rapidity and the capacity to utilise these techniques with a minimum of laboratory equipment and incorporation into a surveillance program would likely be less complicated than a series of biomolecular manipulations. However, they are biochemically pleiomorphic, and while

identifying bacteria to the genus level can be accomplished relatively easily, speciation with biochemical tests can be extremely challenging and confusion between the two genera remains a problem for routine identification in diagnostic laboratories [295-298]. The use of biochemical screening tests before identification can help orient the identification, but final identification to the species level seems need to be confirmed [299, 300]. Identification in this study was attained in a stepwise method starting with five screening tests used to identify presumptive *Aeromonas* sp. and *Vibrio* sp. isolates including: growth on selective media (Aeromonas agar/TCBS), triple sugar iron agar (TSI), oxidase, Gram stain, and mobility. This was followed by biochemical identification and then confirmed using a genetic technique. Several commercial biochemical panels have been applied to the identification of *Aeromonas* and *Vibrio* species and some examples include the API-20 system (bioMérieux), Biolog (Biolog inc.), or Vitek2 (bioMérieux) [301, 302]. These systems use a variable number of miniaturized tests tubes or microwells containing different reagents which determine the metabolic capabilities of bacteria. When these results are compared with a database, the genus and species of the bacteria examined is identified. The Vitek2 was selected here principally because of its availability and extensive battery of biochemical tests. Multiple biomolecular techniques have been developed to help in the speciation of *Aeromonas* and *Vibrio* including: sequencing of housekeeping genes, restriction fragment length polymorphism of selected genes, rep-PCR methods and whole protein analysis [303-307]. In this study, initial attempts were made to speciate *Aeromonas* and *Vibrio* by sequencing an approximately 500 bp fragment of the 16S rDNA as described by Giovannani [308]. However initial trials failed to discriminate isolates in the Genbank database. It is possible that the fragment did not cover a variable region of 16S rDNA permitting identification or perhaps sequencing of the whole gene would have been necessary, as other authors seem to be able to differentiate between isolates and species with success [271, 309, 310]. Following recommendations by Dr Antonella Demarta from the Istituto Cantonale di Batteriologia, Bellinzona, Switzerland, the amplification of the housekeeping *rpoB* gene followed by sequencing, was utilized for definitive speciation of the isolates [269]. The choice of this gene for bacterial identification is also echoed by Mollet (1997) [311]. This choice was verified by the identification of four ATCC strains of *Aeromonas* and *Vibrio*, and in certain cases, the analysis of the *rpoB* sequences permitted identification of not only the species, but the specific ATCC control strain used within the Genbank repository. Two hundred and twelve

and 182 isolates were identified as *Aeromonas* sp. and *Vibrio* sp. respectively by the initial biochemical screening tests previously described, followed by confirmatory *rpoB* and Vitek2® analysis.

The Kappa coefficients for agreement between *rpoB* and Vitek2® were quite low and indicate slight or no agreement between the two tests especially for *Vibrio* (Annex 9). If *rpoB* sequencing were to be considered as the gold standard for identification, when comparing the two identification techniques, the sensitivity (Se) and the specificity (Sp) of the Vitek2® identification can be calculated.

The Se for *Aeromonas* genera identification was quite good at 96%, and is similar to that reported for Gram-negative bacteria by other authors, whereas the specificity would have allowed 57% false positive results [312, 313]. The Se and Sp for speciation of *Aeromonas* were both quite low, and would have resulted in 88% false positive (identifying as *Aeromonas* when it was not) and 68% false negative (identifying as other than *Aeromonas* when it was) results respectively. The Se and Sp values for *Vibrio* identification were 40% false positive and 29% false negative at the genus level and 76% false positive and 29% false negative at the species level. These results are in general higher than that for *Aeromonas* with the exception of *Aeromonas* genera Se (96%).

Other authors have described poor performance of biochemical methods as compared to genetic identification. Borrel (1997) found a 72% agreement at the species level when RFLP analysis of the 16S rDNA was compared with biochemical identification scheme based on Abbotts (2003) [294, 300] and when Castro-Escarpulli's (2003) compared Vitek2 results with the same biomolecular technique, the author found that only 28.5% were speciated correctly, if we accept the genetic technique as gold standard. In a study by Sanjuan (2009) which compared different biochemical identification systems and their capacity to identify confirmed set of *Vibrio vulnificus* isolates, he demonstrated that different API® test strips, API20NE® and API20E®, correctly identified 0 and 60% of the isolates, respectively, whereas Biolog® succeeded in identification of 84% of the isolates [301]. Even though biochemical diagnostic tests have likely evolved since the publication of these studies, the results are similar to what was found here in that, identification to the species level using biochemical methods appears to be unreliable.

Although the sequencing of *rpoB* for speciation is a commonly reported technique in the literature and functioned well here with control strains, it was not validated as a gold standard in this project, and should be, before using as a definitive identification tool. If

however, speciation is a goal, then presumptive identification of isolates using screening tests should be followed by a definitive biomolecular technique, which is a sentiment echoed by other authors [300, 306].

Susceptibility testing of Aeromonas and Vibrio isolates

Since the first recognition of *Aeromonads* resistant to sulfonamides by Snieszko and Bullock in 1957, the presence of AMR in these bacteria in aquaculture, its environment and in aquacultured animals worldwide has been well documented [8, 91, 161, 173, 254, 314-316]. Although this study examined more closely those classes of antimicrobials commonly used in aquaculture (tetracyclines, potentiated sulfonamides, phenicols (florfenicol) and quinolones), phenotypic resistance to all major classes of antimicrobials have been described for *Aeromonas* and *Vibrio* [106, 136, 157, 256, 257, 317].

There are currently no universally accepted epidemiologic cut-off values (ECV's) which have been derived specifically for the *Aeromonas* and *Vibrio* genera. Those that are available have been adapted by CLSI (Clinical and Laboratory Standards Institute) from breakpoints used for *Enterobacteriaceae*, and in consideration of this work, only those concerning sulfamethoxazole-trimethoprim, tetracycline and the quinolones (enrofloxacin) were of interest as they relate to antimicrobials used in aquaculture. Antimicrobial susceptibility testing methods have been proposed by CLSI for aquatic bacteria, but not interpretive criteria [261, 262]. According to CLSI, development of interpretive criteria requires large datasets (300+), rigorous adherence to standardised conditions including control strain measurements, clinical validation and an assumption that MIC measurements are gold standard from which acceptable error is estimated [264]. Our datasets contained 199 *Aeromonas* and 175 *Vibrio* confirmed isolates, and used an incubation temperature of 28°C rather than 35°C as with CLSI published breakpoints (M45-A). Additionally, there is currently a lack of control strain measurements for mobile *Aeromonads* and *Vibrio*, all making interpretive criteria development difficult [263]. To circumvent these limitations, Normalised Resistance Interpretation (NRI), based on disk diffusion distributions of the experimental population [267, 274] was used to evaluate susceptibility. This method, is internally calibrated using the high zone side of the susceptible peak of isolates of the experimental population and is useful when evaluating rarely isolated organisms where numbers may not permit traditional ECV calculation [286]. Even with as few as 10 isolates, susceptibility interpretation could be improved using this technique [286]. When

the bacterial populations were evaluated using the NRI-ECV's, overall resistance was low, with resistance to tetracycline being the highest for both *Aeromonas* and *Vibrio* (see Article 1 and Annex 10). Tetracyclines were among the earliest antimicrobials available for use in aquaculture, and chronic usage may be related to this prevalent phenotype. A recent survey of aquaculture-allied professional (Tusevlak et al, in prep) indicated that tetracycline would still be the most frequently used antimicrobial world-wide, all species confounded [318]. In contrast to our results, several recent publications concerning resistance in marketed seafood show higher levels of resistance to potentiated sulfonamides, tetracyclines and quinolones [302, 319-322]. This discrepancy may have many sources. If cut-off values used for susceptibility interpretation are erroneous, over or under estimation of true AMR prevalence may be the result, as discussed previously. Additionally, the identification of the bacterial isolates could be called into question in light of the results previously discussed. Other obvious explanations for the different AMR prevalences observed certainly include differences in the seafood species investigated, seafood of various origin (country or region, aquaculture versus wild capture, husbandry techniques, etc.), or a biased sampling scheme.

When a sampling scheme requires retaining more than one isolate per sample, adjusted measures of prevalence may be required to avoid overestimating AMR prevalence. In this project, a duplication of AMR phenotypes in isolates of *Aeromonas* or *Vibrio* recovered from the same sample were investigated using rep-PCR (Novakova, 2009) to assess for multiple isolates of the same strain. Nonsusceptible *Aeromonas* isolates were identified in 21 fish/seafood samples. In six of the samples the two isolates retained demonstrated the same AMR phenotype. According to rep-PCR analysis, the isolate pairs from four of the 21 samples were clonal. Similarly, for *Vibrio*, clonal pairs were found in three of 15 fish/seafood samples where the isolate pairs selected exhibited the same AMR phenotype. In consideration our data, if only one isolate was selected for identification and AMR determination and in 50 percent of the cases, (a coin toss), the susceptible isolate was chosen over that presented a resistant phenotype, the overall prevalence of samples exhibiting some type of AMR would drop from 17.2% to 14% for *Aeromonas* and 18% to 10% for *Vibrio*.

The sampling of seafood for this project concentrated principally on shrimp and salmon, although small quantities of other finfish such as trout and tilapia were also taken when salmon was not available. The large scale of geographical origine of the sample, and

the eventuality of manipulation associated cross contamination, may participates to explain the flora on the samples. An effort was made to obtain fresh non-frozen seafood so as to avoid microfloral changes associated with freezing [323]. Within the context of our sampling, 85% of the sampled shrimp were sold as frozen or thawed products, whereas for sampled finfish, only 20% was sold frozen, with 12 and 20% of these products having an unknown preservation status, respectively. The freeze/thaw stress which may occur during transport and store display has been shown to result in curing of plasmids carrying antimicrobial resistance determinants [324, 325]. A decreased resistance to rifampicin was also noted in *Campylobacter jejuni* following freezing due to purported changes in the bacterial membrane [326]. Therefore preservation by freezing could translate to changes in AMR genotypes and phenotypes and may partially explain the low AMR prevalence identified in this work. In contrast however, Escarpulli (2003) was able to demonstrate high levels of resistance to sulfamethoxazole-trimethoprim (49%), tetracycline (44%) and ciprofloxacine (42%) in *Aeromonas* sp. a study of frozen tilapia in Mexico [302]. Therefore, from published information it is difficult to define the importance of freezing on AMR prevalence.

The quality and safety of transported fresh and/or frozen fish and seafood is ensured by the use of temperature controlled supply chains (or “cold chains”) [327]. Failures in this chain may favour the growth of certain *Aeromonas* and *Vibrio* species identified in this study which are capable of growth at temperatures ranging from 0-45°C [10]. These psychrophilic or mesophilic bacteria may increase in numbers in food products, even though their initial concentrations may have been negligible [328, 329]. This was examined by Begum (2010), where the growth of *Pseudomonas*, a bacterial indicator of spoilage, was more frequent in market conditions where there were poor handling, improper storage and sanitary conditions as opposed to modern supermarkets where quality controls are in place.

***Identification of resistance genes in* Aeromonas *and* Vibrio**

In the course of this project the detection of resistance determinants in isolates classified as non-susceptible using the NRI-ECV was achieved using PCR and microarray analysis. The advantage of this approach was the combination of the sensitivity of PCR with the wide spectrum of AMR probes present on the microarray, and interesting differences were noted. As mentioned previously, tetracycline resistance was the most

common phenotype identified for both *Aeromonas* and *Vibrio*. Among 13 *Aeromonas* isolates carrying *tet*(E) identified by microarray analysis, only 46% were identified by PCR and repetition of the simple PCR for *tet*(E) did not detect those genes detected by microarray. In contrast, the presence of *floR*, *sul1* and *intI1* in *Aeromonas* and *sul2* in *Vibrio* were signalled by PCR, but were not found with the microarray. Differences in gene detection by both methods is likely associated with the variability of gene sequences in *Aeromonas*/*Vibrio* and differences between these species and *E. coli*, the species for which the microarray was developed.

Genes in the *bla* family were among the most commonly identified by microarray analysis (Annex 12). A high level of β-lactam resistance is common in *Aeromonas* due to chromosomally located inducible β-lactamases and this was no different in the current study [330]. A resistance phenotype that would normally be associated with an acquired β-lactamase gene would be masked by this innate resistance. A similar high level of phenotypic resistance in *Vibrio* isolates was also recognized which would suggest a similar resistance mechanism. Although β-lactam antimicrobials are not commonly used in the treatment of diseased aquaculture organisms except in the case of certain Gram-positive pathogens, this innate resistance becomes important when discussing bacterial genera of the aquatic environment acting as a reservoir of resistance determinants. For example, the β-lactamase classes B, C and D are commonly associated with innate resistant in *Aeromonas* [48]. The phenotypic resistance conferred by these chromosomally located genes may mask the presence of mobile genetic resistance elements which code for β-lactamases with similar activity. When selected *Aeromonas* and *Vibrio* isolates were subjected to microarray analysis, several putative mobile β-lactamase genes were identified including $bla_{\text{VIM-2}}$, $bla_{\text{FOX-2}}$, bla_{TEM}, and bla_{SME1}, some being novel variants for these genera. Their genomic localisation was not investigated, but these are commonly associated with class 1 integrons or other mobile gene platforms [135, 153, 331, 332]. In seven *Aeromonas* isolates, decreased susceptibility to ampicillin (AM), ampicillin-clavulanic acid (AmC) and cefoxitin (FOX) was associated with the presence of a bla_{VIM2}/bla_{SME1} combination, bla_{FOX2} or bla_{TEM}, whereas 3 isolates containing either bla_{TEM} or $bla_{vim2}/bla_{SME}/bla_{TEM}$ demonstrated a phenotype of decreased susceptibility to AM/AmC. Decreased susceptibility to AM/AmC/FOX and ceftriaxone (CRO) was identified in two *Vibrio* isolates, the presence of where bla_{VIM2}/bla_{SME} could explain the extended spectrum of β-lactamase activity. In *Aeromonas*, the phenotypes could be explained simply by the expression of chromosomal

β-lactamases, even though putative mobile genes were identified. Therefore, innate resistance does not seem to be an impediment for the acquisition mobile resistance determinants and may in fact hide the importance of these genera as gene reservoirs. The use of the microarray platform containing multiple AMR gene probes allowed the identification of important genes which would not have been feasible with PCR. In this case, it would have required prior knowledge of the gene likely present and testing of all isolates phenotypically resistant to the beta-lactams, which with these genera, would have been onerous.

Conclusion

Aeromonas and *Vibrio* are ubiquitous aquatic organisms, easily cultured, can cause diseases in humans and are known carriers of resistance genes. Preliminary identification of isolates to the genus level can be attained with a relatively high level of exactitude using a simple battery of tests and selective media. If definitive species identification is important, confirmation using one or a combination of biomolecular techniques is recommended. In the scope of a surveillance program, those isolates with phenotypic resistance patterns of concern and having the appropriate results for the five biochemical screening tests could then be speciated with genetic methods.

The use of NRI to establish epidemiologic cut-off values was validated here by the presence of resistance genes and agreement between this method and the population distribution and gene presence was excellent. Minimal disagreement was evident between NRI-ECV's, population distribution and/or gene presence for potentiated sulfa. This could likely be minimized for sulfamethoxazole-trimethoprim, by utilizing single antimicrobial agents for NRI calculation. The identification of isolates with partial resistance to SXT (either trimethoprim or sulphonamide resistance), would help identify those where continual SXT therapy would more rapidly result in resistance selection.

There was low occurrence (< 10%) of resistance to antimicrobials in both *Aeromonas* and *Vibrio* spp. isolated from fish and seafood, which seems to be in contrast with published literature concerning AMR in seafood and their aquaculture environment. In light of the low AMR prevalence and complexity of bacterial identification, periodic sampling and assessment of AMR in salmon and shrimps in rotation with other type of seafood, may be more appropriate and cost-effective than routine AMR surveillance of salmon and shrimp as is performed for other commodities in Canada. Finally, the evaluation of resistant isolates for AMR genes using two techniques, simple PCR and microarray, permitted the corroboration of results in some cases and the identification of novel resistance determinants.

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Annex 1: β-lactamase classification

Ambler[a]	Bush[b]	Enzyme type (s)	Substate	Inhibited by	Representative enzymes
A	2a	Penicillinases	penicillins	CA[c]	(Gram positive bacteria)
A	2b	Restricted-spectrum β-lactamase	penicillins and cephalosporins	CA	
A	2be	Extended spectrum β-lactamase	penicillins, narrow and extended-spectrum cephalosporins, monobactams	CA	TEM-3-26, SHV-2-6
A	2br	Inhibitor resistant β-lactamase	penicillins	-	TEM-30-36, TRC-1
A	2c	Carbenicillinase	penicillins, carbenicillin	CA	PSE-1, 2 & 3, CARB
A	2e	Cephalosporinase	cephalosporins	CA	Inducible cesphalosporinases from *Proteus vulgaris*
A	2f	Carbapenemase	penicillins, carbapenems, , cephalosporins, monobactams, sometimes extended-spectrum β-lactams	±CA	NMC-1, SME-1
B	3	Carbapenemase (metallo β-lactamase)	most β-lactams, carbapenems, and cephalosporins (4th gen.)	EDTA[d]	IMP-1-13, VIM-1-7, SPM-1
C	1	Cephalosporinase	penicillins, cephalosporins, cephamycins, and monobactams	-	AmpC, CMY, MIR, FOX
D	2d	Narrow spectrum penicillinase, ESBL, carbapenemase	penicillins, cloxacillin, extended spectrum β-lactams , carbapenems , at times monbactams, and cephalosporins (4th gen.)	CA	OXA (many variants), PSE
*	4	Penicillinase	Penicillins	-	derived from *Pseudomonas cepacia*

[a]Ambler β-lactamase classification adapted from Poole (2004)
[b]Bush-Jacoby-Medeiros β-lactamase classification adapted from Bush (1995)
[c]Clavulanic acid
[d] Ethylenediamine-tetra-acetic acid

Annex 2: MIC's of mobile β-lactamases reported in *Aeromonas* and *Vibrio*[a] (in µg/ml)

Antimicrobials[a]	PER-1 [215]	CTX-M [142]	PER-2,TEM-1 [142]	IMP-19 [152]	VIM-4 [216]	TEM-24 [135]	OXA-1 [137]	OXA-2 [140]	SAR-1 [141]	Carb-7 [146]	AmpC [149]
Ampicillin	-	**>1024**	**>1024**	-	>256	-	-	≥50	**>1000**	**256**	**1024**
Ampicillin/ sulbactam	-	-	-	-	-	-	-	-	-	**16**	-
Amoxicillin	>256	-	-	-	-	>256	>32	-	-	-	-
Amoxicillin - clavulanic acid	8	**4-8**	**16**	-	-	8	-	-	-	-	-
Piperacillin	-	-	-	256	-	32	-	-	-	**32**	**256**
Piperacillin/tazobactam	-	**0.25**	**1**	-	>256	1	-	-	-	-	-
Carbenicillin	-	-	-	-	-	-	-	-	**>1000**	-	**256**
Ticarcillin	>256	**128**	**128**	2049	-	>256	>256	-	-	**512**	-
Ticarcillin/CA	128	-	-	-	-	32	-	-	-	**8**	-
Oxacillin	-	-	-	-	-	-	-	-	-		**32**
Ceftiofur	-	-	-	-	-	-	-	-	-	-	-
Cephalothin	>64	**128-512**	**32**	-	-	-	-	-	-	**2**	-
Cephaloridine	-	-	-	-	-	-	-	-	-	-	**16**
Cephradine	-	-	-	-	-	-	-	-	**16**	-	-
Clavaluanic acid (CA)	-	-	-	512	-	-	-	-	**16**	-	-
Cefazoline	-	-	-	512	-	-	-	-	-	-	-
Cefpirome	-	-	-	-	-	0.5	-	-	-	-	-
Cefoxitin	2	**4-8**	**4**	1024	>256	16	-	-	-	**8**	-
Tazobactam	-	-	-	1024	-		-	-	-	-	-
Ceftazidime (CAZ)	>256	**0.13-0.25**	**8**	1024	>256	32	<0.06	-	-	-	**2**
CAZ-CA	-	**0.13**	**0.13**	-	-	0.06	-	-	-	-	-
Cefotaxime (CTX)	>32	**2-4**	**0.5**	-	>256	0.5	<0.06	-	-	-	**2**
CTX-CA	-	**<0.03**	**<0.03**	-	-	-	-	-	-	-	-
Ceftriaxone	-	-	-	-	>256	-	-	-	-	-	-
Cefepime (FEP)	4	**2**	**1**	-	32	0.25	<0.06	-	-	-	-
FEP-CA	-	**<0.50**	**<0.50**	-	-	≤0.03	-	-	-	-	-
Aztreonam	-	**8**	**128**	8	-	-	-	-	-	-	-
Imipenem	0.25	**1**	**1**	16	32	4	<0.06	-	-	-	-
Meropenem	-	-	-	1	-	-	-	-	-	-	-

[a]MIC's associated with *Vibrio* sp. are annotated in **bold red. Numbers in [] correspond to reference**

Annex 3: Tetracycline resistance genes identified in *Aeromonas* and *Vibrio*

Genus	**Efflux proteins**	**Ribosomal protection proteins**	**Enzymatic proteins**
Aeromonas	*tet*(A)(B)(C)(D)(E)(31)(Y)	-	*tet*(34)
Vibrio	*tet*(A)(B)(C)(D)(E)(G)(35)	*tet*(M)	*tet*(34)

Adapted from Roberts [156]

Annex 4: MIC's associated with the tetracycline resistance genes of *Aeromonas* spp. and *Vibrio*[a] spp.

Resistance gene	**≤4 µg/mL**	**≤8 µg/mL**	**≤16 µg/mL**	**≤32 µg/mL**	**≤ or >64 µg/mL**	**≤128 µg/mL**	**≥256 µg/mL**
***tet*(A)**	**[185]**	[7, 185]	[160] [164, 185]	[85, 161] [164, 185]	[159, 185]	[182]	[91]
***tet*(B)**						**[166, 182, 183] [184]**	**[184]**
***tet*(C)**				[177]	[177] [159]		
tet(D)		[7]		[170]		**[165, 166, 183]**	
***tet*(E)**		[7]	[91, 160] [164]	[161] [170] [164] **[162]**		[91]	[91]
***tet*(G**					**[184]**	**[182]**	**[184]**
***tet*(31/C)**					[177]		
***tet*(34)**							**[174]**

***tet*(35/tetA)**						**[172]**	
***tet*(M)**						**[171]**	**[171]**
***tet*(34/A)**			**[172]**	**[172]**		[**172**]	
***tet*(B,D,M)*;* (A,D,M);(A,B,M) (DEM)**						[165, 217] [164]	
***tet*(A/B)**						**[166, 183]**	
***tet*(A/C)**					[177] [159]	[177]	
***tet*(A/B/D)**						**[166]**	
***tet*(D/E)**						**[166]**	
***tet*(A/E)**		[7]	[160]	[161, 164]	[164]	**[166]**	
***tet*(E/M)**			[164]		[164]		
***tet*(A/M)**			[164]	[164]			
***tet*(D/M)**		[164]				[164]	
***tet*(B/M)**		**[173]**	**[173]**	**[173]**	**[173]**	**[173]**	**[173]**
***tet*(Y)**			[179]				

[a]MIC's associated with *Vibrio* sp. are annotated in **bold** red. **. Numbers in [] correspond to reference**

Annex 5: *Aeromonas* MIC's for *nalidixic acid*, oxalinic acid and ciprofloxacin/enrofloxacin, and associated QRDR mutations

Resistance determinant	≤0.03 µg/ml	≤0.06 µg/ml	≤0.12 g/ml	≤0.25 µg/ml	≤0.5 µg/ml	≤1 µg/ml	≤2 µg/ml	≤4 µg/ml	≤8 µg/ml	≤16 µg/ml	≤32 µg/ml	64 µg/ml	128 µg/ml	≥256 µg/ml
Wild type strains	[222] [62] [62] [228]		[222] [222] *[228]*	*[224]* [224]										
gyrA Se83→Ile				[222] [224]	[225]	[222] [222]	[222]	[225] [222]	[222] [224]			[222] [224]	*[222]*	*[222, 224]*
gyrA Se83→Arg Se83→Val			[222]		[222]	[222]		[222]					*[222]*	*[224]*
gyrA Asp87→Asn				[62]	[62]	[62]	[62]	[62]	[62]					
gyrA Se83→Ile, Ala67→Gly Leu92→Met					[225] [224]	[225] [224]		*[225]*						
gyrA/parC					[228] [224]	[222]	[222]	[222] [228]	[222] [228] [224]		[222, 228]		*[222]* [224]	[222, 224, 228] [224]
qnrS2					[137]		*[137]*	[247]			[247]			*[247]*

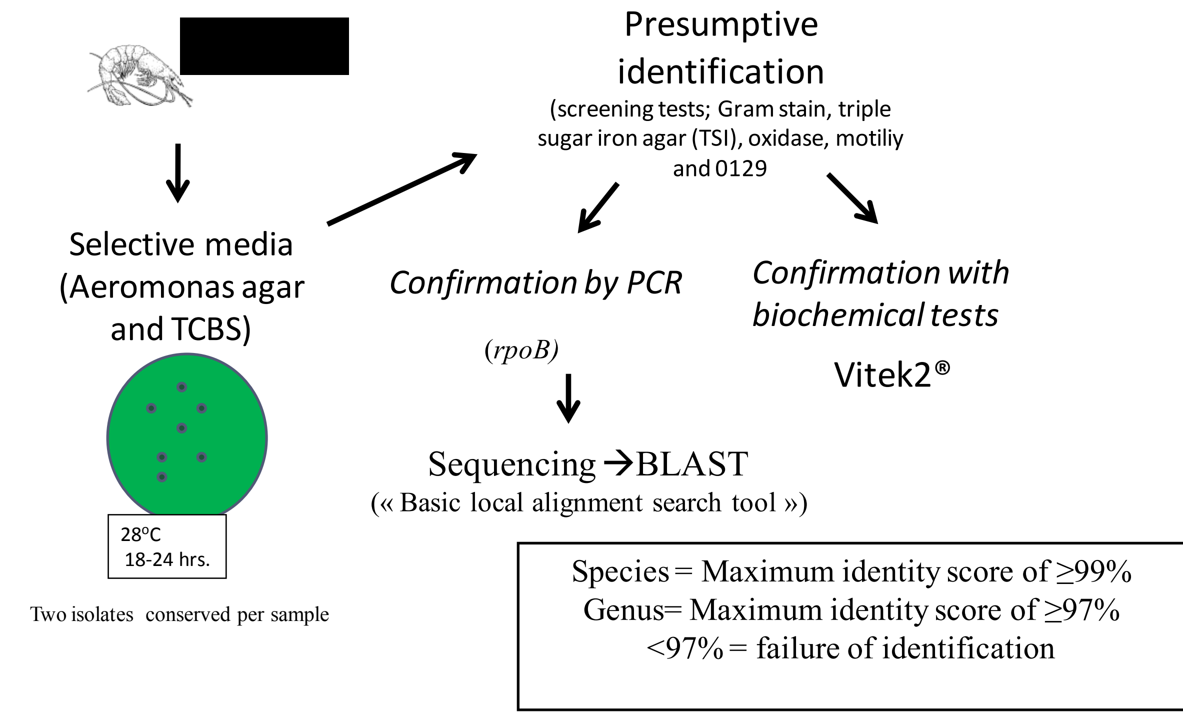
Note: MIC references for naladixic acid and ciprofloxacin/enrofloxacin are underscored in red and italic and black respectively. MIC references for oxalinic acid are not underscored.

Annex 6: *Vibrio* MIC's for *nalidixic acid*, oxalinic acid and ciprofloxacin/enrofloxacin and associated QRDR mutations

Resistance determinant	≤ 0.03 µg/ml	≤ 0.06 µg/ml	≤ 0.12 g/ml	≤ 0.25 µg/ml	≤ 0.5 µg/ml	≤ 1 µg/ml	≤ 2 µg/ml	≤ 4 µg/ml	≤ 8 µg/ml	≤ 16 µg/ml	≤ 32 µg/ml	≤ 64 µg/ml	≤ 128 µg/ml	≤ 256 ≥µg/ml
Wild type strains	*[230, 232]* [233] [234]	[232] [233]	[233] [232]	*[232]* [231, 233]	*[232]* [233] [231, 234]	*[230, 232]*								
gyrA (Se83→Ile)		[230]		[230]	[230]	*[232]*	[232]		[230, 231, 233, 234]	*[232]* [230, 234]	*[232]*	*[232, 234]*		
gyrA (Se83→Arg)						[232]	[232]					*[232]*		
gyrA (Asp87→Tyr)					[234]		[234]	[234]	[234]					
gyrA/gyrB						[232]		[232]					[232]	
gyrA/gyrB/parC						[232]				[232]				*[232]*
gyrA/parC						[230, 232]	[232]		[232] [233, 234]	[230, 233]	[233]	*[231, 232, 234]*	*[230-232]*	[231, 234]

Note: MIC references for naladixic acid and ciprofloxacin/enrofloxacin are underscored in red and italic and black respectively. MIC references for oxalinic acid are not underscored.

Annexe 7: Identification Scheme for *Aeromonas* and *Vibrio*



Daoust, P.Y., Health Canada, culture protocols (non-published)

Annexe 8: Sensitivity and specificity for Vitek2® identification of *Aeromonas* to the genus and species level using an *rpoB* gold standard

	rpoB Aeromonas +	*rpoB Aeromonas* -	Senstivity and specificity	Kappa coefficient (Confidence intervals)
Vitek2 - genus *Aeromonas* sp. +	190	8	Sensitivity (genus); 96%	0.38 (0.176-0.600)
Vitek2 - genus *Aeromonas* sp. -	8	6	Specificity (genus); 43%	
Vitek2 - species *Aeromonas* sp. +	22	23	Sensitivity (species); 12%	-0.214 (-0.268-0.148)
Vitek2 - species *Aeromonas* sp. -	156	11	Specificity (species); 32%	

Annexe 9: Sensitivity and specificity for Vitek2® identification of *Vibrio* to the genus and species level using an *rpoB* gold standard

	rpoB Vibrio +	*rpoB Vibrio* -	Senstivity and specificity	Kappa coefficient (Confidence intervals)
Vitek2 - genus *Vibrio* sp. +	104	2	Sensitivity (genus); 60%	0.056 (¯0.009-0.094)
Vitek2 - genus *Vibrio* sp. -	69	5	Specificity (genus); 71%	
Vitek2 - species *Vibrio species* +	42	2	Sensitivity (species); 24%	¯0.004 ¯0.041-0.017)
Vitek2 - species *Vibrio species* -	131	5	Specificity (species); 71%	

Annex 10: Prevalence of resistance phenotypes in *Aeromonas* and *Vibrio*

Resistance phenotype	***Aeromonas (n=199)***	***Vibrio* (n=175)**
Fluoroquinolone	2.5%	0%
Quinolone	3.5%	2.9%
Florfenciol	0.5%	0%
Sulfamethoxazole-trimethoprim	1.0%	4.6%
Tetracycline	12%	5.7%

Annex 11: Differences in antimicrobial resistance gene detection noted between microarray and simple PCR

Antimicrobial family	AMR gene	Aeromonas		Vibrio	
		PCR	Microarray	PCR	Microarray
Folic acid inhibitors	*sul1*	2[a]	0	-[b]	-
	sul2	-	-	6	0
Phenicols	*floR*	1	0	-	-
Tetracyclines	*tet*(A)	3	3	0	2
	tet(B)	-	-	4	2
	tet(D)	0	1	-	-
	tet(E)	6	13	1	1
Class 1 integron	*intI1*	2	0	0	0

[a]Numbers of isolates containing indicated resistance gene
[b]Hypens indicate comparisons were not possible

Annex 12: Antimicrobial resistance genes identified by microarray

Antimicrobial family	AMR genes	*Aeromonas* (n=18)	*Vibrio* (n=12)
Aminoglycosides	*strA/strB*	2 [a]	2 [a]
	aadA1	1	0
β-lactams	bla_{VIM2}	3	1
	bla_{FOX2}	21	0
	bla_{SME}	3	1
	bla_{TEM}	5	0
Inhibitors of folic acid	*dhfr5*	3	1
	dhfr7	0	3
	dhfr16	1	0
Macrolides	*ereB*	0	4
	ereA2	2	0
Phenicols	*cat*	2	1
	cat2	1	0
	cat3	2	1
Tetracyclines	*tet*(A)	3	2
	tet(B)	0	2
	tet(D)	1	0
	tet(E)	13	1

[a]Numbers of isolates containing indicated resistance gene

Annex 13: Scientific article #2

Characterisation of antimicrobial resistance in *Aeromonas* spp. isolated from seafood produced and imported into Canada

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Abstract

Antimicrobial use in the aquaculture setting is generally considered low as compared to other types of animal production, however, where endemic disease requires frequent therapeutic intervention, or in countries where antimicrobial usage is poorly regulated, the quantities used may be considerably higher. This may lead to increased occurrence of antimicrobial resistance (AMR) in aquaculture products and increased human exposure. Little information is available concerning the occurrence of antimicrobial resistance in finfish and seafood available at the retail level in Canada. In this study, 216 *Aeromonas* isolates were cultured from 281 finfish and shrimp sampled within the framework of the Canadian Integrated Program for Antimicrobial Resistance Surveillance. Bacteria were identified using Vitek2® and confirmed by sequencing of the *rpoB* gene. Epidemiological cut-off values were estimated for the tetracyclines (TET), folic acid inhibitors (SXT), quinolones (ENO and NA) and florfenicol (FFC) using Normalised resistance interpretation of the disk diffusion data. Gene presence associated with resistance phenotypes was evaluated using PCR and microarray analysis. *Aeromonas* prevalence in finfish and shrimp was 58% and 22% respectively, where the *Aeromonas enchelaie/A. salmonicida* was the species most often identified. The prevalence of Non-Wild-Type (NWT) phenotypes/sample of the antimicrobials examined was: 0.5%, 2.5%, 3.5%, 1.0% and 12% for FFC, ENO, NA, SXT and TET respectively. The gene *tet*(E) (74%) and *tet*(A) (21%) were the only *tet* resistance determinants identified in TET-NWT isolates. In two SXT-NWT isolates *sul1* was found in combination with either *dfrA5* or *dfrA5/dfrA16*. Among six of seven isolates demonstrating a NWT phenotype for quinolones, single or dual mutations in *gyrA* were found ($gyrA_{\text{Se83-Ile}}$, $gyrA_{\text{Se83-Ile}}$/$gyrA_{\text{Met92-Leu}}$. $gyrA_{\text{Se83-Val}}$) and in certain cases in combination with substitutions in *parC* ($parC_{\text{Se80-Ile}}$ $parC_{\text{Ala85-Thr}}$, $parC_{\text{Pro98-Ser}}$). The *floR* gene was found in the single FFC-NWT isolate. Plasmid profiling and hybridization revealed that the resistance determinants *tet*, *sul1*, *floR* and *intI1*, were on plasmids ranging in size from 9.5 kb to 20 kb. Overall prevalence of AMR in seafood identified in this study was low and the majority of the AMR phenotypes could be explained by the presence of resistance determinants or gene mutations.

1.0 Introduction

Aquaculture is the fastest growing agri-industry in the world. It currently accounts for 46% of the world fish supply and is poised to overtake wild fisheries as the primary fish source (FAO, 2010). Antimicrobial exposure in the aquaculture setting is generally considered low as compared to other types of animal production, however, in countries where endemic disease requires frequent therapeutic intervention, or in countries where antimicrobial usage is poorly regulated, the quantities used may be considerably higher (FAO/OIE/WHO, 2006). The majority of antimicrobial therapy in aquatic production is administered orally, therefore, in addition to antimicrobial exposure of the bacterial population causing disease, the bacterial flora on fish and in the surrounding environment are also exposed.

Antimicrobial resistance (AMR) has been reported in many species of aquatic bacteria in freshwater and marine environments (Akinbowale et al., 2006; Biyela et al., 2004; Goni-Urriza et al., 2000). *Aeromonas* species are gram-negative, mobile, facultative anaerobic bacteria that are present in aquatic systems worldwide and are frequently found in fish, shellfish and other seafoods (Davies et al., 2001; Hanninen et al., 1997). They are important bacterial pathogens of cultured fish and certain *Aeromonas* species are recognized as zoonotic pathogens. Food poisoning and gastroenteric infections are the most commonly encountered disease in humans and are treated symptomatically, whereas severe systemic infections may require the administration of antimicrobials (Daskalov, 2006; Hsiao et al., 2008). In these cases, AMR could limit the success of antimicrobial therapy. Additionally, multiple laboratory studies have demonstrated that resistant determinants can be transferred between aquatic bacteria which are low pathogen risks to humans such as *Aeromonas salmonicida*, to more pathogenic *Enterobacteriaceae* (Kruse and Sorum, 1994; Schmidt et al., 2001; Sorum et al., 2003). If this exchange occurs, at what frequency and how it happens in the environment or in association with seafood has yet to be elucidated. Recent studies examing *Aeromonas* in retail seafood have shown elevated levels of AMR but have not examined the genetic basis for these phenotypes (Castro-Escarpulli et al., 2003; Kaskhedikar and Chhabra, 2010; Radu et al., 2003; Vivekanandhan et al., 2002). This study examines the antimicrobial resistance phenotypes of *Aeromonas* sp. isolated from

retail seafood in Canada, as well as their associated genetic determinants using MIC, disk diffusion, PCR and microarray techniques.

2.0 Materials and Methods

2.1 Sample collection and bacterial isolation

Two hundred and eighty one retail seafood samples (143 salmon, 128 shrimp, 6 trout and 4 tilapia) were obtained between the 20th of October, 2008 and the 15th of June 2009, within the sampling framework of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). They originated from five different Canadian regions including Québec, Ontario, the Maritimes, Saskatchewan, and British Columbia. Finfish samples taken included filets or steaks (skin-on or skin-off) whereas shrimp samples were submitted whole and the preservation of samples varied from fresh or thawed to frozen. Approximately 100 grams of each sample was placed individually in 7x12inch sterile "Stomacher" bags (VWR International, Mississauga, ON) with 225ml of alkaline peptone water (APW) and homogenized by hand for two minutes with subsequent incubation at 28°C for 18-24 hours. *Aeromonas* species were cultivated and selected by inoculating an *Aeromonas*-selective AA agar plate (*Aeromonas* medium base (Ryan) with ampicillin supplement, Oxoid, Cambridge, UK) with a loopful of the APW enrichment. Four green colonies with darker centers were selected and subcultured on tryptic soy agar (TSA)-Blood + 5% sheep blood for further testing.

2.2 Bacterial identification

Putative *Aeromonas* isolates were subjected to an initial panel of tests including: Gram stain, motility, oxidase, triple sugar iron (TSI) agar and 0129 susceptibility (2,4-diamino. 6,7-diisopropylpteridine phosphate, 150mg) (BD-BBL, Mississauga, Ontario). A putative *Aeromonas* sp. identification was given to those isolates found to be Gram-negative, motile, oxidase positive, resistant to 0129 and demonstrated an acid/acid or alkaline/acid reaction with the presence of gas and absence of H_2S on TSI slant. Two (commenataire PF : comment passe-t- on de 4 colonies à 2 isolats , critères de sélection?) isolates per positive sample were conserved when available in tryptic soy broth (TSB) + 50% glycerol at -80°C for further testing.

Two hundred and sixteen putative *Aeromonas* sp. isolates were then identified using the Vitek2® identification system following protocols outlined by the company. This identification was confirmed via amplification of the ribosomal polymerase subunit *rpoB* as previously described with some modifications (Kupfer et al., 2006). PCR amplification was performed in 25 µl containing; 2 µl 10X PCR buffer, 0.08 mM dNTPs, 0.2 mM $MgCl_2$, 10 pmol of opposing primers, 1.25U Taq polymerase (New England Biolabs, Pickering, Ontario) and from 50-100 ng of DNA template. A temperature of 56°C was used during the hybridization step. Amplicons were evaluated by visualization following migration of an agarose gel (1.7%) stained with ethidium bromide. An E-gel low range semi-quantitative ladder (Invitrogen Canada, Burlington, Ontario) was used as a comparative marker for product sizing, and *A. salmonicida* ATCC33658, *Aeromonas hydrophila* ATCC7966 and *Aeromonas caviae* ATCC15468 were used as controls. The same primers were used for subsequent sequencing reactions of the approximately 540 bp PCR product. Sequencing was performed by the Plate-forme d'analyses biomoléculaires (PAB), Université Laval. Identification was made using the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) (Altschul et al., 1990). Identification to the species level was defined as an *rpoB* sequence 'Maximum identity score' of $\geq$ 99% with that of strain sequences in GenBank whereas identification to the genus level was defined as those isolates having a 'Maximum identity score' of $\geq$ 97%. A score of lower than 97% was considered as a failure of identification (Drancourt et al., 2000).

2.3 Antimicrobial susceptibility testing

All 216 isolates were tested for antimicrobial susceptibility by broth microdilution (MIC) and by disc diffusion using methods for aquatic organisms published by CLSI (M42-A, M49-A). For MIC determination, the ARIS automated system of Sensititre™ (Trek™ Diagnostic System Ltd) with a custom aquatic plate was used, containing (MIC range in µg/ml in parenthesis): enrofloxacin (0.002-1), florfenicol (0.03-16), oxalinic acid (0.004-2), oxytetracycline (0.015-8) and sulfamethoxazole/trimethoprim (0.015/0.3-1/19). Antimicrobials evaluated by disk diffusion included (antimicrobial disc concentration in µg in parenthesis): enrofloxacin (5), florfenicol (30), nalidixic acid (30),

sulfamethoxazole/trimethoprim (23.75/1.25) and tetracycline (30) (BD-BBL, Mississauga, Ontario). Incubation temperature for all testing was 28°C. Quality control for both testing methods was performed using *Escherichia coli* ATCC 25922 and *A. salmonicida* ATCC 33658. The calculation of the Epidemiological Cut-Off Value (ECV) for the interpretation of susceptibility of isolates was accomplished using NRI of disk diffusion data as previously described (Kronvall, 2003; Kronvall et al., 2003). For the NRI calculations, peak values were established using four point rolling means, and a plot of seven probit values versus zone size was used to identify the means and standard distribution of the susceptible population. ECV's were set at 2.5 standard deviations from the mean. Non-susceptible isolates are considered as Non-Wild-Type (NWT) and susceptible isolates, Wild-Type (WT).

2.4 ERIC-PCR

All isolate pairs derived from one sample which demonstrated identical AMR patterns were evaluated for clonality using an ERIC-PCR with conditions as previously described (Novakova et al., 2009) with minor modifications. The amplification reaction was carried out in 25 µl containing: 2 µl of 10X PCR Buffer, 0.064mM dNTPs, 0.16mM $MgCl_2$, 50 pmol of opposing primers, 2 U of *Taq* DNA polymerase (NewEngland Biolabs, Pickering, Ontario) and 100-200 ng of template DNA. Ten µl of PCR product was subsequently evaluated by electrophoresis in a 1.5 % agarose gel for 120 min (7.3 V/cm) in TAE buffer (Tris base, acetic acid and EDTA). Gels were stained with ethidium bromide (1.0 mg/L) for 20 min, destained in deionized water for 20 min, and visualized under UV light. Image analysis of the obtained patterns was carried out using BioNumerics Version 6.0.1 software (Applied Maths, Austin, TX, USA). A clustering analysis was performed using the unweighted pair group method using arithmetic averages (UPGMA) based on the Pearson's correlation coefficient. An optimisation of 1% was allowed, and a threshold of 95% was used to identify bacterial clones.(attention asvez vous une référence qui justifie 95% comme seuil de clonalité)

2.5 Detection of antimicrobial resistance genes

PCR was used to detect individual AMR determinants including *tet*((A), (B), (C), (D) and

(E)), *florR*, *sul1*and *sul2*. A PCR followed by sequencing was used to detect *gyrA/parC* mutations and the primers used are presented in Table 1. Identical amplification reactions were used for *floR, sul1* and *sul2* with 2.0 µl of 10X PCR Buffer, 0.08 mM dNTPs, 0.25 mM $MgCl_2$, 10 pmol of opposing primers, 1 U of *Taq* DNA polymerase (NEB) and 50-100 ng of template DNA in a total volume of 20 µl. Briefly, the PCR reactions used were: *floR*: initial denaturation (94°C, 5 min), 35 polymerization cycles (94°C for 30 s, 60°C for 30 s and 72°C for 30 s), final elongation at 72°C for 7 min; *sul1*: initial denaturation (95°C, 5 min), 25 polymerization cycles (95°C for 30 s, 55°C for 30 s and 72°C for 40 s) final elongation at 72°C for 7 min; *sul2*: initial denaturation (95°C, 5 min), 35 polymerization cycles (95°C for 30 s, 62°C for 30 s and 72°C for 45 s) and final elongation at 72°C for 7 min. All *tet* genes ((A), (B), (C), (D) and (E)) were amplified using identical amplification reactions and PCR conditions. The PCRs consisted of 2.0 µl of 10X PCR Buffer, 0.08 mM dNTPs, 0.14 mM $MgCl_2$ 10 pmol of opposing primers, 1 U of *Taq* DNA polymerase (NEB) and 50-100 ng of template DNA, in a total volume of 20 µl. The PCR conditions included an initial denaturation step (95°C, 5 min), followed by 35 polymerization cycles (95°C for 30 s, 62°C for 30 s and 72°C for 45 s) and final elongation at 72°C for 7 min. Amplification of *qnrA*, *qnrB* and *qnrS* genes was undertaken utilizing a multiplex PCR as previously described (Gay et al., 2006). The *gyrA* and *parC* genes were amplified using conditions described previously using the *gyrm*1-2, *parm*1-2 primer pairs (Okuda et al., 1999). The same primers were used for subsequent sequencing reactions of the approximately 500 bp PCR product. Comparison to phenotypically susceptible isolates of the same genera was performed to characterize mutations in the quinolone resistance determining region (QRDR). Nineteen non-wild type isolates, were further examined for the presence of resistance genes using a microarray previously described by Bonnet (2009) (Bonnet et al., 2009).

2.6 Plasmid extraction and hybridization

Plasmid extraction was performed in selected isolates with confirmed AMR genes using the Plasmid Midi kit (Qiagen, Ontario, Canada) according to the manufacturer's specifications. Subsequently, plasmid extracts were subjected to electrophoresis in 0.7% agarose gel at 7.3 V/cm for 180 minutes followed by staining with ethidium bromide . The supercoiled

DNA ladder (Invitrogen Canada, Burlington, Ontario) and BacTracker BAC-Tracker™ Supercoiled DNA Ladder (Epicentre Biotechnologies, Madison, Wisconsin, USA) were used as molecular weight markers. Probes for Southern blot hybridization were generated for *tet*(A), *tet*(E), *tet*(B), *floR, sul1, sul2* and *intI1* using the PCR DIG probe synthesis kit (Roche-Scientific, Canada) according to the manufacturer's instructions. DNA from plasmid extractions was transferred to positively charged nylon membranes using a Vacuum Blotter Model 785 (Bio-Rad, Mississauga, Ontario, Canada) and revealed with the DIG Nucleic Acid Detection Kit (Roche-Scientific, Canada) as per manufacturer's instructions.

2.6 Statistics

Identification test performance (Kappa, sensitivity and specificity) was evaluated using the 2-way contingency analysis tool found at http://statpages.org.

3.0 Results

3.1 Bacterial isolation

Of the 281 seafood samples examined, 216 putative *Aeromonas* species were isolated from 174 (80.5%) and 42 (19.5%) of the finfish and shrimp samples respectively. Prevalence of *Aeromonas* in seafood based on the *rpoB* identification described below was 58% for finfish and 22% for shrimp. Comment expliquer une prévalence > après rpoB identification qui est présentée dans le matériel et méthode comme intervenant après VITEK2… modifier le M &M?

3.2 Bacterial identification

Among 216 presumptive *Aeromonas* spp. isolates, 199 were identified as *Aeromonas* sp. using the Vitek2® system. Among these, 142 (66.7%) were identified as *A. hydrophila/caviae*, 57 (26.7%) as *A. sobria*, 6 (2.8%) as *Vibrio* sp. and 8 (3.8%) as other species. Confirmation by sequencing of the *rpoB* gene identified 199 isolates as *Aeromonas*, and revealed a larger number of species including: *A. enchelaie/A. salmonicida*, 65 (30.2%); *A. sobria*, 28 (13.0%); *Aeromonas* spp., 30 (14.0%); *A. bestiarum*, 20 (9.3%); *A. molluscorum*, 18 (8.4%); *A. encheleia*, 17 (7.9%); *A.*

salmonicida, 12 (5.6%); *A. veronii*, 6 (2.8%); *A. enteropelogenes*, 2 (0.9%); *A. punctata*, 2 (0.9%); *A. media*, 1 (0.5%). Species other than *Aeromonas* accounted for 6.5% of the above population ou other species in aeromonas genus? Avez vous fait rpoB sur les 17 souches non Aeromonas par Vitek2?. Agreement Attention on ne peut calculer le Kappa que si il y a independance des jugements ce qui n'est pas le cas si on suit le M&M of identification of isolates between Vitek2® and *rpoB* was poor. Kappa coefficient values for agreement at the genus and species level as well as sensitivity and specificity using *rpoB* as the identification gold standard are shown in Table II.

3.3 Antimicrobial susceptibility testing

The distribution of inhibition zone diameters and MIC's for 199 *rpoB* confirmed *Aeromonas* sp. are presented in Figure 1. NWT isolates were identified in 21 samples examined. NWT phenotypes for more than one antimicrobial was noted in five isolates and included : TET/NA (*n*=4) and TET/SXT/FFC (*n*=1) phenotypes. TET was the most prevalent single NWT phenotype (*n*=15) followed by NA (*n*=3) and SXT (*n*=1). Prevalence of NWT phenotypes/sample of the antimicrobials examined was : 0.5%, 2.5%, 3.5%, 1.0% and 12% for FFC, ENO, NA, SXT and TET respectively.

3.4 Evaluation of clonality with ERIC-PCR

Non-susceptible *Aeromonas* isolates were identified in 21 fish/seafood samples and in six of the samples, the two isolates retained demonstrated the same NWT phenotype. According to rep-PCR analysis, the isolate pairs from four of these were culture replicates.

3.5 Detection of antimicrobial resistance genes

All NWT isolates were examined for the presence of resistance genes using PCR, and 19 isolates were further characterized using the microarray and to examine NWT phenotypes for which no resistance genes were identified. Genes detected by PCR and microarray are listed and contrasted in Tables III and IV. Genes coding for tetracycline NWT were detected by both PCR and microarray. The gene *tet*(E) was the most prevalent at 73.7% followed *tet*(A) at 21.1%. In two isolates, no AMR supporting genes were detected. The two NWT isolates identified for folic acid inhibitors were associated with the presence of

sul1 and either *dfrA5* or *dfrA5/dfrA16*. All but one of seven isolates demonstrating a NWT phenotype for quinolones were found to contain single or dual mutations in *gyrA* ($gyrA_{Se83\text{-}Ile}$, $gyrA_{Se83\text{-}Ile}$/$gyrA_{Met92\text{-}Leu}$, $gyrA_{Se83\text{-}Val}$). Certain isolates also harboured *parC* substitutions including $parC_{Se80\text{-}Ile}$ $parC_{Ala85\text{-}Thr}$, and $parC_{Pro98\text{-}Ser}$. The *floR* gene was detected in the single isolate demonstrating a NWT phenotype for florfenicol. The relationship of AMR phenotypes and genotypes are detailed in Table V.

3.6 Plasmid identification and gene carriage

The majority of the isolates examined contained multiple plasmids ranging from 2.5 to 30 kb in size. One isolate harboured a single plasmid of 9.5 kb. Those associated with the resistance determinants *tet*, *sul1*, *floR* and *intI1*, ranged in size from 9.5 – 20 kb (Table V).

4.0 Discussion and Conclusion

Aeromonas species were detected in 58% of the finfish and 16% On parle de 22% plus haut (abstratc et resultats) of the shrimp sampled in this study which is comparable to ranges published in the literature(Castro-Escarpulli et al., 2003; Radu et al., 2003; Vivekanandhan et al., 2002; Yucel and Balci, 2010). Bacterial identification in this study was based on biochemical screening followed by confirmation using a commercial biochemical panel (Vitek2®) and sequencing of the *rpoB* gene. Various authors have demonstrated difficulties of *Aeromonas* identification using biochemical methods and this appeared to be in agreement with the results of this study (Carson et al., 2001; Ormen et al., 2005). The Kappa coefficients for agreement between *rpoB* and Vitek2® were low at the genus and species level, 0.38 and -0.214 respectively même remarque sur la nécessaire indépendance préalable, and indicate slight or no agreement between the two. If *rpoB* sequencing were to be considered as the gold standard for identification, the Vitek2® performs better at the genus level, with a sensitivity (Se) of 96% and specificity (Sp) of 43%, whereas the Se/Sp for speciation is only 12%/32%. In the latter case, a large percentage of *Aeromonas* species would be wrongly identified.

When investigating AMR, the conservation of more than one isolate may be advantageous as it can increase sampling sensitivity in a situation where prevalence is low (commentaire

P Boerlin : à expliquer ou préciser). A duplication of AMR phenotypes in *Aeromonas* isolated from the same sample were investigated in six of 24 samples using the rep-PCR methods described by Novakova (2009). According to rep-PCR anlaysis, the isolate pairs from four of these were clonal. In consideration the data here, if only one isolate was selected for identification and AMR determination and in 50 percent of the cases (a coin toss) it was the sensitive isolate, the overall apparent prevalence of samples exhibiting some type of AMR would drop from 17% to 14%. This decrease must be considered when planning the sampling and isolation protocols with respect to the surveillance goals.

In this study, the level of NWT isolates is low for all antimicrobials studied ranging from 0.5% for FFC to 12% for TET. Higher levels of AMR are reported in the literature for *Aeromonas* isolated from retail seafood with ranges for: fluoroquinlones (10-42%), NA(17-58%), SXT(38-49%) and TET(44-51%) reported (Castro-Escarpulli et al., 2003; Kaskhedikar and Chhabra, 2010; Radu et al., 2003; Vivekanandhan et al., 2002). Differences in sampling and testing methods, bacterial identification, market types, regions/countries and seafood species sampled, among other variables, likely contribute to this disparity.

Commentaire P Boerlin : il serait judicieux de faire une comparaison de correlation genotype/phenotype versus microarray seule /versus PCR seule. Both PCR and microarray techniques were utilised to verify the presence of AMR genes and differences in detection were noted between the two techniques. Only 6 of the 13 *tet*(E) determinants identified by microarray were found by PCR, and conversely, *sul1*, *floR* and *intI1* were only identified by PCR and not by the microarray. Variations in the genes that were used to design primers and probes may be important enough between bacterial genera to explain these differences, as the microarray used here was validated for *E. coli*. When considering the *tet* genes, the use of the microarray was an important complementary tool for identifying resistant determinants responsible for the expressed phenotypes.

The presence of tetracycline as the most frequently identified NWT phenotype is not surprising as tetracycline were among the earliest antimicrobials available for use in

aquaculture and according to a recent survey of aquaculture-allied professional (Tusevlak et al, in prep), tetracycline is the most frequently used antimicrobial world-wide, all species confounded (Tuševljak, In-preparation). The genes *tet*(A) and *tet*(E) were the only determinants identified here and their presence is frequently reported in *Aeromonas* (Agerso et al., 2007; Balassiano et al., 2007; DePaola et al., 1988). All but two of the TET-NWT isolates were correlated to gene presence. The presence of phenotypic TET resistance in the absence of the responsible gene is commonly reported, and may likely suggest the presence of a new or variant tetracycline resistance determinant not present in other species, (Balassiano et al., 2007; Furushita et al., 2003; Schmidt et al., 2001).

Mutations of the *gyrA* and *parC* gene are the most commonly reported cause of decreased susceptibility to quinolones in *Aeromonas* (Alcaide et al., 2010; Goni-Urriza et al., 2002). Substitutions at the 83rd and 92nd codon of *gyrA* and at the 80th codon of *parC* are frequent, and were identified in non-susceptible isolates. In one isolate additional substitutions were noted in *parC* at codons 85 and 98. Although multiple mutations in *gyrA* and *parC* have been cited as being responsible for increased fluoroquinolone resistance, this did not seem to be the case here as all isolates with NWT phenotypes had MIC's ranging from 0.25 to 1µg/ml.

NWT phenotypes for FFC and SXT were found the least frequently. The only two SXT-NWT isolates found were associated with *sul1*, *dfrA5* or *dfrA5*/*dfrA16* and in both cases were identified in association with the *intI1* gene indicating the presence of a Class 1 integron. Although the *intI1* gene was found in five other isolates, it was not found with *sul1* or resistance cassettes which has also been reported by others (Jacobs and Chenia, 2007; Rosser and Young, 1999). The single isolate with a FFC-NWT phenotype contained the *floR* gene. Additional chloramphenicol resistance genes were identified by the microarray, including *cat*, *cat2* and *cat3*. These genes encode for resistance to chloramphenicol through chemical disactivation, but have no effect on florfenicol. If the prevalence of this gene/resistance phenotype is important chloramphenicol should be included in the AMR panel to better target resistant isolates.

Several β-lactamase genes were identified by microarray including *bla*$_{VIM2}$, *bla*$_{FOX2}$, *bla*$_{SME}$, and *bla*$_{TEM}$. The presence of transferable β-lactamase genes is difficult to detect in *Aeromonads* which are intrinsically resistant and normally demonstrate a resistant phenotype to β-lactams. In this study for example, all but one isolate was resistant to ampicillin. Therefore, the acquisition and preservation of these genes does not seem to be dependent on selection of resistant bacterial populations through β-lactam exposure. *Aeromonas* can harbour these genes on mobile genetic elements as in other genera, and therefore, could be considered as a reservoir.

The plasmids identified in this study were of low molecular weight, and have a similar size range as that previously reported (Radu et al., 2003). Those described by Sorum (2003), associated with resistance determinants in environmental or clinical isolates are generally larger, than what was found here, even up to 150 kb (Sorum, 2006). Larger plasmids can be more difficult to isolate due to shearing during the extraction process, however, as the majority of the resistance genes were localised on low-molecular weight plasmids in this study, even if present (6/19) their importance remains unclair as some *tet* determinants were not localised to a specific plasmid. This could indicate a chromosomal location, or perhaps low copy-number and/or larger plasmids.

Overall prevalence of AMR in seafood identified in this study was low and the majority of the AMR phenotypes were explained via gene identification. Although microarray analysis was unable to identify certain genes identified by PCR, it was useful as a complementary tool when investigating AMR phenotypes unobserved by PCR. Identification of *Aeromonad* species using biochemical means appeared to be unreliable, and if bacterial speciation is important, a genetic component should be included in the identification scheme.

6.0 Supporting Information

Table S1: AMR probes present on the microarray and reference genes, used in this study. Found at :

7.0 Acknowledgements

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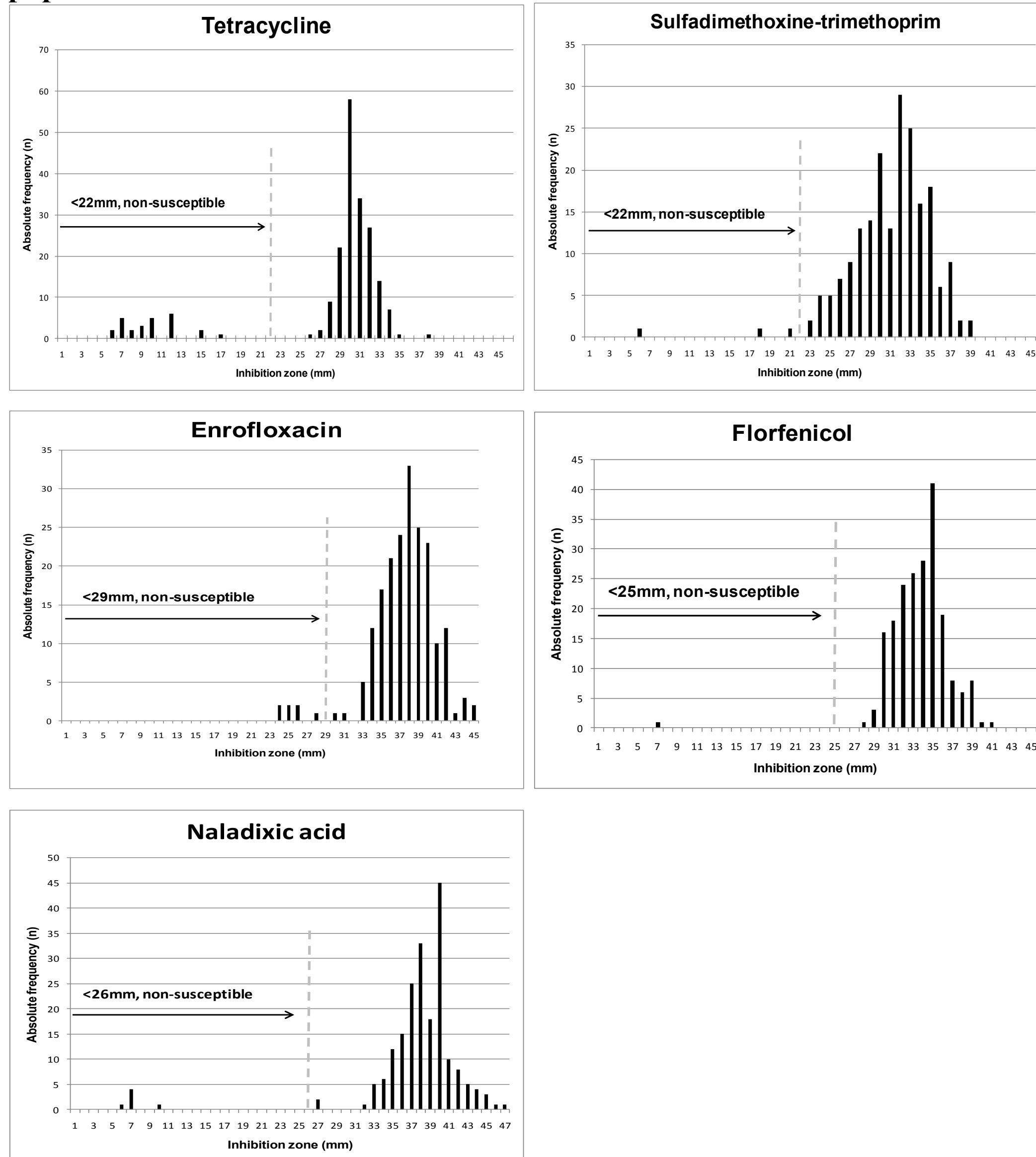
Figure 1: Histogram of inhibition zone diameters of the *Aeromonas sp.* population



Note: The calculated NRI-ECV value for each antimicrobial is indicated by the vertical dotted black line.

Table I: Primers used for PCR analysis of resistance genes

Gene	Primer	Nucleotide Sequence 5'-3'	Product size (bp)	Reference
florR	*floR-F*	GAGATCGGATTCAGCTTTGC	198	This study
	floR-R	TCGGTAGGATGAAGGTGAGG		
gyrA	*gyrA-F*	TCCTATCTTGATTACGCCATG	481	(Goni-Urriza et al., 2002)
	gyrA-R	CATGCCATACCTACCGCGAT		
intI1	*IntI1-F*	GGGTCAAGGATCTGGATTTCG	483	(Mazel et al., 2000)
	IntI1-R	ACATGGGTGTAAATCATCGTC		
parC	*parC-F*	GTTCAGCGCCGCATCATCTAC	225	(Goni-Urriza et al., 2002)
	parC-R	TTCGGTGTAACGCATTGCCGC		
qnrA	*QnrA-F*	ATTTCTCACGCCAGGATTTG	516	(Gay et al., 2006)
	QnrA-R	GATCGGCAAAGGTTAGGTCA		
qnrB	*QnrB-F*	GATCGTGAAAGCCAGAAAGG	469	(Gay et al., 2006)
	QnrB-R	ACGATGCCTGGTAGTTGTCC		
qnrS	*QnrS-F*	ACGACATTCGTCAACTGCAA	417	(Gay et al., 2006)
	QnrS-R	TAAATTGGCACCCTGTAGGC		
sul1	*sul1-F*	CTTCGATGAGAGCCGGCGGC		(Falbo et al., 1999)
	sul1-R	GCAAGGCGGAAACCCGCGCC		
tet(A)	*Tet*(A)-*F*	GTAATTCTGAGCCACTGTCGC		(Schmidt et al., 2001)
	tet(A)-*R*	CTGCCTGGACAACATTGCTT		
tet(B)	*tet*(B)-*F*	CTCAGTATTCCAAGCCTTTG		(Schmidt et al., 2001)
	tet(B)-*R*	CTAAGCACTTGTCTCCTGTT		
tet(C)	*Tet*(C)-*F*	TCTAACAATGCGCTCATCGT		(Schmidt et al., 2001)
	tet(C)-*R*	TCTAACAATGCGCTCATCGT		
tet(D)	*tet*(D)-*F*	TTGCGGCTTCGGTAGTGGCG		
	tet(D)-*R*	CATGCATCGCCCCGAGTCCC		
tet(E)	*tet*(E)-*F*	GTGATGATGGCACTGGTCAT		(Schmidt et al., 2001)
	tet(E)-*R*	CTCTGCTGTACATCGCTCTT		
Class 1 integron variable region	*IC1-F*	TTATGGAGCAGCAACGATGT	variable	(Chang et al., 2007)
	IC1-R	CTGTGAGCAATTATGTGCT		

Table II: Sensitivity and specificity for Vitek2® identification of *Aeromonas* to the genus and species level using an *rpoB* gold standard

	rpoB Aeromonas pos.	*rpoB Aeromonas* neg.	Senstivity or Specificity	Kappa coefficient (Confidence intervals)
Vitek2 - genus *Aeromonas* sp. pos.	190	8	Sensitivity (genus); 96%	0.38 (0.176-0.600)
Vitek2 - genus *Aeromonas* sp. neg.	8	6	Specificity (genus); 43%	
Vitek2 species *Aeromonas* sp. pos.	22	23	Sensitivity (species); 12%	-0.214 (¯0.268-0.148)
Vitek2 species *Aeromonas* sp. neg.	156	11	Specificity (species); 32%	

Table III: Differences in antimicrobial resistance gene detection noted between microarray and PCR(proposition de P Boerlin élimination des résultats Vibrio)

Antimicrobial /integron family	AMR gene	*Aeromonas*	
		PCR	Microarray
Folic acid inhibitors	*sul1*	2[a]	0
	sul2	-	-
Phenicols	*floR*	1	0
Tetracyclines	*tet*(A)	3	3
	tet(B)	-	-
	tet(D)	0	1
	tet(E)	6	13
Class 1 integron	*intI1*	2	0

[a]Numbers of isolates containing indicated resistance gene
[b]Hypens indicate comparisons were not possible

Table IV: Antimicrobial resistance genes identified by microarray in *Aeromonas*

Antimicobial family	Genes	*Isolates* (*n*=18)
Aminoglycosides	*strA/strB*	2[a]
	aadA1	1
β-lactams	bla_{VIM2}	3
	bla_{FOX2}	1
	bla_{SME}	3
	bla_{TEM}	5
Inhibitors of folic acid	*dhfr5*	3
	dhfr16	1
Macrolides	*ereA2*	2
Phenicols	*cat*	2
	cat2	1
	cat3	2
Tetracyclines	*tet*(A)	3
	tet(D)	1
	tet(E)	13

[a]Numbers of isolates containing indicated resistance gene

TableV: Isolates with resistance phenotypes of four antimicrobial families and associated resistance genes

Isolate No.	Identification[a]	Sample type/origin	Resistance phenotype	Resistance genes detected by PCR or microarray	*intI1*	Associated plasmid
6A1	*A. sobria*	Trout	TET	*tet*(E)	-	-
12A2	*A. veronii*	Trout	TET, SXT, FFC	*tet*(A), *sul1, floR, dfrA16,dfrA5*	+ (*dfr16*)	~12kbp
13A1	*A. encheleia*	Salmon	TET, NA	*tet*(E), *gyrA* $_{Se83\text{-}Val}$	-	~12kbp
35A2	*A. veronii or A. sobria*	Salmon	TET	*tet*(A)	-	-
36A2	*A. sobria*	Trout	TET	*tet*(A)	-	-
92A1	*A. enteropelogenes*	Shrimp	NA, ENO	*gyrA* $_{Se83\text{-}Ile}$, *parC* $_{Se80\text{-}Ile}$	-	-
98A1	*A. encheleia*	Salmon	TET	*tet*(E)	-	-
133A2	*A. sobria*	Salmon	TET, NA	*tet*(E), *gyrA* $_{Se83\text{-}Ile}$ *parC* $_{Ala85\text{-}Thr}$, and *parC* $_{Pro98\text{-}Ser}$	-	-
140A1	*A. encheleia*	Salmon	TET	*tet*(E)	-	~12kbp
155A1	*A. sobria*	Salmon	TET	*tet*(E)	-	-
167A2	*A. encheleia* or *A. salmonicida*	Salmon	TET	*tet*(E)	-	-
209A2	*A. encheleia* or *A. salmonicida*	Shrimp	TET, NA, ENO	*gyrA* $_{Se83\text{-}Ile,\ Leu92\text{-}Met}$, *tet*(E)	-	~12kbp
227A1	*A. veronii or A.sobria*	Salmon	SXT	*sul1, dhfr5*	+ (*aadA1*)	~20kbp
234A2	*A. encheleia* or *A. salmonicida*	Salmon	TET	*tet*(E)	-	-
242A2	*A. sobria*	Salmon	TET	*tet*(E)	-	-
252A2	*A.encheleia or A.salmonicida*	Salmon	TET	*tet*(E)	+ (no cassette)	~18kbp
255A2	*A.encheleia or A.salmonicida*	Salmon	TET	*tet*(E)	-	-
259A2	*A. veronii*	Salmon	TET	*tet*(E)	-	-
276A1	*A. encheleia or A. salmonicida*	Salmon	NA, ENO	*tet*(E), *gyrA* $_{Se83\text{-}Val}$	-	-

[a]Isolates were identified by sequencing of *rpoB* and comparison with the Genbank database
[b]Abbreviations for the antimicrobials used: florfenicol (FFC), tetracycline (TET) and trimethoprim-sulfamethoxazole (SXT), naladixic acid (NA), enrofloxacin (ENO)

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