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Microbiological and Chemical Detection of Antibiotic Residues in Livestock and Seafood Products in the Korean Market

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Abstract The microbiological and chemical identification of antibiotic residues was attempted for livestock and seafood products including pork (n=34), beef (n=34), chicken (n=32), flatfish (n=37), armorclad rockfish (n=36), and sea bream (n=27). The meat (n=100) and seafood (n=100) samples were collected from 9 markets in 5 major Korean cities. Antibiotic substances were identified from the classes of tetracyclines, macrolides, penicillins, aminoglycosides, polyethers, peptides, sulfonamides, quinolones, chlorampenicols, and novobiocins using a microbiological assay, the Charm II test and high performance liquid chromatography (HPLC) with ultra violet (UV) and fluorescence detectors. The results showed that 2 tetracyclines (oxytetracycline and tetracycline) and 3 quinolones (ciprofloxacin, norfloxacin, and enrofloxacin) were detected in 4 samples of flatfish among all 100 seafood samples tested. No antibiotic residues were detected in the 100 livestock product samples tested. The amounts (min-max, mg/kg) of the residual antibiotics were as follows; tetracycline 0.78-0.85, oxytetracycline 0.49-0.74, ciprofloxacin 0.09-0.83, norfloxacin 0.01-0.21, enrofloxacin 0.12-2.98. These data indicate that the total detection rate of antibiotics in livestock and seafood products was approximately 2%.

Keywords: microbiological identification, chemical identification, residual antibiotics

Introduction

As the livestock and fishery industries become modernized, antibiotics are intensively used in the treatment and prophylactic control of bacterial infections in veterinary medicine, and as food additives for growth promotion in the modern farming industry. In many countries, antibiotics are used indiscriminately for the treatment of bacterial diseases in domestic animals. Furthermore, in many animal production systems the treatment of groups of animals in advance of clinical symptoms is practiced routinely, for example in connection with weaning, the movement or mingling of animals from different litters or other factors that foster the outbreak of disease (1, 2). When such drugs are administered by laymen, correct dosages are not likely to be used, nor is there a withdrawal period before slaughter. This misuse of antibiotics is a potential hazard to human health (3). In order to safeguard human health, the World Health Organization (WHO) and the Food Agriculture Organization (FAO) have set standards for acceptable daily intakes (ADIs) and maximum residue limits (MRLs) in foods (4).

These limits apply to both the parent drug or chemical and its metabolites that may accumulate and be deposited or stored within the cells, tissues, or organs following the administration of the compound.

Consumers prefer hygienic and safe livestock and marine products as fish is coming into the limelight as an important protein source. As natural marine resources are being depleted, wild fisheries are being replaced by farming. Just as in the livestock industry, fish farming tends to be performed in an overpopulated environment. Such overpopulation has degraded the quality of local waters, and poor water quality has inevitably brought about frequent outbreaks of fish disease. Although the development and propagation of farming technology increases the output of farmed fish, fish farms suffer from a variety of fish diseases that can offset the advantages of the high-speed development of farming technology (5, 6).

To minimize the economical loss and drop in productivity caused by disease, animal drugs are being used. These animal drugs include antibiotics, synthetic antibiotics, hormones, antihelmintic medicines, disinfectants, anesthetics, nutrition-promoting medicines, and the like (7-11). These drugs are orally administered to fish in farms not only to cure but also to prevent diseases (5).

The intense usage of antibiotics has led to a wide distribution of antibiotic resistance among bacterial species, including resistance to tetracycline and penicillin (8). Concerns about food hygiene have arisen regarding the presence of drug residues in livestock products. It is possible for antibiotic residues to have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or to indirectly cause problems through the induction of resistant strains of bacteria (12). In the meantime, the 11th CODEX Committee Report warned that microbiological and clinical evidence indicates that it is harder to cure patients when they are infected with antibiotic-resistant bacteria and resistance-determining factors transferred from animals (13). Thus the use of antibiotics in livestock and fish has become problematic in terms of public health. The control of antibiotic usage in

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animal farming is possible by monitoring antibiotic residues in various biological samples. In addition to limiting the spread of resistance, the monitoring of residues also prevents the introduction of potentially allergenic antibiotics into food products and ensures that such residues do not interfere with food production processes (14).

The aim of this study was to provide a scientific and systematic basis for the implementation of various antibiotic residues in livestock and marine products in domestic markets.

Materials and Methods

Food sample collection All food samples were of Korean origin and collected in 5 South Korean cities and 4 metropolitan areas from January to December 2003. Livestock (beef, pork, and chicken; n=100) and marine samples (flatfish, armorclad rockfish, and sea bream; n= 100) were collected from livestock markets, traditional markets, and marine product wholesale markets. Each sample, typically 200 g, was first minced using a tissue homogenizer, then divided into 20 g portions and stored at -20°C in airtight containers until analysis.

Microbiological analysis ***Standard substances*:** A total of 31 antibiotics were analyzed in the present study: (a) 4 tetracycline antibiotics; oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC), (b) 4 marcrolide antibiotics; tylosin, erythromycin, spiramycin, and tilmicosin, (c) 3 penicillin antibiotics; penicillin, ampicillin, and amoxillin, (d) 3 aminoglycosides; hygromycin B, gentamicine, and spectinomycin, (e) 2 polyether antibiotics; monensin and salinomycin, (f) 2 peptide antibiotics; virginiamycin and bacitracin, (g) 5 sulfa antibiotics; sulfamerazine, sulfamethazine, sulfamonomethoxine, sulfadimethoxine, and sulfaquinoxaline, (h) 6 quinolone antibiotics; oxolinic acid, ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENO), and danofloxacin (DAN), (i) chloramphenicol, and (j) novobiocin.

Microbiological analysis was performed according to the method of Myllyniemi *et al.* (15). The test strains used were *Bacillus megaterium* ATCC 9885, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* var. *mycosides* ATCC 11778, and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149, grown on nutrient agar (Difco®, Voigt Ltd., Kansas City, MO, USA), Mueller-Hinton (MH) agar (Difco®), or A-K No. 2 sporulating agar (BBL®, Voigt Ltd.). Streptomycin of the antibiotic susceptibility test was used with BD Sensi-DiscTM (i.d. 6 mm, 10 μg) (BBL®). Other chemicals used were sodium chloride (Wako Chemicals, Richmond, VA, USA), trimethoprim (Sigma, St. Louis, MO, USA) and methanol (Merck, Darmstadt, Germany).

All samples were screened using a Charm II test antibiotic inhibition assay screening kit (Charm Sciences Inc., Lawrence, MA, USA). The kit tests identified unacceptable residue levels for drugs belonging to any of the following antimicrobial groups; β-lactams (penicillin G), aminoglycosides (streptomycin), and tetracycline, and MSU extraction buffer (Charm Sciences Inc.) and M2 buffer (Charm Sciences Inc.) were used for sample extraction.

***Antibiotic analysis*:** Stock solutions (100 μg/mL) of tetracyclines (OTC, TC, CTC, DC) and quinolones (OFL, NOR, CIP, ENO, DAN) were prepared by dissolving in 10 mL of methanol (100 ppm) and stored at -10°C in low actinic glassware. A mixed working solution (10 μg/mL) was prepared every 2-3 days by combining 10 mL of each stock solution with 100 mL of mobile phase (10 ppm).

B. megaterium, B. substilis, B. cereus, and *B. stearothermophilus* were used as spore suspensions, and overnight cultures of *E. coli* on nutrient agar slants suspended in sterile saline were used (Difco Laboratories, Detroit, MI, USA). The culture media used were MH medium, antibiotic medium No. 2, 5, and 8, (Difco Laboratories). The culture conditions for each microorganism are described in Table 1. Each sample was tested using directed methods. Sliced meat or seafood samples (1×1×0.2 cm) were placed on the surface of each assay plate, kept at 0-4°C for 1 hr, and then incubated for 16-18 hr at 45°C for *B. megaterium*, 37°C for *B. substilis* and *E. coli*, 30°C for *B. cereus*, and 55°C for *B. stearothermophilus*. The degree of antibiotic resistance to each antimicrobial agent was determined by measuring the width of the antibiotic resistance band, with more than 1.0 mm considered to be positive.

The samples of 10 g were homogenized with 30 mL of MSU buffer in 50 mL screwcapped polypropylene centrifuge tubes for 1 min. After heating at 80°C for 45 min, the sample was condensed for 10 min in ice. The homogenate was centrifuged at 1,750×g for 10 min and, to remove fat, filtered through Whatman No. 1 filter paper into a 100 mL beaker. The supernatant was titrated with M2 buffer to pH 7.5. Following analysis using the Charm II test, positive samples were subjected to high performance liquid chromatography (HPLC) analysis. The Charm II system is sensitive to tetracycline at 20 μg/mL in foods. The antibiotic residues in positive samples were confirmed by comparison with spiked samples.

Chemical analysis ***Standards and solvents*:** All antibiotic standards (OTC, TC, CTC, DC, oxolinic acid, OFL, NOR, CIP, ENO, and DAN) were purchased from Sigma Co. All solvents [methanol, oxalic acid, ethylenediamine tetra-acetic acid (EDTA), hexane, ethyl acetate, dichlomethane] were HPLC grade and purchased from Sigma and Merck, and C_{18} powders were obtained from J. T. Baker (Phillipsburg, NJ, USA).

***Tetracycline analysis*:** HPLC analysis for tetracyclines was carried out as follows. Sample (0.5 g) was mixed with 2 g of C_{18} powder, 0.05 g of oxalic acid, and 0.05 g of EDTA. The mixeture was loaded into a syringe (10 mL) with Whatman filter paper, and washed with 8 mL hexane, 8 mL dichlromethane. The filtrate was evaporated to dryness using a vacuum manifold, eluted with 8 mL of methanol containing 0.01 M oxalic acid and dried at 40°C. The residues were dissolved in 1 mL of mobile phase by sonication for 5 min and then centrifuged at 3,000×g for 15 min. The supernatants were filtered using a 0.45-μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA). The extract was analyzed, identified, and quantified using the HPLC methods shown in Table 1.

***Quinolone analysis*:** HPLC analysis for quinolones was carried out as follows. Each sample (0.5 g) was mixed with 2 g of C_{18} powder and 0.05 g of oxalic acid. The mixed sample was loaded into a syringe (10 mL) with

Table 1. Multiple bioassay plates

Microorganism	Medium	Incubation temp. (°C)
Bacillus megaterium	MH medium[1)]	45
Bacillus subtilis	Antibiotic medium No. 5	37
Bacillus cereus. var. *mycosides*	Antibiotic medium No. 8	30
Bacillus stearothermophilus var. *calidolactis*	Antibiotic medium No. 2	55
Escherichia coli	Antibiotic medium No. 2	37

[1)]Miller Hinton (MH) contains trimethoprim (TMP) solution.

Table 2. Operating conditions of HPLC for tetracycline and quinolone antibiotics

Condition	Tetracyclines	Quinolones
Instrumentation	HPLC system	
Column	Shiseido Capcell Pak C_{18} (10 μm, 250×4.0 mm, i.d.)	Zobax-SB (5 μm, 250×4.6 mm, i.d.)
Flow rate	0.5 mL/min	1 mL/min
Mobile phase	0.01 M aqueous oxalic acid/acetonitrile/methanol (v/v, 7/2/1)	(4 mL phosphoric acid + 4 mL triethylamine/1 L,ddw)/ methanol (v/v, 77/23)
Detector wavelength	UV detector 360 nm	Fluorescence detector Ex, 295 nm: Em, 455 nm

Whatman filter paper, and washed with 8 mL hexane, 8 mL ethyl acetate. The filtrate was evaporated to dryness using a vacuum manifold, eluted with 8 mL methanol and dried at 45°C. The supernatants were filtered using a 0.45-μm Acrodisc filter (Gelman Sciences). The extract was analyzed, identified, and quantified using HPLC methods shown in Table 2.

***Validation of HPLC analysis*:** To determine the HPLC recovery rates, tetracycline, and quinolone standard solutions were prepared at a final concentration of 200 and 100 ppb, respectively. The tetracycline test solution was added to a flatfish sample and quinolone test solution was added to flatfish and chicken samples, and the recovery rates were measured. The limit of detection (LOD) and limit of quantitation (LOQ) were estimated as the concentration of analyte which yielded a signal-to-noise (S/N) ratio of at least 3/1 and 5/1, respectively.

Results and Discussion

Screening tests using microbiological and Charm II test assays A microbiological assay was conducted on a total of 200 specimens including 34 beef, 34 pork, 32 chicken, 37 flatfish, 36 rockfish, and 27 sea bream samples as presented in Table 3. Fourteen of a total 200 samples (7%) turned out to be positive for antibiotic residues including 4 pork (11.7%), 4 beef (11.7%), 5 flatfish (13.5%), and 1 sea bream sample (3.7%). The antibiotic residues are presumed to include tetracyclines, quinolones, aminoglycosides, and penicillins based on a microbe estimation table in the Korean Food Code (16). The results of antibiotic assays confirmed that of the 9 positive samples in the microbiological assay, 2 flatfish samples contained tetracyclines (Table 4). Other samples positive for β-lactams, aminoglycosides, and tetracyclines based on the microbiological assay might contain very low concentrations or be false positives. Because quinolones are not detectable with the Charm II test assay, HPLC analysis was carried out on the all quinolone-positive samples based on the microbiological assay.

Table 3. Positive reactions in samples by microbiological assay

Sample	No. of positive	(%)	Remark[1)]
Pork (n=34)	4	11.7	T:3, A:1
Beef (n=34)	4	11.7	Q:2, P:2
Chicken (n=32)	-	-	-
Flatfish (n=37)	5	13.5	T:2, Q:4, P:1
Armorclad rockfish (n=36)	-	-	-
Seabream (n=27)	1	3.7	Q:1
Total (n=200)	14	7	

[1)]T, tetracyclines; Q, quinolones; A, aminoglycosides; P, penicillins.

Table 4. Antibiotics detected by Charm II test

Source	No. of sample	Test antibiotics[1)]		
		β-Lactams	Aminoglycoside	Tetracycline
Pork	4		- (1)	- (3)
Beef	2	- (2)		-
Flatfish	3	- (1)		+ (2)
Total	9	0	0	2

[1)]- (negative), + (positive), ( ) number of samples.

Quantitative analysis using HPLC A total of 9 specimens presumed to include 4 tetracyclines and 5 quinolones were analyzed using HPLC combined with a ultra violet (UV) or fluorescence detector, respectively. The 2 flatfish samples positive for TC had residue levels of 0.49 and 0.74 mg/kg of OTC, and 0.78 and 0.85 ppm of TC as shown in Table 6. Residue levels of the 3 quinolone antibiotics norfloxacin, ciprofloxacin, and enrofloxacin detected in 4 flatfish samples were as follows: 0.01-0.21 mg/kg NOR, and 0.09-0.83 mg/kg CIP, and 0.12-2.98 mg/kg ENO as shown in Table 7. Among the total of 200 specimens, the 100 livestock product specimens had no evidence of antibiotic

Table 5. Recovery rate of tetracyclines and quinolones[1)]

Sample	Fortification level (μg/kg)	Recovery rate (%)								
		Tetracyclines				Quinolones				
		OTC[1)]	TC	CTC	DC	OFL	NOR	CIP	ENO	DAN
Flatfish	200	73.0	57.0	64.5	66.9					
Flatfish	100					64.6	63.4	67.5	64.3	67.2

[1)]OTC, oxytetracycline; TC, tetracycline; CTC, chlortetracycline; DC, doxycycline; OFL, ofloxacin; NOR, norfloxaxin; CIP, ciprofloxacin; ENO, enrofloxacin; DAN, danofloxacin.

Table 6. Residues of tetracyclines in samples by HPLC

Source	Code no. of sample	Result[1)] (mg/kg)			
		OTC	TC	CTC	DC
Flatfish	GJ-15	0.49	0.78	-	-
Flatfish	GJ-18	0.74	0.85	-	-

[1)]OTC, oxytetracycline; TC, tetracycline; CTC, chlortetracycline; DC, doxycycline.
*LOD (LOQ): OTC, TC → 0.05 (0.08) mg/kg, CTC, DC → 0.1 (0.17) mg/kg.

Table 7. Residues of quinolones in samples by HPLC

Source	Code no. of sample	Result[1)] (mg/kg)				
		OFL	NOR	CIP	ENO	DAN
Flatfish	DJ-14	-	0.01	-	0.12	-
Flatfish	DJ-17	-	-	0.09	-	-
Flatfish	GJ-15	-	0.21	0.83	2.98	-
Flatfish	GJ-18	-	0.18	0.56	1.82	-

[1)]OFL, ofloxacin; NOR, norfloxaxin; CIP, ciprofloxacin; ENO, enrofloxacin; DAN, danofloxacin.
*LOD (LOQ): NOR, CIP, ENO → 8 (10) μg/kg, OFL → 10 (16) μg/kg, DAN → 5 (8) μg/kg.

residues and only 4 marine products had evidence of antibiotic residues. Therefore, the total detection rate is about 2% as shown in Table 6 and 7. These data show that the confirmation of TC and quinolone antibiotic residues in 200 livestock and seafood samples can be effectively accomplished with screening tests such as microbiological and Charm II test assays and confirmed using high performance liquid chromatography with UV and fluorescence detectors. These methods were able to provide preliminary identification of the possible residues present in the samples based on retention time (17).

Method validation using HPLC analysis The recovery rates for tetracyclines were 73.0% for OTC, 57.0% for TC, 64.5% for CTC, and 66.9% for DC as shown in Table 5. The LOD (LOQ) for tetracyclines were 0.05 (0.08) mg/kg for OTC and TC, and 0.1 (0.17) mg/kg for CTC and DC as shown in Table 6. The recovery rate and LOD of tetracyclines were similar to results obtained by De Ruyck *et al.* (18) for eggs and broiler meat. The recovery rates for quinolones were 75.2% for OFL, 73.6% for NOR, 74.7% for CIP, 74.8% for ENO, and 62.2% for DAN as shown in Table 5. The LOD (LOQ) values for quinolones were 8 (10) μg/kg for ENR, NOR, and CIP, 10 (16) μg/kg for OF, and 5 (8) μg/kg for DAN as shown in Table 7. The LOD of quinolones was similar to results obtained by Myllyniemi *et al.* (15) for kidney and muscle samples of bovine cattle and pigs.

In conclusion, this study of antibiotic residues in livestock and seafood samples shows that the recommended withdrawal periods for livestock and fish were followed for all antibiotic classes tested with the exception of quinolones and tetracyclines. Therefore, to minimize the impact on public health of antimicrobials in food-producing animals, one should control residues in edible tissues of food animals in which antimicrobials have been used. This study will contribute to the protection of public health and help safeguard the future efficacy of antibiotics in veterinary drugs.

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