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Anti-Microbial Resistance Profiles Of *E. Coli* Isolated From Free Range Chickens In Urban And Rural Environments Of Imo State, Nigeria

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Abstract:
Information on the resistance profiles of normal intestinal flora of extensively reared chickens that hardly receive antibiotics in the developing countries can serve as important means of understanding the human/animal pathogens drug resistance interactions in the zone. Three hundred and fifty *E. coli* isolates, comprising 133 from urban and 217 from rural sites in Imo state, Nigeria, were screened for anti-microbial resistance profile against 10 antibiotics using the disc diffusion method. Overall percentage anti-microbial resistance of the isolates against cotrimoxazole, ampicillin, nalidixic acid, chloramphenicol and nitrofurantoin (72–92%) were very high. The organisms were highly sensitive to other antibiotics, especially gentamicin and ciprofloxacin. The 59.5% overall mean percentage resistance recorded at the urban area was significantly higher than the 46.8% recorded at the rural area ($p<0.05$). With the exception of the figures for cotrimoxazole and ampicillin, resistance values obtained against the other antibiotics at the urban sites were statistically higher than those obtained at the rural sites ($p<0.05$). Zero resistance was recorded against the fluoroquinolones, norfloxacin and ciprofloxacin at all the rural sites except at Enyiogwugwu where a 28.6% resistance was obtained against norfloxacin. Since free-range chickens rarely receive antibiotic medication, it is concluded that the highly resistant *E. coli* organisms isolated from them may be reflecting consequences of human drug use in the study areas.
Key Words: Anti-microbial resistance, *E.coli*, free-range chicken, antibiotics, Nigeria

Introduction:

The development of anti-microbial resistance in many bacterial organisms constitutes serious problem in the control of infectious diseases.(1-3) Anti-microbial use and especially misuse has been found to be the most important selecting force in bacterial antibiotic resistance.(4-6) Although there is no linear relationship between the amounts of anti-microbials used and the development of resistance, increased use of an anti-microbial often results in decreased susceptibility among exposed bacteria.

An anti-microbial selective pressure may arise because of cross-resistance and co-selection of resistance genes and may explain how one anti-microbial selects for another anti-microbial. Again, multiple resistances may confer resistance to several anti-microbials, while virulence and lack of hygiene may account for the survival and spread of resistant bacteria, even in the absence of an anti-microbial selection pressure.(7)

The bacteria belonging to the normal intestinal flora of humans and animals, which constitute an enormous reservoir of resistance genes for potentially pathogenic bacteria may serve as major indictors for selection pressure exerted by antibiotic use in a given animal or human population.(8-10) Investigations, especially on *E. coli* and enterococci, make it possible to understand the prevalence of resistance in different animal populations and to detect a possible transfer of resistant bacteria from animals to humans and vice versa.(11) Nijsten *et al.,* (12,13) for example, found significantly more resistant *E. coli* in the faecal flora of pig farmers than in faecal sample of pig slaughterers and urban residents. Other studies have shown that even when an antibiotic was restricted to veterinary use alone, resistance genes may not only be found in animal isolates or zoonotic bacteria isolates from humans, but also from enterobacteriaceae in the environment, the intestinal flora of farmers and hospital isolates.(14-16)

Although the use of anti-microbial drugs in food animals have been shown to lead to resistant strains of pathogens which may be transmitted to humans through food in the developed countries, the contribution of anti-microbial use in food animals to resistance in bacteria infecting humans or vice versa in developing countries remain unclear. There is however strong evidence that anti-microbial use in humans has not only driven the emergence of multi-drug resistant clones in these countries, but has resulted in an increasingly high prevalence of multiple resistance.(17-19)

There is therefore the need to continue investigating the resistance profiles of normal intestinal flora of extensively reared animals that hardly receive antibiotics in the developing countries as a means of understanding the human/animal drug resistance interaction in the zone. The present study compared anti-microbial resistance profiles of *E. coli* isolates from free-range chickens in urban and rural environments of Imo state, Nigeria.

Imo state is situated in the southeastern rainforest vegetational belt of Nigeria and lies between latitude 5^0 4^1 and 6^0 3^1 N and longitude 6^0 $15^1$and 7^0 34^1 E. The agro-ecological characteristics of the area have been reported.(20) Imo state is divided into 27 local government areas (LGA) for administrative purposes. These LGAs are further grouped into 3 senatorial zones namely, Owerri, Orlu and Okigwe. Livestock farming especially poultry is popular in these zones. There are usually higher concentrations of poultry farms around major cities.

Poultry production in the state could be broadly divided into extensive, semi-intensive and intensive systems. The greatest population of chicken in the study area is made of local breeds reared by rural farming families under the extensive scavenging system.(21) These producers are quite distinct from the owners of small to medium scale commercial poultry farms that are sited in both rural and urban areas.(22) Rearing of started exotic broilers and cockerels has also become an important aspect of this production system.(23)

Commercial intensive poultry productions in southeastern Nigeria include table eggs, broiler, parent stock, hatchery, turkey and started chicken production. These farming operations are distributed over urban, peri-urban and rural sites and have been shown to range from very small operations (50-100 birds), to medium (101-1000 birds) and large scale (above 1000 birds).(24) Small and medium farms are usually family back yard affairs that are predominantly found in urban and peri-urban centers, while most large scale, operations are located in peri-urban and rural environments.

In most of the back yard poultry, hygienic and bio-security measures are usually poor with all the family members being involved in the daily management activities. Usually there is no organized effort at vermin, ferrets and human traffic control.

Materials and Methods:

Identification and selection of sampling sites

The study, conducted in September 2003, consisted of three stages, starting with sample collection from the different sites and preceded by a preliminary field investigation, during which the researcher identified the study sites (rural and urban) and made himself known to the farm operators.

For rural environment studies, 6 sites popular for poultry keeping were purposively selected. These included Obibiezena and Obiangwu in Ngor Okpala LGA, Uzoagba in Owerri North, Amaraku in Isiala Mbano, Umuaka in Njaba and Enyiogwugwu in Abor

Mbaise LGAs respectively. Ubomiri in Mbaitolu LGA, Akabo in Ikeduru, Nekede in Owerri West, Orji in Owerri North and Owerri Urban LGAs (5 sites) were selected for the urban environment study. Free range local fowls, layers, cockerels and broiler roosters were sampled at the different sites.

At each site, the families that own the chicken and the number of chicken to be sampled were determined according to the method previously described by Okoli.(20) Each sampling site was visited twice over a period of three weeks. It was determined that the birds have not received any antibiotic medication in the previous two months, since antibiotic treatment has been shown to compromise resistance results.(25)

Collection of samples, cultivation and isolation of organisms

Cloacae swabs were collected from at least 5 birds randomly selected from free-range flock at each study site, using sterile swab sticks (Antec^R). MacConkey agar (MCA) (Fluka BioChemica^R) was used for selective growth and elucidation of colony characteristics of *E. coli*.(26) The agar was prepared according to manufacturers instruction and each cloacae swab sample streaked directly on MCA and incubated overnight at 37^{O}C. In all cases, the streaking technique described by Cruickshank *et al* (27) was adopted.

After overnight incubation, growths on the MCA plates suggestive of *E. coli* colonies-2-4mm in diameter, opaque and convex with entire edge and rose pink on account of lactose fermentation were further streaked onto eosin methylene blue (EMB) and incubated overnight at 37^{O}C again. Green metallic sheen colonies indicative of *E. coli* were then subjected to biochemical tests, which included Indole, methyl red and Simmons citrate tests for *E. coli* identification as described by Edwards and Ewing.(28)

Susceptibility testing

The isolated *E. coli* were screened for anti-microbial resistance profile using the disc diffusion method (29) according to the methods recommended by the National Committee for Clinical Laboratory Standards Guidelines.(30) This was done by streaking the surface of nutrient agar plates uniformly with the organisms and thereafter exposing them to discs (Poly-Tes Lab^R) impregnated with known concentrations of anti-microbial substances.

Commercial antibiotics discs used in the study were 10 in number and included AM, ampicillin (25µg); CO, cotrimoxazole (50µg); NI, nitrofurantoin (100µg); GN, gentamicin (10µg); NA, nalidixic acid (30µg); TE, tetracycline (30µg); CH, chloramphenicol (10µg); CF, cefuroxime (20µg); NB, norfloxacin (10µg), and CP, ciprofloxacin (5µg).

Statistical analysis

Susceptibility data were recorded quantitatively by measuring the diameters to the nearest whole millimeter using a meter rule. Following the interpretative chat of the Kirby-Bauer Sensitivity Test Method (31), the zones were interpreted as resistant or sensitive. For the purpose of the present study, isolates with intermediate sensitivity were categorized as sensitive. Furthermore, proportions of isolates resistant to individual drugs and having each anti-microbial resistance patterns were computed as averages and percentages across sample sites in the urban and rural environments.

Significant differences in mean percentage resistances were determined across the sample sites using the Student t-Test method.(32)

Results:

Overall, 350 *E. coli* isolates comprising 133 from urban and 217 from rural sites and means of 26.6 and 36.2 isolates/site for both sites respectively were obtained (Table 1)

Table 1: Sites distribution and number of *E coli* isolates from free chickens in urban and rural environments of Imo state, Nigeria.

	Urban/Peri-urban sites			**Rural sites**		
SN	**Location**	**Local Govt. Area**	**No of iso-lates**	**Location**	**Local Govt. Area**	**No of iso-lates**
1	Owerri	Owerri urban	42	Obibiezena	Ngo-Okpala	63
2	Nekede	Owerri west	28	Enyiogwugwu	Abo-Mbaise	49
3	Ubomiri	Mbaitolu	35	Uzoagba	Owerri north	28
4	Orji	Owerri north	14	Obiangwu	Ngo-Okpala	28
5	Akabo	Ikeduru	14	Umuaka	Njaba	28
6				Amaraku	Isiala Mbano	21
Total			133			217
Mean			26.6			36.2
Overall number of *E. coli* isolates = 350						

Figure 1 showed that the overall percentage anti-microbial resistance of the *E. coli* isolates against cotrimoxazole, ampicillin, nalidixic acid, chloramphenicol and nitrofurantoin (72–92%) were very high. The other antibiotics, especially gentamicin and ciprofloxacin were highly sensitive to the organisms.

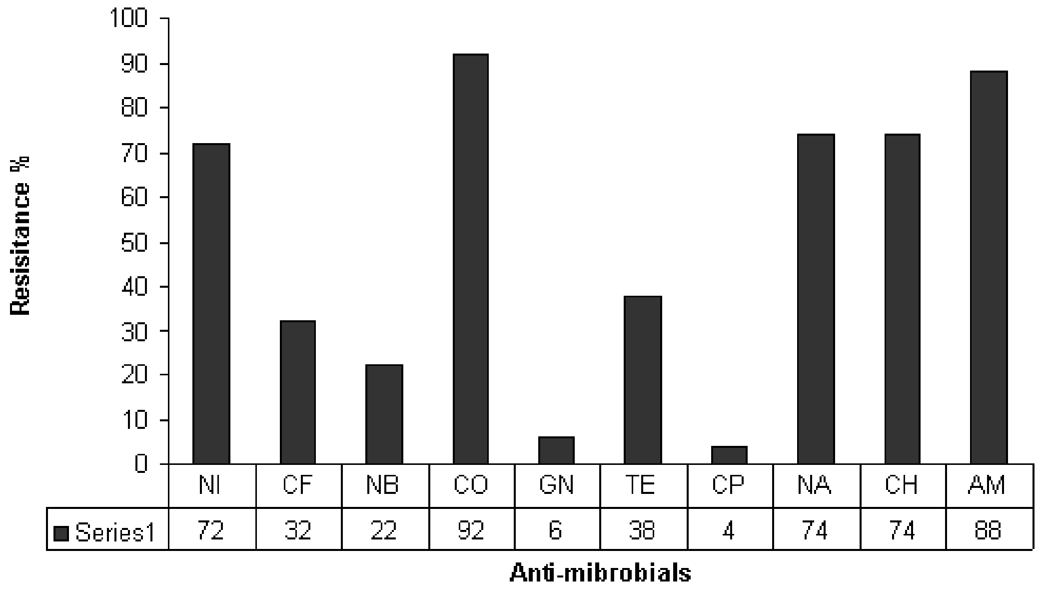
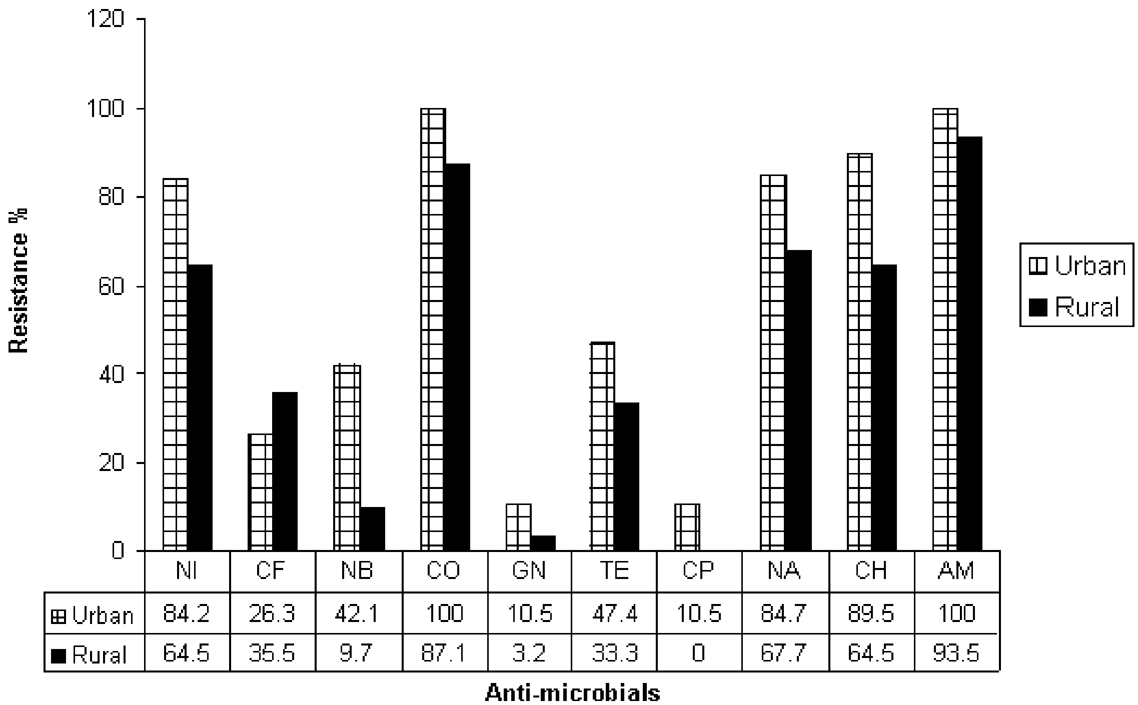


Figure 1: Overall percentage anti-microbial resistances of E. coli isolates from urban and rural environments to various anti-microbial agents. NI, nitrofurantoin; Cf, cefuroxime; Nb, norfloxacin; Co, cotrimoxazole; Gn, gentamicin; Te, tetracycline; Cp, ciprofloxacin; Na, nalidixic acid; Ch, chloramphenicol; Am, ampicillin



	NI	CF	NB	CO	GN	TE	CP	NA	CH	AM
Urban	84.2	26.3	42.1	100	10.5	47.4	10.5	84.7	89.5	100
Rural	64.5	35.5	9.7	87.1	3.2	33.3	0	67.7	64.5	93.5

Figure 2: Comparison of overall anti-microbial resistance in *E. coli* isolates from urban and rural environments in Imo state, Nigeria.
NI, nitrofurantoin; Cf, cefuroxime; Nb, norfloxacin; Co, cotrimoxazole; Gn, gentamicin; Te, tetracycline; Cp, ciprofloxacin; Na, nalidixic acid; Ch, chloramphenicol; Am, ampicillin

Figure 2 showed that *E. coli* isolates from the urban areas were more resistant to any of the tested antibiotics than those from rural areas. For example, while zero resistance was recorded against ciprofloxacin in the rural area, 10.5% was obtained in the urban area. Resistance figures however remained high at both sites for nitrofurantoin, cotrimoxazole, nalidixic acid, chloramphenicol and ampicillin. Statistical treatment of these percentage resistance differences at the two study areas (Table 2), revealed that with the exception of cotrimoxazole and ampicillin, antibiotic resistance obtained at the urban areas were generally statistically higher than those obtained at the rural areas ($p<0.05$). The 59.5% overall mean percentage resistance recorded at the urban area was also significantly higher than the 46.8% recorded at the rural area ($p<0.05$).

Table 2: Comparison of overall anti-microbial resistance in *E. coli* isolates from urban and rural environments in Imo state, Nigeria.			
Anti-microbial	**Urban/Peri-urban**	**Rural**	**p - value**
Nitrofurantoin (NI)	112 (84.2)	140 (64.5)*	.0839
Cefuroxime (CF)	35 (26.3)	77 (35.5)	.0941
Norfloxacin (NB)	56 (42.1)	21 (9.7)	.3558
Cotrimoxazole (CO)	133 (100.0)	189 (97.1)	.0438
Gentamicin (GN)	14 (10.5)	7 (3.2)	.33117
Tetracycline (TE)	63 (47.4)	70 (32.3)	.1192
Ciprofloxacin (CP)	14 (10.5)	0 (0.0)	.5000
Nalidixic acid (NA)	112 (84.2)	143 (67.7)	.0689
Chloramphenicol (CH)	119 (89.5)	140 (64.5)	.1025
Ampicillin (AM)	133 (100.0)	203 (93.5)	.0214
Total number of isolates	**133**	**217**	-
Overall mean % resistance	**59.5**	**46.8**	**.0752**
p <0.05; *values in parenthesis are percentages resistance			

Across the different urban sites (Table 3), resistance against the nitrofurantoin, cotrimoxazole, nalidixic acid, chloramphenicol and ampicillin remained generally high, while for tetracycline, similar high resistance figures were recorded at Owerri, Nekede and Ubomiri. While zero resistances were recorded at gentamicin at all the sites, an abnormally high (100%) resistance was obtained at Orji. Again, moderately high (40 – 50%) resistances were recorded for norfloxacin at Owerri, Nekede and Ubomiri, while at Akabo, 50% resistance was also recorded against ciprofloxacin.

Table 3: Anti-microbial resistance frequencies of *E coli* isolates from urban environments in Imo state.										
Site	**NI**	**CF**	**NB**	**CO**	**GN**	**TE**	**CP**	**NA**	**CH**	**AM**	**n**
Owerri	35(83.3)	7(16.7)	21(50.0)	42(100.0)	0(0.0)	21(50.0)	0(0.0)	35(83.3)	42(100.0)	42(100.0)	42
Nekede	21(75.0)	7(25.0)	14(50.0)	28(100.0)	0(0.0)	14(50.0)	0(0.0)	28(100.0)	28(100.0)	28(100.0)	28
Ubomiri	28(80.0)	14(40.0)	14(40.0)	35(100.0)	0(0.0)	28(80.0)	7(20.0)	28(80.0)	35(100.0)	35(100.0)	35
Orji	14(100.0)	7(50.0)	0(0.0)	14(100.0)	14(100.0)	0(0.0)	0(0.0)	7(50.0)	14(100.0)	14(100.0)	14
Akabo	14(100.0)	0(0.0)	7(50.0)	14(100.0)	0(0.0)	0(0.0)	7(50.0)	14(100.0)	0(0.0)	14(100.0)	14
Total	112(84.2)	35(26.3)	56(42.1)	133(100.0)	14(10.5)	63(47.4)	14(10.5)	112(84.2)	119(89.5)	133(100.0)	133
NI, nitrofurantoin; Cf, cefuroxime; Nb, norfloxacin; Co, cotrimoxazole; Gn, gentamicin; Te, tetracycline; Cp, ciprofloxacin; Na, nalidixic acid; Ch, chloramphenicol; Am, ampicillin											

Across the rural sites (Table 4), resistance against nitrofurantoin, cotrimoxazole, nalidixic acid, chloramphenicol and ampicillin were again generally high with exception of the zero resistance recorded against chloramphenicol at Uzoagba and the 25% recorded against nitrofurantoin at Obibiezena and Uzoagba. An abnormally high 100% resistance was equally recorded against gentamicin at Obibiezena while at the other sites resistance was generally zero. Zero resistances were recorded against norfloxacin and ciprofloxacin at all the sites except at Enyiogwugwu where a 28.6% resistance was obtained against norfloxacin.

Site	NI	CF	NB	CO	GN	TE	CP	NA	CH	AM	N
Obibiezena	7(25.0)	21(75.0)	0(0.0)	28(100.0)	28(100.0)	14(50.0)	0(0.0)	14(50.0)	14(50.0)	14(50.0)	28
Enyiogwugwu	42(85.8)	14(28.6)	14(28.6)	42(85.8)	0(0.0)	28(57.1)	0(0.0)	42(85.8)	49(100.0)	49(100.0)	49
Uzoagba	7(25.0)	0(0.0)	0(0.0)	14(50.0)	0(0.0)	0(0.0)	0(0.0)	7(25.0)	0(0.0)	28(100.0)	28
Obiangwu	42(66.7)	21(33.3)	0(0.0)	56(88.9)	0(0.0)	7(11.1)	0(0.0)	49(77.8)	42(66.7)	63(100.0)	63
Umuaka	21(75.0)	7(25.0)	0(0.0)	28(100.0)	0(0.0)	0(0.0)	0(0.0)	21(75.0)	14(50.0)	28(100.0)	28
Amaraku	21(100.0)	14(66.7)	7(33.3)	21(100.0)	0(0.0)	21(100.0)	0(0.0)	14(66.7)	21(100.0)	21(100.0)	21
Total	140(64.5)	77(35.5)	21(9.7)	189(87.1)	7(3.2)	70(33.3)	0(0.0)	147(67.7)	140(64.5)	203(93.5)	217

Table 4: Anti-microbial resistance frequencies of E coli isolates from rural environments in Imo state.

NI, nitrofurantoin; Cf, cefuroxime; Nb, norfloxacin; Co, cotrimoxazole; Gn, gentamicin; Te, tetracycline; Cp, ciprofloxacin; Na, nalidixic acid; Ch, chloramphenicol; Am, ampicillin

Discussion:

For *E. coli* and other enterobacteriaceae in which asypmtomatic colonization of the intestine typically precedes infection, anti-microbial resistance is usually mediated by the acquisition of one or several new genes, rather than by point mutation in existing genes. For example, segments of wild penicillin binding protein genes could be replaced with alleles whose sequences differ from the wild type at multiple positions. These new mechanisms thus arise and spread in the flock under conditions of antibiotic selective pressure.(33) Organisms or plasmids bearing these types of resistance must be acquired, generally because of cross transformation. Because most *E. coli* strains are not obligate organisms, much of their exposure to antibiotics is during treatment directed at infections caused by unrelated organisms. Antibiotic treatment that changes the incidence or duration of infection in a farm (flock of birds), will affect those birds bacterial contacts.(34) Thus, use of a particular antibiotic in a host for example humans, in an environment may increase the risk of colonization by or infection with resistant organisms in other humans or even animals that have not received that set of antibiotics but are sharing common environment with the treated ones. This indirect effect of ant-microbial use experienced by members of a population has been defined as the enhancement of risk for acquiring a resistant organism, because of the use of anti-microbials in other hosts in the group or population.(33)

The present study on the anti-microbial profiles of *E. coli* isolates from free-range chickens that rarely receive antibiotics recorded very high resistance levels to cotrimoxazole, ampicillin, nalidixic acid, chloramphenicol and nitrofurantoin at both the urban and rural study sites. These high values are similar to those observed in *E. coli* isolates from other sources in the study area, against the inexpensive, first line broad-spectrum, readily available antibiotics (10,25,35-37).

Although free-range chickens hardly receive any modern veterinary attention, they may maintain close contact through a myriad of routes with organisms originating from other important hosts in their environment such as humans and exotic chicken that had been previously exposed to various antibiotics. For example, in many rural communities in southeastern Nigeria, it is common for people to defecate in and around surrounding compound bushes or to urinate just at the corner of the house. Such poor and unhygienic disposal methods of human excrements definitely expose free-range chickens that feed on such excrement to normal human enteric flora that may harbor novel resistant factors. These human *E. coli* organisms may subsequently colonize the intestine of the free-range chicken and become the locus for spread of the resistance factors.

The observed high anti-microbial resistance in the *E coli* isolates from the urban areas probably reflect the high antibiotic usage in the urban environment. This is expected since anti-microbial use has been shown to be the most important selecting force in bacterial antibiotic resistance.(4-6) This is perhaps most vividly highlighted by the resistance results against the fluoroquinolones, norfloxacin and ciprofloxacin in the present study. Fluoroquinolones are relatively recent entrants into drug therapy in Nigeria and are relatively expensive and not readily available for veterinary therapy in Nigeria. It is therefore possible that the very low usage of these drugs by humans at the rural sites is reflected by the equally low levels of anti-microbial resistance in *E coli* isolates from free-range chickens in the areas.

The present data on fluoroquinolone resistance in the urban areas shows that moderate to low resistance rates against these antibiotics have evolved in *E. coli* isolates from the study area even though information on the exact time of fluoroquinolone introduction in the area is lacking. These data sound a warning because indiscriminate use of antibiotics along with poor hygiene and disease control that are part of the risk factors for antibiotic resistance in bacteria are highly prevalent in Nigeria.(4,6,38)

The very low resistance recorded against gentamicin here are also in agreement with results of recent studies in this part of Nigeria.(25,36,37) They are however at variance with the 70 to 100% resistance reported by Uwaezuoke *et al.* (35) in *E. coli* isolates from poultry feeds in Imo state. It is possible that these organisms originated as direct humans contaminants of the feed ingredients through handling. This again may equally account for the abnormally high 100% resistance rates recorded at Orji (urban site) and Obibiezena (rural site). Gentamicin is marked mostly as a 2mL injectable solution in a glass vial. This probably makes the drug unpopular and therefore safe from common abuse in Nigeria. It would seem therefore that low patronage of the drugs by the human population of the study area may be contributing to the low resistance rates recorded here.

Conclusions:

E. coli isolates from free-range chicken roaming the urban environments of Imo state, Nigeria, showed significantly higher anti-microbial resistance profiles than those from rural chickens indicating the higher drug use rates in the urban environment. Since free-range chickens rarely receive antibiotic medication, it is concluded that highly resistant *E coli* organisms isolated from them may be reflecting consequences of human drug use habits in the study areas.

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