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Determination of illegal antimicrobials in aquaculture feed and fish: An ELISA study

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Gentamicin (PubChem CID: 3467)
Fluoroquinolone (PubChem CID: 52948370)
Enrofloxacin (PubChem CID: 71188)
Malachite green (PubChem CID: 11294)
Furaltadone (PubChem CID: 9553856)
Furazolidone (PubChem CID: 5323714)
AMOZ (PubChem CID: 3016406)
AOZ (PubChem CID: 65725)

ABSTRACT

Antimicrobials are added to the feed or drinking water of food-producing animals to reduce susceptibility to infection, accelerate weight gain, or reduce the amount of food required to gain weight. Some compounds have been banned for food safety reasons, for other agents the U.S. Food and Drug Administration (FDA) is implementing a plan with industry to phase out a number of antibiotics.

The concentrations of crystal violet (CRY), chloramphenicol (CAP), gentamicin (GEN), fluoroquinolone-enrofloxacin (FQ), malachite green (MG), and the metabolites of furaltadone (FU) and furazolidone (FZ) antibiotics (respectively AMOZ and AOZ) were determined in 30 samples both feed and fish from an aquaculture farm in eastern Sicily (Italy) using commercial ELISA Kits. Levels exceeding the method's detection capability were found in all feed and tissue samples. Feed contained all the analytes tested; GEN, CRY and CAP showed the highest mean concentrations, respectively 31.8, 4.05 and 3.67 μg kg^{-1}. The mean concentrations of CAP, CRY, FQ, MG, AMOZ and AOZ in muscle were 0.57, 2.05, 0.14, 0.48, 0.29 and 0.09 μg kg^{-1}, respectively (the assay was not certified to determine GEN in muscle). The higher levels detected in feed are explained by the fact that 50% of farmed fish is used to make fish meal, thus compounding bio-accumulation. Our data show that aquaculture feed and fish contain banned antimicrobials. Consumption of farmed fish may therefore involve a risk for consumers, besides contributing to the growth of antibacterial resistance. Surveys of larger feed and fish samples are needed to achieve a more reliable assessment of consumer risk.

1. Introduction

Low fat and high n-3 polyunsaturated fatty acid (PUFA) content make seafood a valuable element in the human diet (McManus, Fielder, Newton, & White, 2011). Several studies have found that regular fish consumption helps prevent chronic conditions such as cardiovascular disease (Albert et al. 1998; Morris et al., 1995), type 2 diabetes (Patel et al. 2009; Wallin et al. 2012), some cancers (Fernandez, Chatenoud, La Vecchia, Negri, & Franceschi, 1999; Torfadottir et al. 2013; Wu et al. 2012), overweight, and obesity (Ramel, Martinez, Kiely, Bandarra, & Thorsdottir, 2010; Thorsdottir, Birgisdottir, Kiely, Martinez, & Bandarra, 2009). Seafood is also essential for the physiological growth and development of newborns (Hunter & Roberts, 2000). These important effects prompted the WHO to hold an expert consultation on the risks and benefits of fish consumption, in January 2010 (WHO, 2010).

Rising consumer demand for seafood has fostered the development of modern aquaculture. However, as with all foods (Sciacca & Conti, 2009; Sciacca, Ferrante, & Conti, 2011), poor quality can involve health risks (Conti et al. 2012; Copat et al. 2012; Copat et al. 2013; Ferrante, Conti, Fiore, Rapisarda, & Ledda, 2013).

Drugs including antibiotics and antifungals are used in modern aquaculture to prevent or treat fish bacterial diseases, which are often the consequence of stress conditions (high fish density, hypoxia, high nitrite and ammonia concentrations) that impair the

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immune system, resulting in increased susceptibility to infection (Myers & Durborow, 2012).

The WHO has long recognized that antibacterial use in industrial livestock farms may entail risks for the consumer, besides contributing to the growing public health problem of antibacterial resistance in human and veterinary medicine (Heuer et al. 2009; Kemper, 2008; Mulcahy, 2011; WHO, 2011).

Some compounds have been banned for food safety reasons, for other agents the U.S. Food and Drug Administration (FDA) is implementing a voluntary plan with industry to phase out or to discourage the use of these drugs, because the antimicrobial resistance may not be completely preventable (FDA, 2013). This approach would be desirable also for the EU countries.

Vaccines and medicated feeds are the mainstays of bacterial infection management and control. Feed consists mainly of fish flour and oil, in percentages ranging from 50% to 80% (FAO, 2012).

The EU list of the antibiotics that can be used in aquaculture includes tetracyclines, penicillins, quinolones, sulphonamides, and trimethoprim (EC Regulation n. 37/2010 of 22 Dec. 2009). Chloramphenicol (CAP) and nitrofurans antimicrobials furazolidone (FZ); furaltadone (FU); nitrofurazone (NFU) and nitrofurantoin (NF) have been banned from use in food production for many years, due to effects related to drug resistance and aplastic anaemia (CAP) and to severe nephrotoxicity and mutagenicity of nitrofurans (EC Regulation no. 1439/1994 of 22 Jun. 1994). The ban on nitrofuran antimicrobials is due to the fact that no safe levels for human health can be set. Since nitrofurans are quickly metabolized and are difficult to detect, their presence is established by seeking their main metabolites, respectively AOZ, AMOZ, SEM (semicarbaide) and AHD (1-aminohydantoin) for FZ, FU, NFU and NF (Xu, Zhu, Wang, Deng, & Zhang, 2006). Indeed, laboratory investigations in EU, Japan and in many other countries use the metabolites for their detection.

Even though nitrofurans have subsequently been banned also in the US, China and most other countries, they are still used in shellfish farms in Asia and Latin America.

Malachite green (MG) and crystal violet (CV) are considered toxic and mutagenic antifungal and antiprotozoan agents (Culp, 2006; FDA 2009; Sivrastava, Sinha, & Roy, 2004; FDA, 2009). MG has been banned from use in EU and US aquaculture farms (2004/25/EC-Article 1) because dietary exposure highlighted significant mutagenic and carcinogenic effects in rats (Culp et al. 2002; Culp et al. 2006); fluoroquinolone (FQ) has been legal in EU until 1990 (Cañada-Cañada, Muñoz de la Peña, & Espinosa-Mansilla, 2009).

Since the use of antimicrobials in aquaculture is still largely unregulated (especially in many Asian and South American countries) and undocumented, unacceptable residues may be found in feed and fish. ELISA is a practical, specific and sensitive method for surveillance purposes (Xing et al. 2009).

In this study the content in the main banned antimicrobials – CRY, CAP, GEN, FQ, MG, AMOZ and AOZ – was determined in feed and muscle tissue from seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) from a Sicilian aquaculture plant.

2. Materials and methods

2.1. Sampling

Thirty seabass and gilthead seabream specimens (15 each) and 30 feed bags (manufactured by italian feed companies) were purchased from an aquaculture plant in eastern Sicily (Italy) in 2013.

No information were provided by feed companies related to origin of raw materials.

2.2. Reagents and kit

Commercial ELISA kits from Bioo Scientific (CAP, CRY, GEN, MG, AMOZ and AOZ) and Gentaur (FQ) were used all consisted of microtiter plates (96 wells) (Table 1).

GEN was determined only in feed, because the kit is not certified for fish muscle analysis.

The kit components were stored at 8 °C, according to the manufacturer's instructions.

2.3. Sample preparation

Fish were filleted and the skin and bones removed. Muscle was minced and weighed (see Table 1) and prepared for ELISA. Feed samples were made representative by quartering method. After grinding and weighing (see Table 1) they were prepared for ELISA. Sample preparation and extraction were carried out according to the kit manufacturers' instructions.

Extraction was performed by adding the kit extraction buffer to feed and tissue; a series of steps common to all ELISA kits were conducted, as follows:

- vortexing for a few seconds;
- centrifugation at 4000 rpm for 4–10 min, depending on kit specifications;
- recovery of supernatant;
- drying of supernatant in a concentrator;
- degreasing of the residue with n-hexane;
- brief centrifugation and removal of the top layer for ELISA

A sufficient number of microtiter wells were used according to the manufacturers' instructions, standards and samples were run respectively in duplicate and triplicate.

2.4. Instruments

The IKA Ultra-Turrax model T25, IKA Vortex model Genius 3 (IKA WERKE GMBH & CO.KG, Germany) and Büchi Syncore® Analyst with Büchi Recirculating Chiller F-105 and Büchi Vacuum Pump V-700 (BÜCHI Labortechnik, Switzerland) instruments were used in sample preparation. Optical density was measured at 450 nm with a Multiskan microplate (Thermo Fisher Scientific Massachusetts, USA) for all antimicrobials tested. Standard curves were constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ppb on a logarithmic curve. The mean relative absorbance values for each sample were used to determine the corresponding concentration of the tested drug.

The performance characteristic of methods were described (see Table 1) and these data have been provided by Quality Assurance & Quality Control offices of suppliers.

2.5. Evaluation of data

One-way ANOVA was applied to compare antimicrobials in feed and fish tissue using SPSS 2.0 (IBM software package, USA).

3. Results and discussion

All antimicrobials tested were found in feed, at significantly different concentrations ($p < 0.001$). GEN, CRY and CAP were the predominant compounds, accounting respectively for 77.8%, 9.90% and 8.97% (Figs. 1 and 2). The mean content in all antimicrobials (Σ) was 40.9 ± 11.6 μg kg^{-1} (Table 2). Subtraction of GEN (Σ minus GEN) resulted in a mean content of 9.1 ± 1.84, where CRY and CAP

Table 1
Information on the ELISA kits used.

Sample weight (g)	CAP	CRY	FQ	GEN	MG	AMOZ	AOZ
Fish muscle	3 ± 0.1	2 ± 0.1	3 ± 0.1	–	3 ± 0.1	3 ± 0.1	3 ± 0.1
Feed	2 ± 0.1	2 ± 0.1	3 ± 0.1	1 ± 0.1	3 ± 0.1	3 ± 0.1	3 ± 0.1
ELISA information	**CAP**	**CRY**	**FQ**	**GEN**	**MG**	**AMOZ**	**AOZ**
Assay type	Competitive	Competitive	Competitive	Competitive	Competitive	Competitive	Competitive
Kit mean recovery rate[c]	>80%	80–95%	75–95%	>80%	80–95%	80–95%	80–95%
CCα (μg kg^{-1})[a] Fish muscle	0.025	0.1	0.1	–	0.2	0.1	0.1
CCα (μg kg^{-1})[a] Feed	0.25	0.2	0.2	6.25	0.1	0.2	0.2
CCβ (μg kg^{-1})[b] Fish muscle	0.075	0.5	5.0	–	0.5	0.3	0.3
CCβ (μg kg−1)[b] Feed	0.75	0.1	5.0	10.75	1.0	0.1	1.0

[a] CCα is verified by using fortified samples. This method is able to detect/identify the target component in 50% of the cases at CCα. CCα is defined as the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.
[b] CCβ is verified by using fortified samples. CCβ is defined as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (5%).
[c] Mean recovery was derived from 20 samples spiked at CCβ for each sample type. Coefficients of variation (CV) for recovery rate are within 9%.

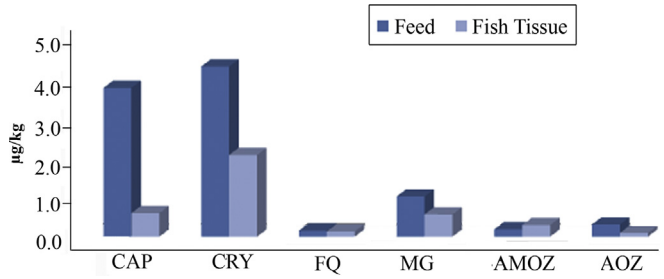

Fig. 1. Mean antimicrobial content in feed and tissue (μg kg^{-1}).

accounted respectively for 44.5% and 40.3% (Figs. 1 and 2). Fish tissue was also positive for all drugs tested, again with significantly different concentrations ($p < 0.001$). The mean concentration (Σ) was 3.62 ± 0.73 μg kg^{-1} (Table 3). CRY and CAP accounted for 56.6% and 15.7% respectively, followed by MG at 13.3% (Figs. 1 and 3).

In particular, even though CAP and CRY were the agents predominantly found in feed, more than 80% of FQ and AMOZ found in feed were also detected in fish tissue (Fig. 3).

The favourable effects of seafood consumption entail a consumer demand that is only partially met by traditional fishing, hence the development of modern aquaculture. Regular fish consumption is recommended for a healthy diet, but poor fish quality can adversely affect health. Drugs are essential in industrial livestock production, including aquaculture; antimicrobials are those used most commonly. However drugs are not free of risks, and the large amounts of antimicrobials commonly used in aquaculture may adversely affect both animal and consumer health by causing new diseases related to the compounds used besides contributing to antibiotic resistance. The management and control of bacterial infections rely on vaccines and/or medicated feeds. Aquaculture feeds contain 50–80% of fish flour and fish oil. In this study the

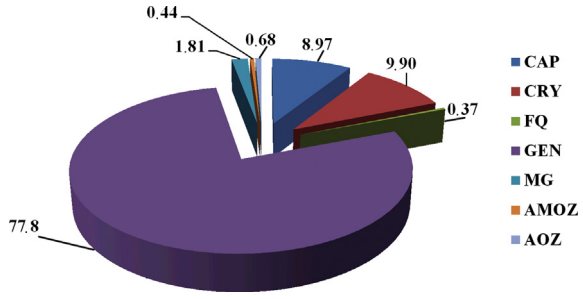

Fig. 2. Percentages content of each antimicrobial in feed.

Table 2
Antimicrobials detected in feed: mean and range (μg kg^{-1} wet weight).

Feed samples	CAP	CRY	FQ	GEN	MG	AMOZ	AOZ	∑	∑ minus GEN
Min	2.90	2.08	*<0.2*	*<6.25*	*<0.1*	*<0.2*	*<0.2*	11.9	5.68
Max	4.57	6.35	0.31	53.3	2.72	0.22	0.88	68.4	15.1
Mean	3.67	4.05	0.15	31.8	0.74	0.18	0.28	40.9	9.1
SD	0.47	0.82	0.07	7.34	0.58	0.03	0.13	11.6	1.84

Italic signifies the detection limit.

concentrations of the main banned antimicrobials were determined in feed and fish from a Sicilian aquaculture farm.

Our data show that feed, and predictably fish tissue, contained all the antimicrobials tested. Indeed 50% of farmed fish is dehydrated and used to make fish meal, leading to bio-accumulation of drugs in the muscle of aquaculture fish. The dehydration explains the higher drug content detected in feed.

GEN was detected in all feed samples, but the scarce literature entails that our findings cannot be compared with those from other studies.

CAP concentrations were high both in feed and muscle (respectively 3.67 μg kg^{-1} and 0.57 μg kg^{-1}). Tissue concentrations of 0.3 μg kg^{-1} have been reported in Norway (Bjørn-Tore, 2012) as well as in Ibadan, Nigeria (Olusola, Folashade, & Ayoade, 2012).

MG concentrations in muscle were below the legal minimum required performance limit of 2 μg kg^{-1} (EC Decision 2004/25/EC). Despite the ban from use in animals, MG residues have been reported in the past decade in food from nearly all countries including various foodstuffs from India, and farmed fish from Croatia (2009–2011), Denmark (2000–2005), China (2003) and the UK (2001–2010) (Bilandzic, Varenina, Kolanovic, Oraic, & Zrncic, 2012). MG therefore remains a cause for consumer concern throughout the world.

Close, effective surveillance is thus essential. Since none of these drugs are banned in Asia and Latin America, fish and fish products originating from these countries require even closer control.

A Canadian Total Diet Study analysing the residues of 39 veterinary drugs in fish, seafood and seafood preparations was

Table 3
Antimicrobials detected in fish tissue: mean and range (μg kg^{-1} wet weight).

Fish muscle	CAP	CRY	FQ	MG	AMOZ	AOZ	∑
Min	0.23	0.52	*<0.1*	*<0.2*	0.14	*<0.1*	1.29
Max	0.83	3.79	0.25	1.21	0.38	0.45	6.91
Mean	0.57	2.05	0.14	0.48	0.29	0.09	3.62
SD	0.12	0.49	0.05	0.23	0.06	0.08	0.73

Italic signifies the detection limit.

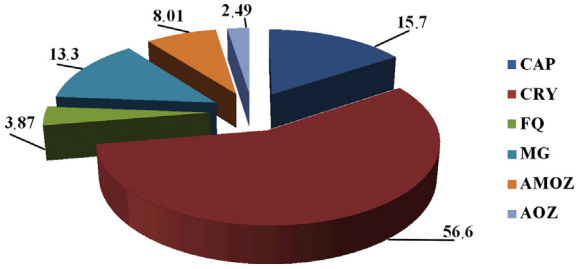

Fig. 3. Percentages content of each antimicrobial in fish tissue.

undertaken to obtain baseline data that could be used to estimate Canadians' dietary exposure to these residues. The residue found most commonly was AOZ (0.50–2.0 μg kg^{-1} wet weight); AMOZ (0.40 μg kg^{-1}) was detected in a single sample and CAP (0.40 μg kg^{-1}) in another (Tittlemier et al. 2007). In line with our data, these findings indicate that Canadians and many other individuals are exposed to variable concentrations of some banned veterinary drugs through consumption of some aquaculture products.

In conclusion, the use of illegal antimicrobials in aquaculture is widespread and largely unregulated and undocumented all over the world, resulting in consumer exposure to residues and contributing to selection of resistant bacteria. The strength of our study is that it provides an accurate assessment of the residues of banned antimicrobials detected in aquaculture fish and feed. The fact that we evaluated a single Sicilian aquaculture plant, albeit possibly a weak point, did however provide baseline data in view of a larger and more exhaustive study. Surveys of larger samples of fish and feeds are needed to achieve more reliable consumer risk assessments. Consumer protection policies aimed at reducing drug residues in aquaculture products must therefore involve the entire supply chain through wider and more frequent monitoring.

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