

## The Endocrine Disruption Induced by Ampicillin and Amoxicillin in Japanese Medaka (*Oryzias latipes*)

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**Abstract:** The study was designed to determine the estrogenic effect of some penicillins on endocrine function in adult Japanese medaka (*Oryzias latipes*). Vitellogenin (Vtg) produced in male fish has been used for a biomarker to study endocrine disrupters. 17 $\beta$ -estradiol (E<sub>2</sub>) was used a positive control that was induced Vtg in male fish. Result of total protein quantification and ELISA for female and male fish were exposed to 17 $\beta$ -estradiol 10 ng/ml for 3~5 days. As a result, male fish exposed to amoxicillin respectively appeared 0.75, 0.23, 8.21 and 9.36‰ of 1, 10, 100 and 1000 ppm respectively, that value was elevated compared with control male fish. Male fish exposed to ampicillin respectively appeared 1.85, 4.68, 0.85 and 39.59‰ of 1, 10, 100 and 1000 ppm respectively, that value was elevated compared with control male fish. This study is one of the first reports suggesting potential endocrine disruption of some penicillins in aquatic ecosystem. These results suggest that vitellogenin and estrogen receptor induction patterns alter in male medaka treated with selected estrogenic compounds, and that these results may be useful molecular biomarkers for screening estrogenic EDCs (endocrine-disrupting chemicals) in the shortest possible time.

**Keywords :** penicillin, ampicillin, amoxicillin, endocrine disruption, vitellogenin, Japanese medaka (*Oryzias latipes*), ELISA, Western blot

### Introduction

Medicines have an important role in the treatment and prevention of disease in both humans and animals. But it is because of the very nature of medicines that they may also have unintended effects on animals and microorganism in the environment. Although the side effects on human and animal health are usually investigated in thorough safety and toxicology studies, the potential environmental impacts of the manufacture and use of medicines are less well understood and have only recently become a topic of research interest (Boxall, 2004a; Kang *et al.*, 2005). The powerful antibiotic penicillin was discovered by Alexander Fleming in 1928 when he observed, by chance, that bacterial growth was inhibited by a contaminating mold (*Penicillium* spp.). Since then, many synthetic derivatives of penicillin have been made and used for a wide spectrum of applications. Ampicillin is one of the

most useful of these derivatives and serves as a highly effective medication to quench many bacterial infections. Ampicillin is prescribed to treat or control infections caused by susceptible bacteria. Side effects include fever, joint pain, swelling, skin rash, hives, and itching. It is suspected to cause certain types of cancer in animals, including humans. Do not use ampicillin in rabbits, guinea pigs, chinchillas, or hamsters since it will affect the normal bacteria in the gastrointestinal tract and possibly cause a fatal diarrhea. Thus, ampicillin is not effective against infections caused by viruses or parasites such as worms (Lullmann *et al.*, 2000).

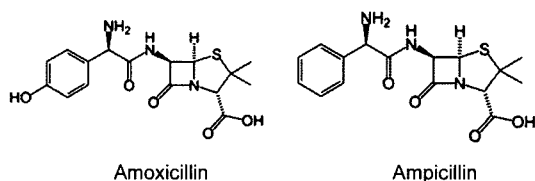
Amoxicillin is an antibiotic in the class of drugs called penicillins. It fights bacteria in the body. Amoxicillin is used to treat many different types of infections, such as tonsillitis, pneumonia, ear infections, bronchitis, urinary tract infections, gonorrhea, and infections of the skin. Amoxicillin is a broad spectrum penicillin with a bactericidal effect. Amoxicillin inhibits the normal development in the bacterial cell wall of the peptidoglycan network structure. Amoxicillin has the important property that it can reach the peptidoglycan network more quickly than ampicillin. When tissue fluid

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**Table 1.** Veterinary medicines by sales amount in 2005

Ingredient	CAS No.	Use	Sales amount* (kg)
Ampicillin	69-53-4	antibiotics	208,300
Amoxicillin	268787-78-0	antibiotics	811,910

\*KAHPA 2005 (Korea Animal Health Products Association).

**Fig. 1.** Structures of compounds used in this study.

concentrations are similar to those of ampicillin, bactericidal concentrations of amoxicillin reach the required site much more quickly. In all animals, amoxicillin, administered orally, is absorbed rapidly and completely from the gastrointestinal tract and distributed to the various tissues. Absorption is not inhibited by the presence of food particles, so that amoxicillin is much better absorbed than ampicillin. Amoxicillin is acid-resistant. A fraction of the administered dose can be metabolized to penicilloic acid derivatives, and may cause allergic reactions. Amoxicillin is excreted in the kidney by glomerular and active tubular filtration. However, most of it is excreted via the bile (Lullmann *et al.*, 2000).

The aim of this study was to determine the effects of penicillins on several aspects of endocrine function in Japanese medaka, specifically vitellogenin production from the male medaka. An understanding of the nature and magnitude of the response to ampicillin and amoxicillin will act as a baseline for further investigations, which will focus on the estrogenic potential of the environment and human health.

## Materials and Methods

Animals used in this study were d-rR medaka (*Oryzias latipes*), which contain a red pigment color marker on the male Y chromosome. Fish with an XY chromosome have an orange-red phenotype and XX are white. Medaka (d-rR) of about 2 months was kindly provided by the Korea

Institute of Toxicology (KIT), in Korea. First-generation medaka eggs obtained from the Laboratory of Freshwater Fish from the Bioscience Center of Nagoya University, in Japan. The fish were placed under a summer photoperiod (16:8-hour light:dark) and fed exclusively with commercial food (Tetramin<sup>®</sup>) twice a day. Water temperature was maintained at  $25 \pm 1^\circ\text{C}$ . The test equipment and glass aquarium containing were cleaned at least once a week to prevent any dense bacterial or algal growth. Residual food and feces in the glass aquarium containing were removed daily. Every 7 days all groups of experimental animals were placed into new aquarium with complete renewal of tank water.

Fish were thawed on ice and weighed. Whole body was minced and individually homogenized in ice-cold phosphate-buffered saline (PBS; pH 7.3) with a 1 g : 10 ml (weight : volume) ratio of wet mass to buffer volume in a glass homogenizer. The homogenate was centrifuged at  $4^\circ\text{C}$ ,  $8,000 \times g$  and collect the supernatant. The supernatant was withdrawn and immediately frozen at  $-80^\circ\text{C}$  until use. Protein contents were determined at 595 nm with spectrophotometer by Bradford assay. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sample and medaka Vtg standard (Biosense BATCH-0501) were used at this assay (Kang *et al.*, 2006).

SDS-PAGE samples, whole body sample of male, female and  $E_2$ -treated male medaka, were electroblotted by use of a Western Blot System (Bio-Rad, Mini Trans-Blot,) to a transfer membrane (nitocellulose membrane, Bio-Rad 162-0115). After shaking with primary antibodies (1:500; mouse anti-striped bass Vtg monoclonal antibody; Biosense CK-4B3) and the membrane was incubated shaking with secondary antibody (1:1000; Peroxidase-Labeled Affinity Purified Antibody to Mouse IgG KPL 04-18-18). The membrane was incubated shaking with ABC reagent and DAB substrate reagent (InnoGenex A-0401). (Kang *et al.*, 2005). The microtiter was set up with 96-well microtiter plates (EnbioTec IBTM-3500, Japan). Absorbances were determined at 450 nm with spectrophotometer (Kazuto *et al.*, 2002).

The vitellogenin concentrations were calculated

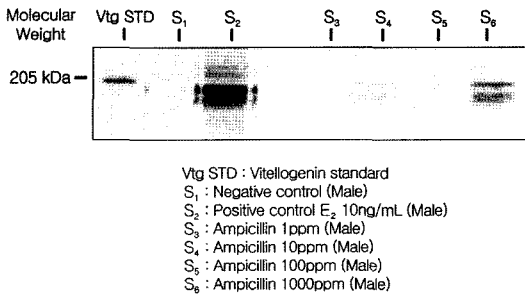
by this equation  $Vtg\ Con. (\%) = C/D \times 1,000$  at A : ELISA (Enzyme-Linked Immunosorbent Assay) testing value, B : Protein quantitative analysis value, C : Vitellogenin= Values calculated that injected A into a ELISA standard curve, D : Total protein= Values calculated that injected B into a Protein quantification standard curve.

One-way analysis of variance (ANOVA) was performed to detect differences between treatment groups. Pairwise differences were determined using Tukey-Kramer post-hoc test.

**Results**

Male and female fish were exposed to 1, 10, 100, and 1000 ppm of ampicillin. Western blot results showed approximately 205 kDa, that is similar to Myosin at High Molecular Weight Range Sigma Maker. Vtg band was showed fainted to 1000 ppm for ampicillin (Fig. 2). Vtg concentration of ampicillin was quantified by total protein quantification and ELISA (Table 2, Fig. 3). Exposure of the male fish to ampicillin of 1, 10, 100, and 1000 ppm produced Vtg concentrations of 1.85, 4.68, 0.85 and 39.59‰, respectively, that value was elevated than control male fish (0.14‰).

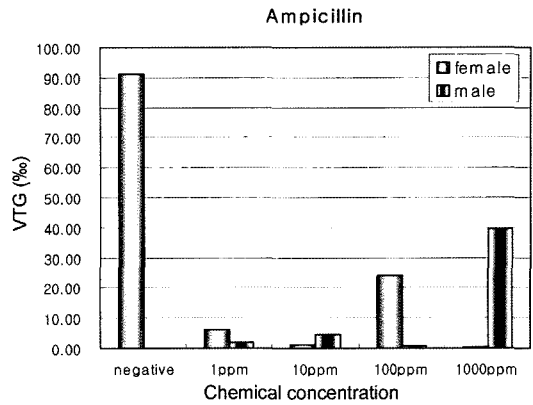
Male and female fish were exposed to 1, 10, 100



**Fig. 2.** Result of Western blot for male fish which were exposed to ampicillin 1, 10, 100, and 1000 ppm for 3~5 days.

**Table 2.** Vitellogenin contents at female and male which were exposed to ampicillin 1, 10, 100, and 1000 ppm for 3~5 days

Ampicillin		Exposure concentration (ppm)			
		1	10	100	1000
Vtg (%)	Female	6.26	1.35	23.86	0.43
	Male	1.85	4.68	0.85	39.59

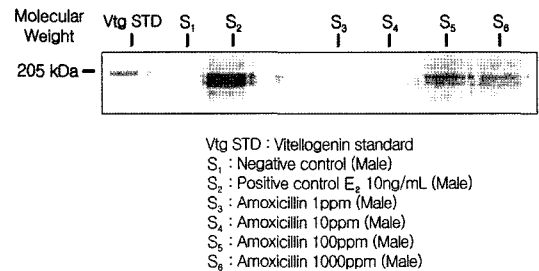


**Fig. 3.** Vitellogenin induction by ampicillin in Japanese medaka.

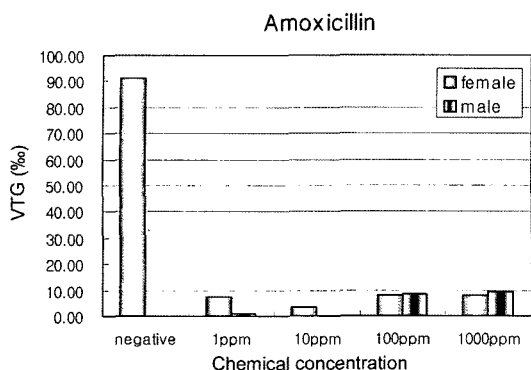
and 1000 ppm of amoxicillin. Western blot results showed approximately 205 kDa, that is similar to Myosin at High Molecular Weight Range Sigma Maker. Vtg band was showed fainted to 100 and 1000 ppm for chlortetracycline (Fig. 4). Vtg concentration of amoxicillin was quantified by total protein quantification and ELISA (Table 3, Fig. 5). Exposure of the male fish to amoxicillin of 1, 10, 100 and 1000 ppm produced Vtg concentrations of 0.75, 0.23, 8.21 and 9.36‰, respectively, that value was elevated than control male fish (0.14‰).

**Table 3.** Vitellogenin contents at female and male which were exposed to amoxicillin 1, 10, 100, and 1000 ppm for 3~5 days

Amoxicillin		Exposure concentration (ppm)			
		1	10	100	1000
Vtg (%)	Female	7.13	3.27	8.05	8.05
	Male	0.75	0.23	8.21	9.36



**Fig. 4.** Result of Western blot for male fish which were exposed to amoxicillin 1, 10, 100, and 1000 ppm for 3~5 days.



**Fig. 5.** Vitellogenin induction by amoxicillin in Japanese medaka.

**Table 4.** Initiation concentrations of some penicillins which were inducing endocrine disruption in male medaka

Antibiotics	Initiation concentrations (ppm)
Ampicillin	1,000
Amoxicillin	100

The vitellogenin induction by penicillins in Japanese medaka were showed. The initiation doses of two penicillins were showed at Table 4. The vitellogenin induced by ampicillin from the concentration 1,000 ppm. Amoxicillin was showed the vitellogenin induction from 100 ppm. Amoxicillin was more stronger vitellogenin inducer at fish than ampicillin.

## Discussions

Many of man-made chemicals, so-called endocrine disruptors, are widespread in the environment in connection with human life, and seem to have a serious affect on the health and reproduction of people or wildlife (Boxall, 2004b). Vitellogenin has been proposed as an ideal biomarker for the preliminary screening of the estrogenic effects of the endocrine disruptors and medaka Vtg bioassay would be a useful tool for mechanistic studies of such substances (Kazuto *et al.* 2002).

However, female medaka did exhibit significant differences in Vtg induction (Yamaguchi *et al.* 2005). Exposure of the female fish to ampicillin of 1, 10, 100 and 1000 ppm, produced Vtg concentrations of 6.26, 1.35, 23.86 and 0.43%,

respectively. After termination of exposure by ampicillin, the Vtg concentration dropped to 1/4~1/200 of in non-exposed fish. Exposure of the female fish to amoxicillin of 1, 10, 100 and 1000 ppm, produced Vtg concentrations of 7.13, 3.27, 8.05 and 8.05%, respectively. After termination of exposure by amoxicillin, the Vtg concentration dropped to 1/10~1/30 of in non-exposed fish.

This study demonstrated that the endocrine disruption induced by antibiotics in male and female medaka are affected by treatment with selected estrogenic compounds, and suggests that these results may be useful molecular biomarkers for screening EDCs in the shortest possible time.

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