

Complexities Involved in Source Attribution of Antimicrobial Resistance Genes Found in Aquaculture Products

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ISSN: 0116-6514
E-ISSN: 2073-3720
<https://doi.org/10.33997/j.afs.2020.33.S1.003>

Abstract

Aquaculture is contributing to nearly half of food fish production and the growth of the sector is the main contributor to the increase in fish production for the last three decades. Detection of antibiotic resistance in food-associated bacteria including those associated with products of aquaculture has been causing great concern. Often, this is directly linked to antimicrobial use in aquaculture. However, attributing the source of antimicrobial resistance in bacteria found in aquaculture products is complicated. In this study, we look at the origin, evolution and spread of antibiotic resistance documented in literature. The results indicate that antibiotic resistance observed in aquaculture environment has multiple sources. Antibiotic resistance is a natural phenomenon and existed in bacteria before the human use of antibiotics. Some bacteria have intrinsic resistance to certain antibiotics. Resistance to antibiotics is found in bacteria in the deep sea and in high seas, where it is unlikely to have any exposure to antibiotics. However, extensive use of antibiotics in different sectors has contributed to the enrichment of antibiotic-resistant bacteria in hospital environments, animal farms, and the aquatic environment. Aquatic bodies receive effluents from hospitals and animal farms that carry antibiotic-resistant bacteria from these sectors. These bacteria may end up in aquaculture farms and in fish harvested from these farms. Distinguishing resistance that has been selected in different sectors is extremely difficult and caution is needed while trying to attribute the source of antibiotic resistance in bacteria in the aquatic environment.

Keywords: AMR, antibiotics, fish production, aquaculture

Introduction

Aquaculture is one of the fastest-growing food production sectors in the world according to FAO estimates (FAO, 2020), aquaculture had an annual growth rate of 7.5 % since 1970, compared to only 0.9 % for capture fisheries and 2.6 % for terrestrial farmed meat production systems over the same period.

Global aquaculture production (including aquatic plants) in 2018 was reported to be 114.5 million tonnes by volume with a value of USD263.6 billion. Aquaculture contributes to about half of the global food fish production. Interestingly, 57.8 % of the total volume and 58.6 % of the total value of aquaculture production comes from the People's Republic of China; other top producers for 2018 were Indonesia,

India, Viet Nam, Bangladesh, Philippines, South Korea, Egypt, Norway and China (FAO, 2020). Fish is also one of the highly traded commodities and, in 2018, 67 million tonnes of fish (live weight equivalent) were traded internationally for a total export value of USD164 billion. While developing countries in Asia are major producers of aquaculture products, developed countries constitute the major market of consumers. European Union accounted for 34 % of global seafood imports in 2018, while the United States accounted for 14 % and Japan, 9 %.

There are very stringent regulations in the EU, USA, and Japan regarding the use of antimicrobials in aquaculture and as per the World Trade Organization (WTO) Sanitary and Phytosanitary (SPS) Agreement, imported products also need to comply with these

regulations. The EU has an elaborate system of verifying, whether outside countries exporting to their member countries have regulations on par with EU regulations. Additionally, the EU Food and Veterinary Office (FVO) conducts inspections to verify whether the Competent Authorities in fish exporting countries can provide guarantees regarding compliance with these regulations. These measures have led to significant improvements in aquaculture practices in producing countries. Nevertheless, there is a perception among some consumers and other non-governmental organisations, and also in some members of the scientific community, that there is heavy use of antibiotics in the aquaculture sector. Cabello (2006) indicated that the heavy use of prophylactic antibiotics in aquaculture is a serious problem for humans, animal health, and the environment. But many improvements have been made over the last decade, and more recent studies indicate that only 3 % of shrimp grow-out farmers in Viet Nam used antibiotics from 2011 to 2012 and that prophylactic treatments are rare (Rico et al., 2013). Antibiotic usage per ton of product in many species produced by aquaculture is much lower than that of other food-producing animals, even in developed countries. Usage in shrimp production in Viet Nam was 1.44 g.ton⁻¹ of production, and the corresponding figures for China and Thailand are 1.67 and 4.53 g.ton⁻¹ respectively. For tilapia culture, antibiotic usage was 1.32 g.ton⁻¹ of production in China and 7.31 g.ton⁻¹ of production in Thailand. The highest usage of antibiotics was 92.9 g.ton⁻¹ of Pangasius production in Viet Nam. This is still lower than the antibiotic usage of 144 g.ton⁻¹ of food-producing animals in the EU (ECDC/EFSA/EMA, 2015).

Issues Associated with Antimicrobial Use in Aquaculture

The Joint FAO/OIE/WHO Expert Meeting on Antimicrobial Use and Antimicrobial Resistance in Aquaculture identified that the two hazards to be considered are antimicrobial residues and antimicrobial resistance (AMR) (FAO/OIE/WHO, 2006).

While residues found in animal tissues may be directly related to the use of antimicrobials in the respective sector, the issue of AMR is more complicated in the case of aquaculture as illustrated in Figure 1 (Karunasagar, 2012). Detection of antimicrobial residues (e.g. chloramphenicol, nitrofurans, malachite green) in internationally-traded shrimp has resulted in the slowdown of imports, causing economic losses among producers and governments. This has also led to tightened national regulations on the use of antibiotics and the implementation of national residue control programs in many countries. As a result of these tightened regulations, the number of cases of rejections and alerts related to seafood due to antimicrobial residues has drastically decreased in recent years, although some cases of alerts and rejections still occur for some exporting countries. At the international level, the maximum residue limit (MRL) acceptable is set by the Codex Alimentarius Commission based on the scientific evaluation of the drugs by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Currently, there are MRLs for only a few antimicrobials in Codex. There are also additional national and regional MRLs in seafood importing countries, but these may or may not be consistent between countries. Hence, there is a need to have Codex MRLs for antimicrobials that are approved for use in aquaculture.

It is to be recognised that antibiotic-resistant genes are often carried on mobile genetic elements that can be transferred to other bacteria and that there are no phylogenetic or geographical barriers for such transfer. (FAO/OIE/WHO, 2006) Furthermore, because animal farm and hospital effluents reach the aquatic environment, antibiotic resistance determinants selected in other environments can also be found in the aquatic environment and may reach products of aquaculture. Rowe et al. (2016) demonstrated that the abundance of AMR genes (ARGs) in effluents entering a river catchment area is higher than that of the receiving environment. This review discusses the various aspects that need to be considered while making any conclusions about AMR detected in aquaculture products.

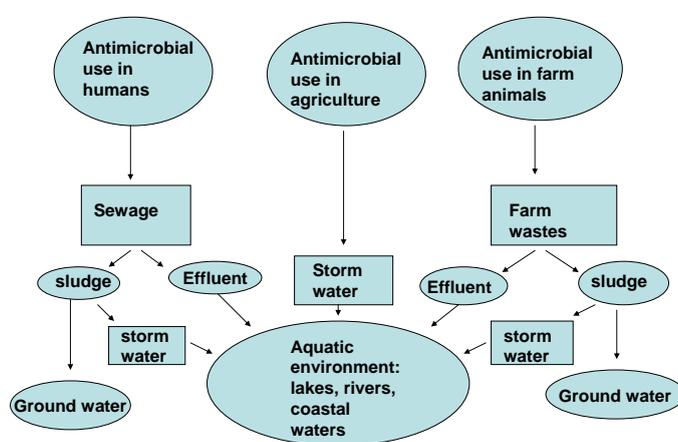


Fig. 1. Pathways for the spread of antimicrobial residues and resistant bacteria in the aquatic environment (Karunasagar, 2012).

Antibiotic Resistance is Natural, Ancient, and Predates Anthropogenic Use of Antibiotics

It is now well accepted that antibiotic-resistant genes are of ancient origin and can be found in bacteria from the pre-antibiotic era. D'Costa et al. (2011) noted that genes encoding resistance to β -lactam, tetracycline, and glycopeptide antibiotics could be found in bacteria from 30,000-year-old Beringian permafrost sediments. Viable multidrug-resistant bacteria were cultured from the Lechuguilla Cave in New Mexico, USA that was isolated for more than 4 million years (Bhullar et al., 2012). These bacteria were resistant to at least one antibiotic and often seven or eight antibiotics, including β -lactams, aminoglycosides, and macrolides, as well as newer drugs such as daptomycin, linezolid, telithromycin, and tigecycline. It has been estimated that the earth is inhabited by 5×10^{30} bacterial cells, with only about 1 % of these culturable and most of them are non-human pathogens (Monier et al., 2011); hence, we know very little about environmental bacteria and the genes that they carry. At least some of the resistance determinants presently circulating among human pathogens have been thought to originate from environmental bacteria. For example, *qnr* genes encoding quinolone resistance and found in plasmids of *Escherichia coli* and *Salmonella* might have originated from aquatic organisms like *Shewanella* or members of aquatic *Vibrionaceae*, where the *qnr* gene is found in the chromosome (Poirel et al., 2012). The plasmid-borne extended-spectrum beta-lactamase (ESBL) gene of CTX-M group (*bla*CTX-M) found in *E. coli* is thought to have originated from the environmental organism *Kluyvera* (Poirel et al., 2012). Thus, many environmental bacteria harbour resistance determinants that may not be related to the exposure to the antibiotics.

Recent molecular biological studies on antibiotic resistance genes provide interesting insights into the evolution and ecology of antibiotic-resistant genes. Tetracycline resistance is mediated by ribosomal protection proteins (RPPs) in Gram-positive and Gram-negative bacteria. Kobayashi et al. (2007) noted that RPPs are derived through duplication and divergence of GTPase before the divergence of the three superkingdoms, Bacteria, Archaea, and Eucarya. This suggests the extant function of RPPs occurred even before the evolution of *Streptomyces* which produce tetracyclines. They propose that RPPs evolved independently of tetracyclines and possibly serve a function other than antibiotic resistance. β Lactamases are enzymes involved in resistance to the penicillin group of antibiotics. Fevre et al. (2005) provided evidence to show that β lactamase genes in *Klebsiella oxytoca* were evolving for over 100 million years in this host, without concomitant evolution of an AMR phenotype. In addition to being involved in the hydrolysis of β lactam ring, metallo- β lactamases are involved in various basic cellular processes such as

hydrolysis, DNA repair, and RNA processing, and these enzymes can be found in all the three domains of life, i.e. Bacteria, Archaea, and Eucarya (Garau et al., 2005). The ancient evolution of antibiotic-resistant genes is further supported by the observation of antibiotic resistance in bacteria trapped in deep Greenland glacier ice cores for at least 120,000 years ago (Miteva et al., 2004).

Dutta and Hughes (1983) studied conjugative plasmids in enteric bacteria isolated before the medical use of antibiotics and compared them with plasmids found in more recent isolates. They noted that the 'pre-antibiotic' plasmids belonged to the same groups, as defined by incompatibility tests (Inc groups), as modern R plasmids. This suggests that the acquisition of antibiotic resistance in the past 50 years seems to have been by the insertion of new genes into existing plasmids rather than by the spread of previously rare plasmids.

Need to Differentiate Intrinsic Resistance and Acquired Resistance

Many Gram-negative bacteria have intrinsic resistance to the beta-lactam group of antibiotics. *Aeromonas* spp., commonly found in freshwater aquaculture environments, have been reported to have intrinsic resistance to ampicillin and amoxicillin and possess at least four chromosomally borne beta-lactamase genes (Janda and Abbot, 2010). This intrinsic resistance has been the basis for the development of the starch-ampicillin agar medium for quantitative detections of *Aeromonas* in foods (Palumbo et al., 1985). *Aeromonas hydrophila* strains possess the Ahe ABC efflux pump that contributes to intrinsic multidrug resistance (Hernould et al., 2008). Therefore, when attributing resistance of *A. hydrophila* isolated from aquaculture environments, it would be important to focus on the acquired resistance. Antibiotic-resistance genes have been reported to be present in mobile genetic elements such as plasmids, transposons, and associated with integrons (Piotrowska and Popowska, 2015).

When microorganisms that were once sensitive to an antimicrobial agent become resistant to a particular antibiotic, the resistance is acquired. The acquired resistance could be due to genetic changes such as mutations or to the acquisition of genes contributing to resistance through horizontal gene transfer. Antibiotic resistance genes may be transferred through mobile genetic elements such as plasmids, transposons, bacteriophages, genomic islands, or integrons. Though integrons are not self-mobile, they contain gene cassettes that are mobile.

Mechanisms of AMR

Bacteria resist the action of antimicrobial agents through different mechanisms (Alekhun and Levy,

2007; Blair et al., 2015). Some of the common mechanisms are:

- **Inactivation of the drug:** Bacteria acquire gene encoding enzymes that inactivate the antibiotic before it can reach the target, e.g. beta-lactamases inactivate beta-lactam antibiotics like penicillins and cephalosporins; carbapenemases inactivate carbapenems; aminoglycoside modifying enzymes such as N-acetyltransferases, O-adenyltransferases and O-phosphotransferases modify the antibiotics of this class.
- **Prevention of drug access to targets in bacterial cells:** Reduced access can happen due to: (a) reduced permeability to the drug: e.g. carbapenem resistance due to reduced or altered porin production by mutations. It has been reported that selective pressure due to carbapenems favours the emergence of mutations in porin genes or in genes that regulate porin expression; and/or (b) increased efflux: bacterial efflux pumps actively transport antibiotics outside the cell. Some efflux pumps may show narrow substrate specificity, e.g. Tet pumps, but others show broader activity such as multidrug efflux pumps.
- **Modification of drug targets:** Antibiotics act on specific bacterial targets (e.g. ribosomes) and modification of drug targets would render the antibiotic ineffective. For instance, the erythromycin ribosome methylase (*erm*) family of proteins methylate 16SrRNA and alter binding targets for macrolide antibiotics. The *qnr* family of genes encodes pentapeptide repeat proteins that bind to and protect topoisomerase IV and DNA gyrase from the lethal action of quinolones. Polymyxin antibiotics like colistin bind to lipopolysaccharides in Gram-negative bacteria and the antibacterial activity are due to the disruption of the cell membrane by the hydrophobic chain. Overexpression of *pmrC* in colistin-resistant bacteria leads to the addition of phosphoethanolamine to lipid A, leading to decreased binding of colistin.
- **Bypass targets:** Methicillin resistance in *Staphylococcus aureus* is due to the acquisition of chromosomal cassette *mec* element. The *mecA* gene encodes the β -lactam-insensitive protein PBP2a that enables cell wall biosynthesis to occur despite the native PBP being inhibited in the presence of the antibiotic. Resistance to sulphonamide and trimethoprim could be due to bypass mechanisms. These drugs interfere in different steps in de novo synthesis of tetrahydrofolic acid, an essential precursor for several amino acids and nucleotides. Bacterial resistance could be due to the production of drug-resistant

dihydrofolate reductase or dihydropteroate synthesis. Plasmid borne *sul1* and *sul2* genes in sulphonamide resistant bacteria encode drug-resistant dihydropteroate synthase.

Genetic Mechanisms of AMR

Detected phenotypic AMR could be due to different genetic mechanisms. Tetracycline is commonly used in the treatment of diseases in aquatic animals. When resistance to tetracycline is detected, it could be due to: (a) overproduction of efflux proteins, (b) production of ribosomal protection proteins, or (c) production of tetracycline inactivating proteins (Chopra and Roberts, 2001). Therefore, to understand the emergence and spread of AMR in aquaculture and the relation between antimicrobial use (AMU) and AMR in different sectors, it is important to have information on the genetic determinants related to the resistance.

In addition, information on the genetic type of resistance determinants in the study of the epidemiological aspects of the spread of AMR is also an important consideration. Extended-spectrum beta-lactamases could be of different molecular types and the genes encoding these show considerable variations. There are four classes based on molecular types and these different types are based on functions where some are inhibited by clavulanic acid while others are not. Some are serine-based enzymes and others are metalloenzymes.

AMR in Bacteria Associated with Aquaculture

AMR in pathogens of aquatic animals has been reported from different systems. In shrimp hatcheries, the presence of antibiotic-resistant luminous bacteria has resulted in mass mortalities. (Karunasagar et al., 1994). Acquired resistance in *Aeromonas salmonicida* causing furunculosis in temperate waters has been reported from various countries (FAO/OIE/WHO, 2006). Several mobile genetic elements such as plasmids, transposons, and integrons carrying AMR genes have been detected in *Aeromonas* spp. from aquaculture sites in different parts of the world (Piotrowska and Popowska, 2015). Over 80 % of *Vibrio harveyi* from finfish aquaculture systems in Italy showed resistance to amoxicillin, ampicillin, and erythromycin, while 76 % of strains of *Vibrio* spp showed resistance to sulphadiazine (Scarano et al., 2014). AMR in pathogens of aquatic animals could impact disease management in these systems and the resistance determinants could be transferred to human pathogens in aquatic systems.

Though AMR is observed in aquatic bacteria associated with aquaculture systems, it is difficult to find a direct link between the resistance profile and AMU. Culture-independent studies in the Baltic Sea show the presence of resistance genes encoding

resistance to sulphonamides, trimethoprim, tetracycline, aminoglycoside, and chloramphenicol. In addition, genes encoding multidrug efflux pumps were discovered in sediments below fish farms, even though some antibiotics like tetracyclines, aminoglycosides, and chloramphenicol are not used in this area (Muziasari et al., 2017). Some of these might represent a natural reservoir of resistance genes in the aquatic environment. Antibiotic-resistant marine bacteria have been found as far as 522 km offshore and in the deep sea at depths of 8,200 m (Aminov, 2011).

Public Health

From a public health perspective, AMR in aquatic bacteria of zoonotic potential would be significant. Studies done in Korea show that all *Vibrio parahaemolyticus* isolated from oysters were resistant to ampicillin and vancomycin and half of the number of isolates exhibited resistance to cephalothin, rifampin, and streptomycin (Kang et al., 2016). However, there was no linkage to the use of these antibiotics in aquaculture. There may be geographical variation in the prevalence of resistance. Studies in China with isolates from crustaceans and shellfish showed much higher (over 90 %) resistance to rifampin and 78 % showed resistance to streptomycin (Hu and Chen, 2016). Most *Vibrio vulnificus* strains isolated from Dutch eel farms showed resistance to cefoxitin, even though this antibiotic was not used in eel aquaculture (Haenen et al., 2014). Thus, the detection of antibiotic resistance in bacteria isolated from aquaculture cannot be directly linked to the use of antimicrobials in aquaculture. Therefore, the detection of antibiotic resistance in aquaculture systems needs to be interpreted with caution, considering that resistance determinants are naturally present in these environments and that ARGs also come from other sectors. But often in literature, we can see the simplistic linking of any resistance found to AMU in aquaculture, even when there is no use of antimicrobials in that system. For example, Akinbowale et al. (2006) attributed resistance found in aquaculture environments in Australia to significant off-label use. Implementation of an integrated surveillance program within the framework of One Health, which includes the study of AMU and ARGs in different sectors (human, agriculture, veterinary, aquaculture), could improve our understanding of the drivers leading to the selection and spread of AMR in the aquatic environment.

AMR in Bacteria Associated with Products of Aquaculture in Retail Markets

When farmed fish are handled and processed, there can be significant changes in the microflora. Therefore, bacteria found in farmed fish at the retail level may not represent the microflora coming from

the aquatic environment. For example, farmed fish such as Vietnamese catfish and tilapia are filleted before reaching the market. Aquacultured shrimp are handled and processed (beheaded, gutted, peeled) before being sent to export markets. Uddin et al. (2013) examined the microflora of raw-cultured and wild-caught shrimp imported into Denmark and concluded that the flora changes considerably during processing. They suggested that it is not possible to pick up any indicator bacteria representative of the aquaculture environment at this stage. This calls for caution in the interpretation of AMR found in fish and shrimp at the retail level or import control points and in linking resistance found in bacteria at this stage and AMU in aquaculture. Data from aquatic products at the primary production stage is essential to understand any linkage between AMU and AMR in aquaculture.

Conclusion

Antibiotic resistance is a natural phenomenon and exists in bacteria from environments even without exposure to antibiotics. Nevertheless, extensive use of antibiotics in different sectors has led to the selection and spread of multidrug resistant bacteria. However, it is very difficult to link AMR in bacteria found in aquaculture systems with the use of antibiotics in this sector, since the aquatic environment receives effluents from hospitals and terrestrial animal farms, consequently leading to the spread of resistant bacteria from all sectors through aquatic systems. The processing of fish leads to change in microflora and resistant bacteria found in processed fish may not be derived from aquaculture. Therefore, there is a need to be cautious while drawing conclusions about the source of antibiotic resistance in bacteria associated with aquaculture.

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