Annual trend in the occurrence of antimicrobial drug residues particularly Chloramphenicol using a comparative detection methods in Federal Capital Territory (FCT), Abuja, Nigeria

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Abstract: *The use of chloramphenicol is highly restricted in many countries of the world because of its toxic effects. Several disease conditions of poultry have varied incidences between seasons of the year. Higher incidences may attract wrong application of the drug amongst farmers. This study consequently assessed the misuse of antimicrobial drugs, particularly chloramphenicol across seasons. Out of 2000 chicken egg sampled in 12 months, 370(18.5%) and 120(6.0%) were positive for antimicrobial drug residues (AMDR) and chloramphenicol residues (CAPR) respectively. Occurrences between the seasons showed no significant difference (P > 0.05). Mean concentrations however showed significant difference (P < 0.05) between the seasons with dry windy/harsh and dry humid seasons showing highest and lowest mean concentrations of (0.338±0.097 and 0.165±0.057)μg/L respectively with wet rainy season showing an intermediate mean concentration of 0.273±0.020 μg/L. Both analytic methods showed good correlation with correlation coefficient, r^2 of 0.998. Accuracy was determined as average recovery rate of 84.0%.*

Keywords: *Chicken eggs, Drug residue, ELISA, FCT, Nigeria, Spectrophotometry*

I.   Introduction

Chloramphenicol belongs to amphenicol-class of antibacterial. It is an antibiotic first isolated from cultures of Streptomyces venequelae in 1947 but now produced synthetically (Aliu, 2007; Switala et al., 2007). It has a relatively simple structure and was the first broad-spectrum antibiotic to be discovered. It acts by interfering with bacterial protein synthesis and is mainly bacteriostatic. It binds to the 50s ribosomal sub-unit to inhibit peptidyl transferase of activities with the resultant inhibition of peptide bond formation and the subsequent depletion of microbial protein synthesis (Cannon et al., 1990).

CAP has been proven to posses both reversible and irreversible toxic effects in humans. The p-nitro group of CAP is responsible for the bone marrow toxicity. This toxicity could manifest in form of dose-dependent reversible bone marrow suppression. It reverses at the withdrawal of the causal agent. This form of toxicity is considered mild or less severe. The other form is the dose-independent irreversible bone marrow suppression resulting in a condition known as aplastic anaemia (Lynas et al., 1998; Papich et al., 2001). This form is fatal and could lead to death of an individual. It has a greater public health impact as it is predetermined by the genetic composition of an individual. Reduction or the withdrawals of the causal agent have little or no ameliorative impact on the subject. By extension, this attribute of the drug explains such phenomena as, no established acceptable daily intake (ADI) or no maximum residue limit (MRL) in tissues or matrices meant for public consumption. Such established phenomena have led to the placement of ban on CAP administration to food animal (WHO, 1998).

Quality control measures are frequently advocated in regulating food contaminations as a result of microbes, agro-chemicals and drugs. These measures entail continuous or routine monitoring of ex-farm and on-farm products; and it is highly dependent on the availability of sophisticated technologies and supporting legislations. These have been major sources of limitation to researches of such kinds, especially in the underdeveloped and developing nations, thus creating dearth of information regarding the occurrence of CAPR, a necessary prerequisite for global management of international trade. This study also aims at assessing the utilization of relatively cheaper, simple and available technologies in determining the extent of misuse of CAP in Nigeria. The present study was therefore undertaken to screen commercial eggs of antimicrobial drugs particularly CAP by using a combined comparative methods of ELISA and spectrophotometry.

II.   Materials and methods

2.1 Study location

Abuja is the Federal Capital Territory (FCT) of Nigeria, having a population of 1,857 million people as at last census carried out in 2006. Abuja is one of the fastest growing cities in Africa, centrally located in the

heart of the nation with links to its suburbs. Abuja is located in the north central geographical zone of Nigeria with coordinates of 9° 4 ' 0" N, 7° '29' 0" E and a total land mass of 713km² (275.3m²).

2.2 Egg sampling
Commercial chicken eggs were randomly sampled from four major markets in Abuja. These include: Gwagwalada, Kuje, Zuba and Deidei markets. A total of 2000 chicken egg samples were collected (500 samples from each of the market) between the month of March, 2012 and February, 2013. Sampling was carried out at intervals of at least 2days. The major markets were selected based on established high activities of sales of chicken eggs. At the market level, a systematic random sampling technique was adopted to select the crates from which eggs were sampled using simple random sampling technique. Samples collected were transported in crates to the laboratory for immediate preservation at +4°C till analysis.

2.3 Sample preparation
2.3.1 Homogenization of egg yolks
Egg samples were prepared for antimicrobial drug residue screening according to previous method (Omeiza et al., 2012b). Briefly, a 75% solution of ethanol was used for egg surface disinfection before breaking to separate yolk from albumen. The yolk was homogenized using Stomacher blender and about 10ml was transferred aseptically into universal bottles for onward storage at -20°c till further analysis.

2.3.2 Fast and rapid antimicrobial screening
Premi®test kits were used in this respect and strict adherence to manufacturer's recommendations was observed. Briefly, an aliquot amount of 100ml of the yolk homogenate was aspirated, using a 100μl syringe, on to the surface of the premi®test ampoules. These ampoules were incubated at 80°C for 10 minutes. Thereafter, they were transferred to already pre-heated automated incubator at 64°3°c for a period of 2h 45min before the results were read

2.3.3 Extract preparation
Crude extract of the positively identified Premi®test samples were prepared according to the method previously reported (Omeiza et al., 2012a). To every 3g of the positive egg yolk sample, acetonitrile was added drop-wise while vortex mixing. The resulting mixture was centrifuged at 4000×g and the supernatant was collected as the extract.

2.3.4 Extract purification
This was meant to remove interfering substances from the extract. The process strictly adopt the method reported by Arnold and Somogyi (1985) modified by Omeiza et al. (2012a). A 6ml of n-Hexane and 3ml of distilled water were added sequentially to every 4ml of the extract. The process was repeated using 4ml n-Hexane and 2ml of distilled water respectively. Then, 6ml of ethyl acetate was added to the lower aqueous phase (after a second centrifugation at 1700×g). The separated organic phase, resulting from the repeated centrifugations, was collected and evaporated to dryness by passing it through a stream of nitrogen at 55°C.

2.4 Spectrophotometric method
The dry product was resuspended in methanol to a volume of 0.5mL. Solutions of zinc and calcium chlorides were both prepared at concentrations of 1% respectively. These concentrations were sequentially added to the suspension until a volume of 0.2mL is exhausted. This is to cause chemical reduction of any available aromatic nitro compounds present in the suspension. The resultant products were then allowed to react with trisodium pentacyanoferrate to give purple products having an absorbance maximum (λmax) between 480nm and 540nm (Divya et al., 2013).

2.4.1   Method validation
2.4.1.1 Linearity
The absorption spectrum of the purple colored product with λmax of 468nm was used in the study of CAP. The reagent blank practically showed negligible wavelength. Under the experimental condition, absorbance is linearly proportional to concentration over the range of $0.2\text{-}2.0\mu gL^{-1}$ for CAP (Figure1). Slope, intercept, and correlation coefficient were obtained by the method of least squares which is described by the equation: $Y = a + bX$ (where Y = absorbance, a = intercept, b = slope and X = concentration in μgL^{-1}).
2.4.1.2 Accuracy
To determine the accuracy, recovery study was carried out by standard addition technique at three different concentrations (0.5, 1.0, and 1.5 μgmL^{-1} for CAP). Accuracy was calculated as the average percentage recoveries of the drug in their standard concentrations.

2.5 Enzyme Linked Immunosorbent Assay
2.5.1 Sample preparation
Sample was prepared according to the manufacturere's recommendation. To 2 g of homogenized whole Egg (raw) sample, 12mL of ethyl acetate was added and agitated vigorously for 10 minutes. It was then centrifuged for phase separation for 10 minutes at 3000 g (room temperature). An amount of 6 mL of the upper ethyl acetate phase was transferred to a clean glass vial and the solvent evaporated at 50- 70°C under a nitrogen stream to dryness. n-hexane was added in quantity of 1mL to the residue. Also 1 mL sample diluent was added to the mixture and agitated vigorously for 1 minute. For phase separation, the mixture was centrifuged for 10 minutes at 3000 g (room temperature). The lower aqueous phase was then tested in the ELISA.

2.5.2 ELISA procedure
Sterile pipette was used to pipet 100μL standards and prepared egg samples in duplicate into the appropriate wells of the microtiter plate. Immediately 50μL anti-chloramphenicol antibody was introduced into each well. The microtiter plate was covered with a plastic foil and incubated for 30 minutes at room temperature. Without preceding washing 50μL chloramphenicol-peroxidase conjugate was added into each well.
The microtiter plate was again covered with a plastic foil and incubated additional 15 minutes at room temperature. The plate was now washed three times as follows: Discarded the contents of the wells (by dumping or aspiration). Then 300μL of diluted washing solution was pipetted into each well. After the third repetition, the wells again were emptied and the residual liquid removed by striking the plate against a paper towel. The wash procedure was critically done as insufficient washing would result in poor precision and falsely elevated absorbencies. Now, 100μL of substrate solution was pipette into each well which and the reaction was allowed to develop in the dark (cupboard was used to achieve this as the chromogen is light-sensitive) for 15 minutes at room temperature. Enzyme reaction was stopped by adding 100μL of stop solution (0.5M H_2SO_4) into each well. The blue color then turned yellow upon addition. After thorough mixing, absorbance was measured at 450 nm using an ELISA reader. The color was stable for 30 minutes.
The average optical density (OD 450 nm) for each set of reference standards and samples was determined from the reader. A standard curve was constructed by plotting the mean optical density obtained for each reference standard against its concentration in ng/mL on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis. Using the calibration curve developed from reference standards, corresponding concentrations of CAPR in ng/mL were determined.

III.   Results
3.1 Occurrence of antimicrobial drug residues among sampled commercial eggs
The occurrence of antimicrobial drug residues is given in table 1 and 2. The level of occurrence is considerably high. Out of a total of 2000 egg samples analyzed, 370 were positive for antimicrobial drug residues (AMDR) out of which 120 were positive for Chloramphenicol residues (CAPR), making 6% of the total samples analyzed. Table 2 shows clear-cut disparities in the occurrence of AMDR between seasons even though there was no statistically significant difference ($P > 0.05$) in the occurrence of the AMDR between dry humid, dry windy/harsh and wet/rainy seasons. The seasons showed significant influence on the levels of occurrence of antimicrobial drug residues. The mean values of occurrences (40, 60 and 110) of antimicrobial drug residues were characteristic of both wet/rainy and dry windy/harsh seasons respectively as shown in Table 1.

Table 1: Annual Distribution of Antimicrobial Drug Residues

Month	Range of occurrence	Frequency of occurrences		Mean occurrence
		AMDR	CAPR	
Mar	40-100	4	1	60.0
Apr	00-20	1	0	20.0
May	20-60	3	1	30.0
Jun	10-70	4	2	40.0
Jul	30-90	2	2	30.0
Aug	20-60	4	1	40.0
Sep	00-40	2	0	20.0
Oct	00-60	2	0	20.0
Nov	30-60	3	0	30.0
Dec	20-40	4	2	40.0
Jan	60-80	4	1	70.0
Feb	80-140	4	2	110.0

AMDR= Antimicrobial drug residue
CAPR= Chloramphenicol residue

3.2 Proportion of CAPR among the AMDR incriminated egg samples

Table 3 presents the number of analyzed samples and proportion of contaminated samples with CAPR

Sample source	Season	No. positive Antimicrobial drug residues (%)	No. positive Chloramphenicol residues (%)	No. negative samples (%)	Total samples
A	Dry humid[(a)]	22	2	97.0	119
	Dry/windy [(b)]	32	11	140.0	172
	Wet/rainy [(c)]	39	19	171.0	210
B	Dry humid[(a)]	25	3	80.0	105
	Dry/windy [(b)]	51	15	163.0	214
	Wet/rainy [(c)]	43	12	138.0	181
C	Dry humid[(a)]	16	3	127.0	143
	Dry/windy [(b)]	15	10	119.0	134
	Wet/rainy [(c)]	25	11	198.0	223
D	Dry humid[(a)]	17	2	67.0	84
	Dry/windy [(b)]	22	13	86.0	108
	Wet/rainy [(c)]	63	19	246.0	309
Total		**370 (18.5)**	**120 (6.0)**	**1630 (81.5)**	**2000**

among the AMDR positive egg samples. Out 370 chicken egg samples incriminated with AMDR and were analyzed, 120 (32.4%) were confirmed positive for CAPR using ELISA and spectrophotometric techniques respectively, making a proportion of 6.0% of the total chicken egg samples analyzed.

3.3 Trend in the occurrence of AMDR across seasons

The trend of the overall occurrence of AMDR with particular reference to CAPR in this study is given in Figure 1. The occurrence of AMDR in dry windy/harsh season was significantly higher ($P < 0.05$) than the respective occurrences during wet/rainy and dry humid seasons. It was observed that there was no significant difference ($p > 0.05$) in the occurrence of these residues between dry windy/harsh, wet/rainy and dry humid/non-windy seasons, although the dry windy/harsh and wet/rainy seasons showed higher level of residue contaminations. In the overall, the pattern of occurrences of AMDR and CAPR showed some levels of variations between seasons.

3.4 Determined concentrations of Chloramphenicol residues

The determined concentrations of CAPR spread across 3 different seasons ranged between 0.12-0.22μgL^{-1}, 0.24-0.30μgL^{-1} and 0.32-0.36μgL^{-1} as presented in (Table 4). Mean concentrations ± STDs were also determined for each season with $P < 0.05$ between them (Table 4). The limit of detection (LOD) and the limit of quantification (LOQ) were 0.12 and 0.37μgL^{-1} respectively. Recovery rate was averagely calculated to be 84%. Correlation coefficient, r^2 was determined to be 0.998.

Table 2: Seasonal occurrence of CAPR amongst AMDR

There is no statistically significant difference between (a), (b) and (c) Fisher's Exact Test, $P > 0.05$

Table 3: Comparative analyses of CAPR using spectrophotometry and ELISA

Season	Comparative analysis		
		Spectrophotometry	ELISA
	No. sample analyzed	No. CAPR	No. CAPR
Dry humid	10	10	10
Dry windy/harsh	49	49	49
Wet/rainy	61	61	61
Total	**120**	**120**	**120**

Table 4. Determined concentration range of CAPR-positive samples

Season of sampling	No. samples analyzed For CAPR	Concentration range (μg/L)	Mean concentration ± STD (μg/L)
Wet /rainy	10	0.24-0.30	0.273±0.020[(a)]
Dry windy/harsh	49	0.32-0.36	0.338±0.097[(b)]
Dry humid	61	0.12-0.22	0.165±0.057[(c)]

Statistically significant difference exists between (a), (b) and (c)
Student's t-test, $P<0.05$

IV.   Discussion

The samples utilized in this study were obtained from selected markets in FCT where chicken eggs are supplied by marketers across different geo-political zones of the country. The study also focuses on the misuse of antimicrobial agents particularly Chloramphenicol, a drug with broad-spectrum of activity against both Gram-positive and Gram-negative microorganisms, and had been used widely in treating several infectious diseases of humans and animals. It is however a known fact that such effects of the drug, in many instances, do not occur without adverse effects (Impens, 2003; Yibar et al., 2011). The current study also considered the flow pattern of occurrence of these antimicrobial residues across one year span, as previous reports in Nigeria did not address this (Mbodi et al., 2014). However, few reports earlier indicated that season could affect the incidences of disease causing agents (Nwanta 2008) and this was further supported by a claim which explained the possible implication of seasons on drug usage in animal production and the attendant drug residues (Omeiza et al., 2012b).

Fig 1: Trend in the occurrence of antimicrobial drug residues
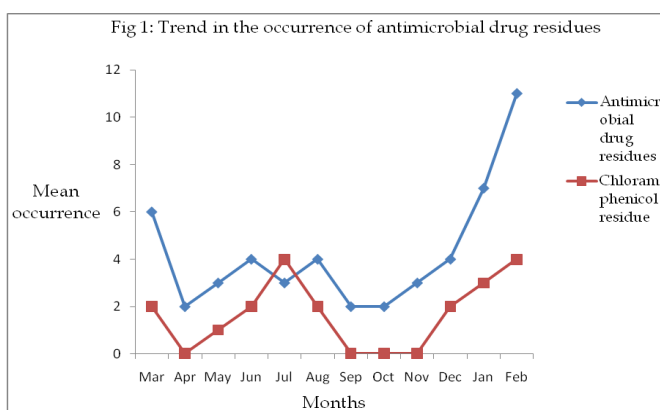
Findings from the study demonstrated significant level of occurrence of 18.5% for AMDR. Both methods employed in the analysis of CAPR agreeably showed significant levels of 6.0% occurrence. These values present public health concern. Most antimicrobial agents used in animal production have established withdrawal periods and residue limits. However, with respect to CAP, there is no established limit in tissues/matrices of exposed food-animals, therefore exposing consumers of eggs and other foods of animal origin to potentially harmful effects (Rocha Sigueira et al., 2009). This has ultimately led to the prohibition of the use of CAP in food–producing animals in many countries (Council of the European Communities, 1990).

Previous reports have shown different levels of occurrences of AMDR in Nigeria. Occurrences of 1%, 3.6% and 7.6% were reported in 2004 (Kabir et al., 2004); 2010 (Fagbamila et al., 2010) and Omeiza et al., (2012a). Recently, Mbodi et al. (2014) reported higher values of occurrences (5.6% and 6.0% for CAPR from states bordering FCT and the FCT respectively. All these, harmonized with the current study, may be interpreted as an increasing trend in the occurrences of antimicrobial drug residues over time and by extension implying increasing wrong application of antimicrobial agents in animal production in this part of the world. This may not be unconnected to poor or lack of legislation which empowers professionals or stakeholders in their respective fields of training to exhibit their due franchise of enforcing monitoring, surveillance and perhaps prosecution of defaulters. Also, Poor husbandry practices and biosecurity measures could be considered as another reason for this increasing trend. Omeiza et al. (2012b) demonstrated the practical effect of poor biosecurity amongst small scaled farmers as a responsible factor favoring the upsurge of drug misuse among farmers. This was attributed highly to low level understanding in separating basic farming principles towards achieving a healthy production and society. The different occurrence profiles revealed in this paper, even though from different parts of the country, when compared with the current study which shows significant increased rates of 18.5% for AMDR and 6.0% for CAPR, are clear indicators of increasing poor biosecurity and husbandry management.
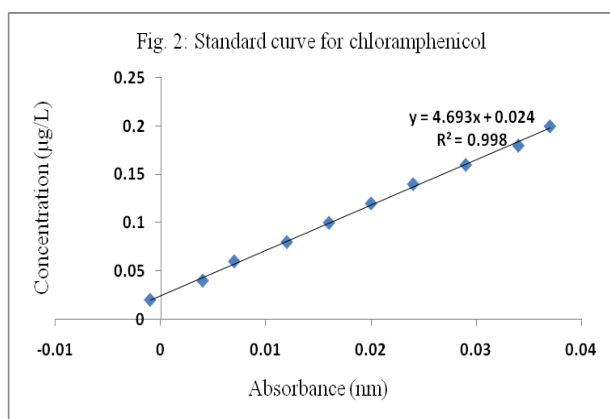
In this study it is clear that major egg suppliers pool their eggs from different small scale farmers in different part of the country before supplying them to main marketers in the FCT. This could be reasoned to contribute to the high level of residue occurrences in eggs in the FCT. This finding agrees with the previous report (Mbodi et al**.,** 2014) that the CAPR prevalence rate of 7% in FCT majorly results from the influx of CAP–positive eggs (5.6%) from states bordering FCT and beyond.

Fig. 2: Standard curve for chloramphenicol

$$y = 4.693x + 0.024$$
$$R^2 = 0.998$$

Of importance in this study is the observed pattern of distribution of the analyzed residue samples across a span of one year. Higher mean occurrences ($\times \leq 40 \leq 110$) were observed during the dry windy/harsh season, when compared with other seasons with low mean occurrences ($\times \leq 20 \leq 40$). Even though there was no statistically significant difference (P > 0.05) between seasons in the occurrence of residues in all the sample locations, it is however, worthy of mention that data generated in this study show varied pattern in the occurrences of AMDR and CAPR across seasons in all the locations. This pattern was however well substantiated with the calculated (mean concentrations ± STDs) which showed statistically significant difference (P < 0.05) between seasons. This trend clearly indicates seasonal influence on the use or misuse of antimicrobials in animal production, with their attending consequences of residues in foods of animal origin. In 2012, Omeiza et al. (2012b) reported a scenario where high prevalence rate of drug residues was observed during harsh windy climate. However this report was not assertive as more extensive study was needed to establish the claim. Harsh environmental pressure on producing animals may allow high expression of diseased state, with resultant drug misuse amongst unqualified managers of farms. Nwanta (2008) asserted the direct bearing of season on disease causation mostly among poultry rearers. Omeiza et al. (2012b) in a different study observed that such seasonal influence on disease causation could adversely affect the pattern of drug use in animal production. These assertions agree with the findings in the current study which demonstrates varied drug residue responses across seasons in one year.

The observed trend in the occurrences of drug residues in this study and reported prevalent cases of drug residues in different parts of the country (Kabir et al.,2004; Olatoye et al., 2012; Omeiza et al., 2012 and Mbodi et al., 2014) have pointed to a deficient systems of monitoring and surveillance coupled with poor awareness of the health implication of the subject matter amongst farmers and consumers.

Researchers in the developing world are faced with challenges especially in areas of analytical research. One of such great challenges is the quantitative studies using sophisticated technology. However, in situation where prompt and regular monitoring of ex-farm consummated products are advocated (Cannavan, 2014), most researchers are limited due to inaccessibility and high cost of obtaining them. Choma (2003) cited instances in which inaccessibility to sophisticated equipment could hinder researches of credible impacts. This has informed the need, in the current study, to employ a combined relatively cheaper and easily accessible methods of Enzyme linked Immunosorbent Assay and sensitive spectrophotometry. Results from ELISA which were confirmed by spectrophotometric means showed high level of agreement. Accuracy, which was determined by level of recoveries of the drug standard, showed high (average) recovery of 84%. The high recovery rates achieved in this study was not unconnected to the extraction method employed. Arnold and Somogyi (1985) attributed high recoveries to the use of acetonitrile as a pre-treatment which assists in denaturing tissues for effective release of bound drugs and cleaner extracts due to defattening and complete deproteination by n-hexane and ethylacetate. Limit of detection (LOD) and limit of quantification (LOQ) as parameters of determining precision, were determined at different levels and put at 0.12μg/L and 0.37μg/L respectively. This finding agrees with the previous report (Ronning et al**.,** 2006) which puts LOD of CAPR at 0.1μg/L using sophisticated equipments (based on the EU validaiton methods (2002/657/EC). The absorbance spectrum of the purple colored product with max of 486nm was used in the study of CAPR. In this study, and under the experimental conditions, absorbance (OD) was linearly proportional to concentration over a range of 0.2-2.0 mgmL^{-1}. The linearity obtained in this study was near perfect with r^2=0.998 (Fig. 2). This was achieved using regression analysis in which slope, intercept and correlation coefficient were obtained by the method of least squares, described by the equation :Y = a + bx (where Y= absorbance, a= intercept, b= slope and c= concentration in μgL^{-1}). This result agrees with the previous report (Divya et al., 2013) which utilized sensitive spectrophotometer in quantification studies on paracetamol and protriptyline HCl.

V.   Conclusion

This study came up with findings which revealed that chloramphenicol as a drug is still being used in poultry production by some curious and non-challant farmers in this part of the world. More so, the study revealed varied patterns of drug residues occurrences in different seasons of the year. Higher concentration was a consistent finding associated with dry windy and harsh weather. It is therefore rational to believe that seasons with higher incidences of poultry diseases encourage antimicrobial drugs (antimicrobial drugs with no maximum residue levels, MRLs, inclusive) misuse with the attendant incidences of higher residue concentrations. It is concluded that such seasons be monitored with increased effort by the relevant agencies to forestall public health risks associated with the consumption of eggs.

Adequate legislations be put in place towards implementing the FAO/WHO Codex Alimentarius recommendations which enforce prompt and adequate monitoring and total restriction of use of antimicrobial drugs with no MRLs in animal production.

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