Screening of antimicrobial residues in poultry meat in Enugu metropolis, Enugu State, South East Nigeria

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Antimicrobials, Residues, Organs, Poultry, Matrix.

Summary
The aim of this study is to determine the prevalence of antimicrobial residues in poultry in Enugu Metropolis, Enugu State, Nigeria. Four organs – kidney, liver, muscle, and gizzard – were harvested from 100 commercial broiler birds and tested using the Three Plate Test (microbiological method) with *Bacillus subtilis* as test organism. Of the 100 sampled birds, 64 were positive for antimicrobial residues, with a prevalence of 64%. Out of 400 organs, 155 were positive for antimicrobial residues, with different prevalence values observed in the harvested organs. Our findings indicate an association between the type of organ and the occurrence of antimicrobial residues, with the kidney having the highest prevalence (p value: < 0.0001, Chi Square test). Overall, in this study, commercial poultry were found to contain multiple antimicrobial residues, which strongly suggest the indiscriminate use of antimicrobials in livestock production.

Screening dei residui antimicrobici nel pollame della città di Enugu, sud-est della Nigeria

Parole chiave
Antimicrobici, Residui, Organi, Pollame, Matrice.

Riassunto
Scopo di questo studio è determinare la prevalenza di residui antimicrobici nel pollame nella città di Enugu Metropolis in Nigeria. Rene, fegato, muscolo e ventriglio sono stati prelevati da 100 volatili da allevamento e testati con il *Three Plate Test* (metodo microbiologico); come prova è stato usato il *Bacillus subtilis*. Dei 100 volatili analizzati, 64 sono risultati positivi a residui antibiotici con una prevalenza del 64%. Dei 400 campioni, ne sono risultati positivi 155, con valori di prevalenza differenti negli organi indagati. Dallo studio emerge un'associazione tra il tipo di organo e l'occorrenza dei residui con una prevalenza maggiore nel fegato (p value:< 0.0001, Chi Square test). Complessivamente, questo lavoro dimostra che il pollame commerciale contiene residui antimicrobici, probabilmente per l'uso indiscriminato nella produzione zootecnica.

Introduction

Veterinary drugs, specifically antimicrobials, are widely used in poultry production for the treatment of infections, management of stress, and as additives for prophylaxis and growth promotion. These drugs tend to accumulate in tissues forming residues with concentrations above their Maximum Residue Limits (MRL) if withdrawal periods are not observed. Antimicrobial residues in food are potential allergens and may result in severe reactions in sensitised individuals. They can influence also the microbial composition and metabolic activity of the intestinal micro flora (Vollard and Clasener 1994, Nisha 2008). It is known that a link between the use of antibiotics in food animals and the development of bacterial resistance to these drugs exists (Stark 2000, Reig and Todra 2008). Other recorded pathological effects induced by antimicrobial residues in food include autoimmunity, carcinogenicity, mutagenicity, and bone marrow toxicity (Pavlov *et al.* 2008, Nisha 2008).

To buttress the importance of drug residues,

tolerance limits or maximum residue limits (MRLs), for antimicrobial residues have been set in most developed countries. Countries that do not have MRLs have, generally, adopted those established by Codex Alimentarius, which have also been adopted by the World Health Organisation (WHO). Several related programmes addressing food safety and drug residues in foods of animal origin have been developed. These include the Residue Avoidance Programme (RAP), which was initiated in 1981 by the Extension Service and Food Safety and Inspection Service (FSIS) of The United States Department of Agriculture (USDA) with the goal of monitoring food supply in order to ensure that antibiotic residue concentrations do not exceed Maximum Residue Limits. The results from RAP are then disseminated to farmers and people working in the livestock industry through an educational programme, thus helping to prevent the occurrence of drug residues. The European Union has similarly well-developed abattoir-based programmes for the surveillance and monitoring of antibacterial residues in meat (Myllyniemi 2004). In sharp contrast, Nigeria has no national programme in place for monitoring drug residues in food animals in farms and abattoirs and no strict regulations on the use of antimicrobials in livestock.

Several methods to detect antimicrobial residues in different sample matrices are available, but many of these methods are relatively expensive and time consuming. Antimicrobial residues in animals are conventionally detected through microbiological tests, including *Bacillus stearothermophillus* Disc assay (BsDA), the European four Plate Test (FPT), the German Three Plate Test (TPT), the Premi® test, and a number of other commercial kits. These tests, which are essentially qualitative, i.e only able to screen for the presence of antimicrobial residues, are based on bacterial inhibition and are used primarily as screening tools for the presence of antimicrobial residues in meat, milk, and eggs. The TPT, like the FPT, determines the presence of antimicrobials in a sample and also identifies the specific antimicrobial group/class (Haasnoot *et al.* 1999; Javadi *et al.* 2009). The test is prepared with *Bacillus subtilis* as test organism at pH 6, 7.2, and 8, hence the name TPT. The pH 6 plates detect, in particular, beta-lactams and oxytetracyclines; pH 7.2 plates detect sulphonamides; while aminoglycosides are detected by pH 8 (Chang *et al.* 2000). The only variation from the conventional EU FPT is the fourth plate, which contains Micrococcus luteus at pH 8 in order to detect beta-lactams and macrolids. The aim of this study is to determine the prevalence of antimicrobial residues in poultry in Enugu Metropolis, Enugu State-South East Nigeria, using a conventional microbiological method (TPT) with locally sourced *Bacillus subtilis*.

Materials and methods

The 3-Plate Test was used for the antimicrobial residues detection with *Bacillus subtilis* as test organism.

Isolation and identification of *Bacillus subtilis*

Five grams of soil sample from a cassava waste dump-site was weighed into 45 ml of ringer solution to form a stock mix. This was then heated at 90 °C for 1 hour to encourage the formation of spores as well as for eliminating other unwanted microorganisms. Then, 0.1 ml of the mix was inoculated in 0.4% dextrose nutrient agar and incubated at 37 °C for 24 hours.

Biochemical tests

Isolated colonies were subjected to API 50 CH biochemical tests (Biomeriuex, UK) for identification. The API 50 CH test is a standardised commercial system of 50 biochemical tests in microtubes designed for the study of carbohydrate metabolism, substrate utilisation, and enzyme production of microrganisms. It is used in conjunction with API 50 CHB/E medium for the identification of *Bacillus* species.

A suspension of the organism was prepared from a 24-hour culture of the isolated *B. subtilis* in API 50 CHB/E medium and compared with 0.5% McFarland standard. The organism was inoculated into the 50 tubes and incubated at 24-30 ºC for 24 hours. Fermentation was revealed by a colour change in the tube. The API 50 results were read using the API web software (https://apiweb.biomerieux.com/servlet/Identify).

Molecular detection of *B. subtilis*

Colonies of *B. subtilis* were emulsified in 200 µl of Tris EDTA (TE) buffer. Nextec bacterial DNA extraction kit (Nextec UK) was used to extract genomic DNA from the emulsified isolates. Primers specific to a 595 bp amplicon of *Bacillus subtilis* group's 16s rRNA gene were used at a final concentration of 0.4 M to detect the organism genome. The primer sequences and thermal profile we used have already been published (Wattiau *et al.* 2001). The PCR products were electrophoresed at 100 V for 30 minutes using 2.0% Agarose gel and compared with a DNA ladder (Promega, UK).

Antimicrobial sensitivity test

Antibiogram of the *B. subtilis* isolate was determined using the disc diffusion technique (CLSI 2011) with

commercially available discs (Oxoid, thermofisher, UK). A total of 16 antibacterial agents belonging to the following classes were used: fluoroquinolones, beta-lactams, aminoglycosides, tetracyclines, amphenicols, glycopeptides, and macrolides. A suspension of a 24-hour fresh culture of the isolate was made in 1 ml of distilled water to correspond to 0.5 McFarland standards, poured into freshly prepared Mueller Hilton agar, and then the excess was tilted off. Inoculated plates were allowed to dry for approximately 3-5 minutes and the antibiotic discs applied aseptically to the surface of the inoculated agar. The plate was incubated at 37 °C for 24 hours and read afterwards; the diametre of zone of complete inhibition was measured in millimetre scale.

Sample collection

A total number of 100 birds from the 3 major poultry markets in Enugu metropolis (Artisan market, Ogbete Main market, and Gariki market) in South East Nigeria , were randomly selected and purchased. Each market was visited twice a week for 4 weeks, and 4 birds were purchased per visit. Samples of muscle, liver, kidney, and gizzard were harvested from birds after slaughter.

Sample preparation

For each organ, a 5-g piece was weighed and macerated with equal volume of distilled water at a 1:1 ratio. The mixture was centrifuged at 5,000 rpm for 10 minutes and the supernatant decanted and stored frozen at -20 °C for analysis.

The Three-Plate Test

Nutrient agar was prepared and adjusted to pH 6, 7.2, and 8 with NaOH and HCl; the media were poured onto sterile petri dishes and seeded with *Bacillus subtilis*. Each agar plate was bored 5 times. About 100 µl of the organ extracts were then inoculated in 4 holes, each hole representing an organ. The remaining hole was inoculated with 100 µl of distilled water as negative control. Plates were incubated at 30 °C for 24 hours. A clear zone of inhibition with annular diameter ≥ 2mm indicated a positive result for antimicrobial residues (Myllyniemi *et al.* 2001).

Statistical analysis

Using Graphpad Statmate 2.0, a sample size of 100 at 90% power was calculated and significance was accepted at $p \leq 0.05$. Data from the study was analysed in GraphPad Prism Statistical software version 5.02 (www.graphpad.com).

Results

Cultural and morphological characteristics

Large, flat undulated colonies with ground glass appearance on nutrient agar, showing gram-positive spore forming small rods, occurred singly and in short chains. These were suspected to be *Bacillus subtilis*.

Biochemical and molecular tests

The organism fermented glycerol, D-glucose, D-fructose, D-Manose, D-sorbitol, inositol, and 15 other carbohydrates. The organism was identified as *Bacillus subtilis* with 99.9% accuracy using API WEB. The PCR amplified a 595 bp product of the 16S rRNA, confirming the presence of *B. subtilis*.

Antibiogram sensitivity test

The isolate, *Bacillus subtilis*, was sensitive to all tested antimicrobials except cefixime, which is not used in poultry production in Nigeria.

Antimicrobial residues in poultry meat and organs

Out of 100 birds sampled for antimicrobial residues, 45 (64%) were positive while the remaining 25 (36%) were negative.

Table I shows the ratio of antimicrobial residues in descending order of frequency in the organs as follows: kidney (60%), liver (54%), gizzard (30%), and muscle (11%). A total of 155 organs out of 400 tested positive for antimicrobial residues. A strong association was found between the occurrence of antimicrobial residues and the organ type (χ^2 value = 64.5, p value < 0.0001); the kidney had the most positive cases.

Out of the 155 positive organ samples, 25 were detected only at pH 6.0 (best detects tetracycline and β-lactams), 26 at pH 7.2 (best detects

Table I. *Antimicrobial residues in the tested organs.*

Matrix (organ)	Sample	No of positive (≥ 2 mm)	No of negative (< 2 mm)	Percentage (%) positive
Muscle	100	11	89	11
Liver	100	54	46	54
Kidney	100	60	40	60
Gizzard	100	30	70	30
Total organs	**400**	**155**	**245**	**38.8**

≥ 2 mm = inhibition zones more than or equal to 2 mm;
< 2 mm = inhibition zones less than 2 mm.

Table II. *Antimicrobial positive samples according to pH.*

Matrix	No positive	pH						
		6.0	7.2	8.0	Multiple			
					6.0/7.2	6.0/8.0	7.2/8.0	All three
Muscle	11	2	0	5	1	1	0	2
Liver	54	6	11	15	2	3	3	14
Kidney	60	11	10	9	6	3	3	18
Gizzard	30	6	5	6	1	1	4	7
Total	**155**	**25**	**26**	**35**	**10**	**8**	**10**	**41**

sulphonamides), and 35 at pH 8.0 (best detects aminoglycosides). These results are shown in Table II. Residues were detected in 69 samples with multiple pHs: 10 by pH 6.0 and 7.2; 8 by pH 6.0 and 8.0; 10 by pH 7.2 and 8.0, and 41 by the 3 pH levels.

Discussion

Up to 64% of the investigated commercial broilers in this study had detectable levels of antimicrobial residues. A previous study done in Northern Nigeria by Kabir and colleagues (Kabir *et al.* 2004) reports a prevalence of 15.7%. This disparity may be due to the fact that they only used faeces as sample in their study, which can be related to the 11% prevalence of muscle matrix tested in this study. The overall prevalence is also consistent with the findings of Ezenduka and colleagues (Ezenduka *et al.* 2014), who demonstrated a 60% prevalence rate in a similar study at Enugu State. A significant presence of different antimicrobials in chickens in the Western part of the country has also previously been reported (Dipeolu and Alonge 2002, Dipeolu and Dada 2005). Similar to other developing countries, a high prevalence of antimicrobial residues in broilers have been recorded: 39.4% and 70% were reported in Pakistan by Jabbar (Jabbar 2004) and Muhammad and colleagues (Muhammad *et al.* 2007), respectively; 52% in Iraq by Shareef and colleagues (Shareef *et al.* 2009), and 70% in Tanzania by Nonga and colleagues (Nonga *et al.* 2009). The high prevalence may indicate the excessive prescription, overuse, and abuse of antimicrobial drugs in Nigeria, similar scenario observed in most developing countries. This problem may be largely due to the unrestricted availability of antimicrobial drugs and the practice of self-medication by poultry farmers and animal handlers. The unauthorised and unprofessional exposure of poultry to veterinary drugs without adherence to recommended doses and/or withdrawal time promotes an accumulation of residues in meat and eggs.

The kidney and liver had the highest concentration of residues, at 60% and 54% respectively. These findings are similar to previous studies by Aerts and colleagues (Aerts *et al.* 1995) and Myllyniemi and colleagues (Myllyniemi *et al.* 1999), and are, indeed, used as matrix in many countries in which the level of antibiotics in meat needs to be assessed. Kidney is generally used as sample matrix as it is the organ responsible for excreting most drugs. However, other reports show that kidney may give false positive results due to the presence of natural inhibitors of bacterial growth such as lysozymes, which are often present in kidneys (Kirbis 2007), and so should be used along with other matrixes, as it was done in this study.

The detection of antimicrobials at different pH levels in the same organ implies that different classes of antimicrobials are being administered to a bird at the same time. The concurrent use of different antimicrobials that were observed in this study has been also reported by Shareef and colleagues (Shareef, *et al.* 2009) and Ibrahim and colleagues (Ibrahim *et al.* 2010). *Bacillus subtilis* at pH 6.0 generally detects oxytetracycline (OTC), which is the most widely used drug in poultry production in Nigeria (Kabir *et al.* 2004, Ezenduka *et al.* 2011), but the detection of tetracyclines (25) alone was lower in this study when compared with sulphonamides (26) detected at pH 7.2 and aminoglycosides (35) detected at pH 8. This could be explained by the fact that most positive pH 6.0 samples may have fallen into multiple pH categories.

Generally, microbiological methods are basic screening methods for detecting the presence of antimicrobial residues in food and are able to differentiate antibiotic groups. They were the earliest methods used for the detection of antibiotic residues and are still widely used. They are very cost-effective and, in contrast to immunological or receptor-based tests, have the potential to cover the entire antibiotic spectrum in only 1 test. It is however necessary to confirm the presence and concentration of antibiotics and identify specific substances within the different antibiotic groups with chemical or chromatographic methods, particularly High Performance Liquid Chromarography (HPLC) and immuno-enzymatic method like ELISA (Mitchell *et al.* 1998, Kirbis 2007).

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Antimicrobial use is widespread in Enugu State, Nigeria. This behaviour underpins the indiscriminate use of these drugs along with the lack of observation for recommended periods of withdrawal. The implementation of best practices in combination with adequate legislation regarding drug use in veterinary practice and livestock production is crucial to establish surveillance programmes for detecting drug residues in meat and other foods of animal origin. Based on findings, kidney and liver are recommended as sample matrices for screening drug residues in poultry products.

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