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Target Analysis of Antibiotic Drugs in Poultry Feedstuff by Solid Phase Extraction and Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract. Seventy-five poultry feed samples of two feed types were analysed for antibiotic drugs using a simple generic solid phase extraction procedure with dichloromethane-acetonitrile after delipidation with n-hexane. Analytical separation was performed on a Waters Acquity C18 column with gradient elution consisting of water and acetonitrile. Liquid chromatography–tandem mass spectrometry (LC-MS/MS), with positive and negative electrospray ionization methods in the multiple reaction monitoring modes (MRM), was used for the quantification of 21 compounds from six classes including fluoroquinolones, sulfonamides, lincosamides, anthelmintics, macrolides and the β-lactams in a single chromatographic run of 14 minutes. All the six classes of the drugs were found in the two feed types at concentration ranging between 0.22 – 1505 ng/g. Sulfadimethoxine, sulfaguanidine, sulfamerazine, and sulfamethoxazole were the major sulfonamides in the two feed types with concentration at the part per million levels. Albendazole, penicillin-G, sulfadiazine, sulfaquinoxaline and sulfixosazole were not detected in the layers mash exclusively fed to laying birds; also, sulfamethazine and sulfamozole were the only two drugs not detected in the growers mash meant for birds raised for meat. Ciprofloxacin, sulfadimethoxine, sulfamethoxazole, sulfamerazine, and sulfaguanidine were the most prominent antibiotic drugs in the two feed types. Results from the present study suggest that feed millers surreptitiously fortify their feeds with antibiotics without declaring same, thus exposing poultry chickens to sub-therapeutic dosages of the drugs. It is evident that self-regulation for safety in the poultry industry should be discouraged thus relevant authorities must take steps to reduce and control the use of antibiotics to protect public health.

Keywords: Poultry feedstuff, Nigeria, Antibiotic drugs, LC-MS/MS

1. Introduction

The use of antibiotics in poultry feeds results in increased growth rate, improvement in egg production and hatchability [1]. Poultry birds, however, retain bacteria strains resistant to the antibiotics they are fed thus flourishing in the intestinal flora as well as the muscle and final transfer to human along the food chain [2]. Several countries have put a ban on the use of antibiotics in feeds because it portends a long-term health hazard to humans by promoting antibiotic-resistant pathogens, an important threat to modern medicine [1,3]. As part of efforts to raise awareness on the incidence of antimicrobial resistance and deliver solutions to fight antibiotic-resistant pathogens, this study examined antibiotic drugs in poultry feeds since the food chain is a significant source of antimicrobial resistance and drug allergic reactions [4].

2. Materials and method

2.1 Standards

Stock solutions of targeted analytes were prepared from commercially available standard antibiotics. 10 mg of each pure standard was dissolved in 10 mL of solvent to prepare 1 mg/mL solution. Working solutions of antibiotic mix were prepared from the stock stored at 4^{o}C.

2.2 Sample Preparation

The samples were prepared in a three stage previously validated method as described by Mu et al. [5]. 0.50 g of feedstuff sample was placed in a mortar separately with 0.05 g Na_2-EDTA and 0.05 g oxalic acid was added to the mortar and gently ground with the sample to obtain a uniform blend.

2.3 SPE Clean-up

Samples were cleaned-up with an ENVI-8® SPE cartridge that had been previously conditioned with 5 mL analytical grade n-hexane (EMSURE®). The sample blend was afterwards introduced onto the cartridge and drained with 6 mL of the n-hexane, and the analytes eluted with 8 mL acetonitrile (LiChrosolv®) – dichloromethane (1:1, v/v). The eluate was evaporated to near dryness under a gentle stream of nitrogen, and the residue re-dissolved in 1 mL 10% methanol (LiChrosolv®) and vortexed. The final solution was filtered with a 0.45 µm disposable syringe (Acrodisc®) and 10 µL of the filtrate injected into the Nexera UHPLC system [5].

2.4 Chromatographic Separation

The chromatographic separation was performed on a Nexera UHPLC system with a Waters Acquity UPLC® BEH C18 column (2.1 mm X 100 mm, 1.7 µm, particle size) with the column compartment maintained at 40^{o}C. Water and acetonitrile with 0.1% formic acid were the mobile phases in gradient elution with a flow rate of 0.2 mL/min as listed in Table 1.

Table 1: Liquid Chromatography gradient

Time (min)	A%	B%
0	98	2
10	0	100
12	0	100
14	88	12

2.5 MS/MS Detection

Sample analysis was performed on Shimadzu Triple Quadrupole Mass Spectrometer (LC-MS 8040®) system using electrospray ionization (ESI) + and – polarity switch. Analytes were monitored in the MRM mode in a dwell time of 100 ms for each channel, and an event time of 0.309 sec to achieve optimal peak shapes and sensitivity.

Table 2: Analytes, retention time (RT) and MRM transition with collision energies (CE)

Antibiotics	RT(Min)	Transition 1 (amu) Ch 1	Transition 2 (amu) Ch 2	Transition 3 (amu) Ch 3
Albendazole*	7.243	264.5/232.15(8.0)	264.5/264.50(26.0)	264.50/264.50(35.0)
Ampicillin	7.447	351.10/160.05(-14.0)	351.10/106.15(-30.0)	351.10/107.00(-25.0)
Azithromycin	6.076	749.50/591.30(-32.0)	749.50/116.10(-48.0)	749.50/158.10(-42.0)
Ciprofloxacin	3.194	333.10/315.15(-21.0)	333.10/232.10(38.10)	333.10/289.20(-17.0)
Erythromycin	6.969	734.60/158.15(-33.0)	734.60/576.30(-21.0)	734.60/83.00(-53.0)
Levofloxacin	5.594	362.50/261.15(-29.0)	362.50/318.15(-19.0)	362.50/219.05(-43.0)
Lincomycin	5.606	407.50/126.15(-30.0)	407.50/359.30(-18.0)	-
Mebendazole	7.419	297.10/265.05(-35.0)	297.10/256.20(-6.0)	297.10/105.05(21.05)
Mebendazole*	7.382	294.50/262.20(9.0)	294.50/294.50(40.0)	294.5 /294.5 (52.0)
Penicillin G	4.856	367.50/160.0(-16.0)	367.50/91.10(-48.0)	367.50/114.10(-36.0)
Phebendazole	8.294	300/268.00(-21.0)	300/159.0(-37.0)	300/131.05(-50.0)
Phenbendazole*	8.294	298.2/266.10(11.0)	298.2/189.10(31.0)	298.2/160.05(50.0)
Sulfadiazine	5.875	252/156.0(-15.0)	252/157.0(-16.0)	252/93.10(-29.0)
Sulfadimethoxine	7.148	311.90/156.05(-23.0)	311.90/157.05(-22.0)	311.90/108.05(-30.0)
Sulfadimethoxine*	7.372	309.5/66.15(38.0)	309.5/154.0(27.0)	309.5/122.20(45.0)
Sulfaguanidine	5.162	216.0/93.10(-26.0)	/216.0/157.0(-13.0)	216.0/60.15(-17.0)
Sulfamerazine	6.104	266.0/156.95(-16.0)	266.0/ 93.10(-34.0)	266.0/64.95(-49.0)
Sulfameter	6.398	282.0/92.15(-30.0)	282.0/93.20(-30.0)	282.0/157.0(-18.0)
Sulfamethazine	6.578	280.0/187.0(-17.0)	280.0/186.0(-17.0)	280.0/125.05(-23.0)
Sulfamethoxazole	6.578	255.0/93.05(-30.0)	255.0/157.0(-16.0)	255.0/92.15(-30.0)
Sulfamozole	4.696	269.0/156.90(-16.0)	269.0/155.95(-15.0)	269.0/92.05(-30.0)
Sulfaquinoxaline	6.024	301.90/156.05(-18.0)	301.90/108.25(-26.0)	301.90/92.10(-30.0)
Sulfaquinoxaline*	5.534	299.5/144.15(35.0)	299.5/142.20(34.0)	299.5/208.30(25.0)
Sulfasalazine	7.706	400.0/382.10(-20.0)	400.0/224.05(-30.0))	400.0/118.90(-45.0)
Sulfasalaxine*	7.706	398.1/198.15(24.0)	398.1/197.20(35.0)	398.1/92.00 (50.0)
Sulfixozazole	2.639	269.00/156.95(-13)	269.00/93.00(-27.0)	269.00/155.90 (-14.0)
Sulfixozazole*	2.254	267.2/172.1(21.0)	267.2/240.1(17.0)	267.2/171.05(19.0)
Tylosin	6.952	916.50/174.05(-41.0)	916.50/101.05(-51.0)	916.50/145.0(-39.0)

Precursor (amu)/Product (amu) *Negative electrospray ionization (CE is positive in MeV)

3. Results and discussion

The presence of 21 antibiotic drugs from different classes were established in two poultry feed types collected between May and September, 2017 in Ogun State, Nigeria.Albendazole, penicillin- G, sulfadiazine, sulfaguanidine and sulfixozazole were in the growers mash at mean concentrations, ranging between 1.33±1.50 and 56.18±1.99 ng/g with albendazole and penicillin-G at both extremes. Sulfamethrazine and sulfisoxazole were present in the layers feed at mean concentrations of 105.68±63.99 and 120.05±206.32 ng/g, respectively. All other 17 antibiotics were in both the growers and layer mash at varying concentrations (Table 3).

Ciprofloxacin, sulfadimethoxine, sulfaguanidine and sulfamerazine were the most prevalent drugs in the two feed types and they occurred at concentrations above 200 ng/g except sulfaguanidine that was 104.96±19.72 and 140.74±10.28 ng/g in the growers and layers mash, respectively. Ciprofloxacin and sulfadimethoxine had higher mean values in layers mash compared with the growers mash. Only sulfamerazine occurred more in the growers mash among the major drugs that were determined. Sulfamethoxazole with a mean concentration of 241.51±206.32 ng/g was higher in the layers mash compared with the 31.53±41.04 ng/g in the growers mash. Sulfameter was 77.47±60.45 and 61.19±22.26 ng/g, in the growers and layers mash, respectively.

The maximum residue limit for tylosin in feedstuff is 100 mg/kg and has been reported as the most abundant residue in chicken liver [6]; meanwhile, some EU members including Sweden have banned the use of antibiotics in feeds arising from concerns linked to multidrug resistance [7]. Some reports have linked the presence of antibiotic-resistant organisms in poultry products to the use of feed supplemented with antibiotics [8], and tylosin, erythromycin, penicillin, sulfonamides, fluoroquinolones and lincomycin have been reported to be present at 1 – 200 g per ton of poultry feed [9]. Tylosin was reported to be available in poultry feedstuffs from Belgium at a concentration of 0.85 – 6.32 mg/kg by Huebra and Holst [10], while Poucke et al. [11] had 0.29 – 0.41 mg/kg tylosin in feed samples from the same area. Tylosin is usually used as a growth promoter. Even though, tylosin has been banned by the EU[12], it is allowed as feed additive in China and the United States [13]. Tylosin is, however, present in feedstuffs examined in the present study. According to Diarra et al. [14], supplementation of broiler feedstuff with penicillin resulted in increased body weight among broiler chickens. The drug reportedly improved feed efficiency with reduced feed intake and this improvement was significant, thus, the addition of different antibiotics into poultry rations as seen in the present study may lead to the same effects on the birds. Meanwhile, various types of bacteria have been reported to be resistant against penicillin in chicken and the drug had refused to kill E. coli. Due to increase in antibiotic resistance in foodborne pathogens, there is a campaign to reduce the use of antibiotics at sub-therapeutic levels as growth promoters [15], and approval for the use of antibiotics as growth promoters has been withdrawn in the European Union [16].

The use of antibiotics in feeds could result to selectivedevelopment of resistanceby disease-causing bacteria, and when used as growth-promoters it imposes a selection pressure for bacteria that are resistant to antibiotics that may be used in clinical or veterinary practice, thus compromising the continued use of antibiotics for therapeutic purposes [15,17,18]. Boix et al. [19] reported the presence of α – nandrolone, chlortetracycline, tetracycline, trimethoprim and salicylic acid in poultry feedstuff from Spain. Chlortetracycline, oxytetracycline, and doxytetracycline were found in poultry feedstuffs from Poland [3]. Poultry feedstuff analysed from the Valencia Region in Spain as part of a residual control plan also showed the presence of growth promoting agents [20]. Growth promoting agents were, however, not determined in the present study. Ampicillin residue was reported in tissues of poultry fed ampicillin medicated drug at 40 mg/kg after a 2-day withdrawal period suggesting that drug residues in animal tissues could arise from their presence in the feedstuff. However, the concentration of the drug was below the 30 ng/g acceptable maximum residue limit for the drug in Japan [21].

Table 3: Distribution of Antibiotic Residues (n=34) in Poultry Feeds by LC-MS/MS

Antibiotics	Growers mash			Layers mash				
	Mean±SD	Min	Max	Mean±SD	Min	Max	f	p
Albendazole	1.33±1.50	0.22	2.52	ND	-	-	-	-
Ampicillin	41.49±25.65	4.31	58.54	48.49±9.30	37.55	58.54	0.26	0.63
Azithromycin	10.87±1.0	10.29	12.02	23.79±28.59	1.8	56.11	0.61	0.48
Ciprofloxacin	383.89±410.95	35.51	836.67	546.12±358.20	145.9	836.67	0.27	0.63
Enrofloxacin	46.26±13.23	32.11	58.33	63.33±7.64	56.67	71.67	3.75	0.13
Lincomycin	7.73±3.45	4.3	11.2	6.43±1.45	4.77	7.44	0.3	0.58
Mebendazole	22.67±7.22	12.5	28.39	25.74±23.00	12.26	60.11	0.07	0.81
Phebendazole	4.1±1.10	2.85	4.89	7.63±3.10	4.72	10.89	3.47	0.14
Penicillin-G	56.18±1.99	53.88	57.33	ND	-	-	-	-
Sulfadiazine	10.98±0.92	10.26	12.01	ND	-	-	-	-
Sulfadimethoxine	603.56±786.76	61.311	1505.92	628.31±458.37	126.97	1025.62	0.002	0.97
Sulfaguanidine	104.96±19.72	90.18	127.35	140.74±10.28	129.22	148.79	7.77	0.05
Sulfamerazine	206.66±29.00	173.61	227.9	105.79±85.37	29.83	221.29	3.7	0.11
Sulfameter	77.47±60.45	19.46	140.1	61.19±22.26	44.57	86.48	0.19	0.68
Sulfamethazine	ND	-	-	105.68±63.99	35.52	160.84	NA	NA
Sulfamethoxazole	31.53±41.04.52	6.6	78.9	241.51±206.32	4.89	385.67	3.00	0.16
Sulfamozole	ND	-	-	120.05±5.84	113.49	124.67	-	-
Sulfaquinoxaline	18.96±15.66	8.74	36.99	ND	-	-	-	-
Sulfasalazine	15.54±8.07	6.22	27.14	15.82±9.59	0.801	24.45	0.003	0.96
Sulfixozazole	16.97±1.02	16.25	17.69	ND	-	-	-	-
Tylosin	4.04±1.7	2.75	5.04	3.3±0.19	3.09	3.42	1.18	0.34

4. Conclusions

Results from the present study showed the presence of antibiotic drugs in poultry feeds at sub-therapeutic levels. To address the exposure to these drugs, feed millers must reduce and refine the use of antibiotics, replace antibiotics with alternatives to reduce the threat of antibiotic resistance. Control measures including improvements in food hygiene must be taken by poultry farmers to reduce the spread of zoonotic bacteria to humans via the food chain.

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