Antibiotic resistance and resistance genes in Escherichia coli from poultry farms, southwest Nigeria

Adelowo, Olawale O.; Fagade, Obasola E.; Agersø, Yvonne

Published in:
Journal of Infection in Developing Countries

Link to article, DOI:
10.3855/jidc.4222

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Original Article

Antibiotic resistance and resistance genes in E*scherichia coli* from poultry farms, southwest Nigeria

Olawale O Adelowo[1,2], Obasola E Fagade[2], Yvonne Agersø[3]

[1] *Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria*
[2] *Environmental Microbiology and Biotechnology Laboratory, Department of Botany and Microbiology, University of Ibadan, Nigeria*
[3] *Department for Microbiology and Risk Assessment, National Food Institute Technical University of Denmark, Lyngby, Denmark*

Abstract

Introduction: This study investigated the mechanisms of resistance in 36 *E. coli* isolated from waste, litter, soil and water samples collected from poultry farms in Southwestern Nigeria.

Methodology: Minimum inhibitory concentration (MIC) distributions of the isolates were determined using the methods of the Clinical and Laboratory Standard Institute and resistance genes detected by PCR.

Results: A total of 30 isolates (94%) showed resistance to more than one antimicrobial. Percentage resistance was: tetracycline 81%, sulphamethoxazole 67%, streptomycin 56%, trimethoprim 47 %, ciprofloxacin 42%, ampicillin 36%, spectinomycin 28%, nalidixic acid 25%, chloramphenicol 22%, neomycin 14%, gentamicin 8%, amoxicillin-clavulanate, ceftiofur, cefotaxime, colistin, florfenicol and apramycin 0%. Resistance genes found among the isolates include *bla-$_{TEM}$* (85%), *sul2* (67%), *sul3* (17%), *aadA* (65%), *strA* (70%), *strB* (61%), *catA1* (25%), *cmlA1* (13%), *tetA* (21%) and *tetB* (17%). Class 1 and 2 integrons were found in five (14%) and six (17%) isolates, respectively, while one isolate was positive for both classes of integrons. Seven out of eight isolates with resistance to ciprofloxacin and MIC ≤ 32 mg/L to nalidixic acid contained *qnrS* genes.

Conclusions: Our findings provided additional evidence that the poultry production environment in Nigeria represents an important reservoir of antibiotic resistance genes such as *qnrS* that may spread from livestock production farms to human populations via manure and water.

Key words: Farm waste; livestock production environment; *qnrS.*

J Infect Dev Ctries 2014; 8(9):1103-1112. doi:10.3855/jidc.4222

(Received 10 September 2013 – Accepted 14 May 2014)

Introduction

Antimicrobial drug use in livestock production has been implicated as a risk factor in the development and dissemination of drug resistance from livestock production farms [1,2]. Food animals and their production environments are reservoirs of both resistant bacteria and resistance genes that could be transferred to humans either by direct contact between animals and humans or indirectly via the food production chain [3,4]; or as a result of the spread of animal waste on land [5,2]. In Nigeria, antimicrobial agents are routinely used in livestock production especially as additives to feed and water [6]. This may result in a selective advantage and a consequent increase in the abundance of resistant bacteria in animals, their wastes and surrounding environment [5]. Antibiotic resistant bacteria have been detected in poultry waste [7,8,6,9], commercial poultry production environments [10,11] and poultry products [12,13].

Most food animal production farms in Nigeria have no waste treatment facility. Wastes generated on these farms are either dumped in heaps on farmlands or at remote locations, often inside or close to water bodies. Wastes are also used as organic fertilizer or as feed supplements, especially in fish ponds. This raises the fear that human beings resident in the vicinity of the farm and/or animal waste dump may be exposed to antibiotic resistant bacteria in the waste. In addition, close interaction between human and animal ecosystems is a common occurrence in Nigeria with livestock kept in close proximity to or inside human residences. This practice has been shown to favour high rates of transmission of zoonotic *E. coli* between livestock and humans in rural Uganda [14]. Although *E. coli* is a normal inhabitant of the intestinal tract of

warm blooded animals, commensal *E. coli* from humans and animals can cause extra intestinal infections and are a potential reservoir of antimicrobial resistance genes [15]. These bacterial strains, especially when they carry mobile genetic elements such as plasmids, transposons and integrons, can play important roles in the dissemination of transmissible resistance genes [16].

Little is known about the factors contributing to the problem of antibiotic resistance in Nigeria. This is partly because there is a dearth of information on the types, quantity and frequency of antibiotics used in farm animal production system in Nigeria. Very little is also known about the antibiotic resistance profile of bacteria (such as *E. coli*) commonly associated with animal production systems in Nigeria despite the growing global concern about the transmission of antibiotic resistant bacteria through the food animal production chain. The few studies that have investigated antibiotic resistance in *E. coli* from the poultry production system in Nigeria stopped at phenotypic level without a corresponding investigation of the molecular mechanisms of resistance [17]. Yet such studies are warranted because of the potentials of *E. coli* as reservoirs of transferable antibiotic resistance genes that could be disseminated into human populations as a result of the contact with animal wastes. This study therefore investigated antimicrobial susceptibility and molecular mechanisms of resistance in *E. coli* isolated from eight poultry

Table 1. Description of study farms

Farm/City:	Age (yrs):	Description:	Antimicrobial use:	Animal population:
Ogbomosho:				
OG1	15	Teaching and research farm, poultry, swine and fish operation. Swine and pond not operating at time of study.	Furazolidone, nitrofurantoin, ampicillin, neomycin, streptomycin, sulphonamides, tetracyclines, chloramphenicol, erythromycin.	2000 birds
OG2	6	Research and demonstration farm, poultry operation.	Furazolidone, nitrofurantoin, ampicillin, neomycin, streptomycin, sulphonamides tetracyclines, chloramphenicol, erythromycin.	750 birds
OG3	9	Poultry operation in a medium density residential area. Farm separated from adjoining building by a brickwall.	Furazolidone, nitrofurantoin, ampicillin, neomycin, streptomycin, sulphonamides tetracyclines, chloramphenicol, erythromycin.	2500 birds
OG4	unknown	Integrated poultry, swine and aquaculture operation in a rural agricultural community. Uses poultry waste as feed supplement in pond. Pond operation suspended about 5 months before sample collection.	Furazolidone, nitrofurantoin, ampicillin, streptomycin sulphonamides, doxycycline, chloramphenicol.	1200 birds, 120 pigs
Ibadan:				
IB1	>20	Teaching and research farm, poultry operation	Furazolidone, nitrofurantoin, ampicillin, neomycin, streptomycin, sulphonamides tetracyclines, chloramphenicol, erythromycin.	2500 birds
IB2	17	Integrated poultry, swine and aquaculture operation in a rural agricultural community. Swine and fish pond not operating at time of study. This is the only farm in this study that mentioned using enrofloxacin and tylosin.	Furazolidone, nitrofurantoin, ampicillin, neomycin, streptomycin, sulphonamides tetracyclines, chloramphenicol, enrofloxacin, tylosin.	2500 birds
Osogbo:				
OS1	5	Poultry and swine operation**,** swine operation relocated due to protests from surrounding residents	Furaltadone, nitrofurantoin, streptomycin, sulphonamides, tetracyclines, chloramphenicol, erythromycin.	1000 birds
OS2	unknown	Poultry operation in a rural agricultural community. People living around this farm use water from a well located in the farm for domestic purposes	Furazolidone, nitrofurantoin, ampicillin streptomycin, sulphonamides, tetracyclines doxycycline, chloramphenicol.	750 birds

-a brief profile, including types of antibiotics used in each of the farms sampled in this study. OG1- OG4, represent the four farms sampled in Ogbomoso, IB1 – IB2, represent the two farms sampled in Ibadan, OS1-OS2, represent the two farms sampled in Osogbo

production farms in Ogbomoso, Ibadan and Osogbo in Southwestern Nigeria. Four of the farms combined swine and/or fish ponds with poultry operations. The farms with aquaculture operations use the wastes generated from the poultry pens as feed supplement in the ponds. Our primary aim is to assess the potential of the poultry production environment in Nigeria as a reservoir of antibiotic resistance genes that can be mobilized into human populations.

Methodology

Bacterial strains

Thirty six *E. coli* isolated from samples of faecal materials, litter, farm soil, water and fish pond sediment collected from the study farms in May and June 2008 were included in the study. The water samples were collected as previously described [18] while samples of soil; litter and faecal materials were collected in sterile polythene sample bags as described [6].The eight farms included in the study were selected from Ogbomoso and Ibadan in Oyo State (8^{o} 0′ N 4^{o} 0′ E) and Osogbo in Osun State (7 ^{o} 46′ N 4 ^{o} 34′ E) of Southwestern Nigeria. The farms are those who voluntarily agreed to participate in the study and are described in Table 1. Among the farms, only one has a comprehensive record of drug usage, it was also the only farm that admitted using enrofloxacin. The remaining farms admitted using one or more of furazolidone, furaltadone, nitrofurantoin, ampicillin, neomycin, streptomycin, sulphonamides, tetracyclines, doxycycline, chloramphenicol and erythromycin regularly on the farms for animal health management. The drugs are usually part of formulations used as feed supplements, commonly referred to as "premix" which often contained mixtures of antimicrobials. The farm with combined aquaculture operation suspended aquaculture operations about 5 months before the period of sediment collection from the fish pond.

The organisms were isolated from the samples within 24 hours on MacConkey agar as previously described [6] and were identified at the Unit of Antimicrobial Resistance and Molecular Epidemiology, National Food Institute, DTU, Copenhagen, Denmark by streaking on Blood agar, MacConkey agar and CHROMagar orientation Medium (Bencton Dickinson GmBH, Heigelberg, Germany). Thirty-six non duplicated isolates confirmed as *E. coli* were randomly selected from the samples and stored at -80^{o}C prior to further analysis for Minimum inhibitory concentration (MIC) determination and molecular characterization.

Susceptibility testing

The susceptibility of the isolates to antimicrobial agents was tested at the National Food Institute, DTU, Denmark using commercial dehydrated Sensititre panels (Trek Diagnostic Ltd, East Grinstead, UK) containing the antibiotics listed below at the stated test concentrations in mg/L. Amoxicillin (2-32 mg/L) – clavulanate (1-16 mg/L), ampicillin (1-32 mg/L), apramycin (4-32 mg/L), cefotaxime (0.125-4 mg/L), ceftiofur (0.5-8 mg/L), chloramphenicol (2-64 mg/L), ciprofloxacin (0.015-4 mg/L), colistin (1-16 mg/L), florfenicol (2-64 mg/L), gentamicin (0.5-16 mg/L), nalidixic acid (4-64 mg/L), neomycin (2-32 mg/L), spectinomycin (16-256 mg/L), streptomycin (8-128 mg/L), sulphamethoxazole (64-1024 mg/L), tetracycline (2-32 mg/L) and trimethoprim (1-32 mg/L). Panels were incubated aerobically at 37^{o}C for 18-20 hours. MIC distributions were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. The MIC results were interpreted using the European Committee for Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values and *E. coli* ATCC 25922 was used as a positive control strain.

Molecular mechanisms of resistance

Polymerase chain reaction (PCR) amplification of class 1 and 2 integrons and antimicrobial resistance genes were performed as described previously [20]. One to four colonies of the tested isolates on blood agar were suspended in 100µl of TE buffer and lysed as previously described [20]. The lysates were used as template in PCR to detect *strA, strB* and *aadA* in streptomycin resistant isolates, *bla-$_{TEM}$* in ampicillin resistant isolates, *sul2* and *sul3* in sulphamethoxazole resistant isolates, *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G) and *tet*(39) in tetracycline resistant isolates, *cmlA, catA1* and *floR* (primers for *floR:* Flor-1, 5'-ATGGCAGGCGATATTCATTA-3'; flor-2: 5'-AAACGGGTTGTCACGATCAT-3') in chloramphenicol resistant isolates, acc*(3)-IV* in gentamicin resistant isolates, *qnrA*, *qnrB*, *qnrC*, *qnrD, qnrS*, *aac(6')Ib* and *qepA* in isolates resistant to ciprofloxacin (≥ 0.125mg/L) but sensitive to nalidxic acid with MIC ≤ 32 mg/L [20, 22-25]. The variable regions of Class 1 and Class 2 integrons were amplified and representatives of the different sized amplicons sequenced to characterise their gene contents as described by Peirano *et al* [21]. The following were used as positive control strains in PCR: *E. coli* (*tet*(A)), *Salmonella Typhimurium* DT104 (*tet*(G)), *E. coli* CSH50::Tn10 (*tet*(B)), *E. coli*

DO7pBR322 (*tet*(C)), *E. coli* C600psl106 (*tet*(D)), *E. coli* HB 101 psl 1604 *tet*(E)), *E. coli* YA5605 pUC19::tet(39) (*tet(39)*), *E. coli* K13 (acc*(3)-IV*), *Salmonella* Weltevreden TA428/97 (*catA1*), *S.* Typhimurium P502212 DT104 (*floR*), Paratyphi B63.48 (*bla-TEM*), *S.* Typhimurium DT104 (*strA, strB, aadA1*), *Salmonella* B (*sul2*), *Salmonella* C (*sul3*), *S.* Typhimurium DS611 (*cmlA*, *int1, sul1*), *Salmonella* (int2).

Results

Antimicrobial susceptibility

Tables 2 and 3 showed the distribution of MICs and phenotypic patterns of resistance among the isolates. Resistance to tetracycline (81%), sulphamethoxazole (67%), streptomycin (56%), trimethoprim (47%), ciprofloxacin (42%) and ampicillin (36%) was the most commonly detected among the isolates tested while phenotypic resistance to spectinomycin (28%), nalidixic acid (25%), chloramphenicol (22%), neomycin (14%) and gentamicin (8%) was less commonly detected. In contrast, all the isolates were sensitive to amoxicillin-clavulanate, ceftiofur, cefotaxime, colistin, florfenicol and apramycin. Among the 15 ciprofloxacin resistant isolates, eight isolates had MICs to nalidixic acid ≤ 32 mg/L. While this study was not designed to investigate the relationship between antibiotic use and resistance on the study farms due primarily to the non-availability of antibiotic-use information as mentioned earlier, it is important to report that tetracycline, streptomycin, sulphonamides, and ampicillin are among the antimicrobials used on all the study farms.

Among isolates of the present study, resistance to two or more antibiotics was detected in 30 of 36 (83%) isolates. When looking at isolates from poultry droppings/litter, swine waste, soil (including fish pond sediment) and well water, 93% (14/15), 63% (5/8), 89% (8/9) and 75% (3/4) of the isolates respectively showed resistance to two or more antimicrobials. Four isolates from pig manure (EC137, EC146), farm soil (EC162) and well water (EC116) were fully susceptible to all antimicrobials tested (Table 3).

Mechanisms of resistance

Table 3 showed the phenotypic patterns of resistance and resistance genes profiles of the isolates. Among the 36 isolates tested, six (17%) carried class 2 integrons, five (17%) of 24 sulphamethoxazole resistant isolates carried class 1 integrons, while only one isolate (3%) (EC3) carried both classes of integrons. The variable region of the class 1 integrons of one representative isolate (EC117) selected from among the most common sized amplicon (1500 bp found in four isolates) was partially sequenced (935 bp). The partial sequence showed 99% sequence identity with position 695 to 1629 containing *aadA5* in *E. coli* GL1 (GenBank accession number FJ807902). As expected the sulphonamide resistance gene *sul1* was detected in all class 1 integron-positive isolates as part of the integron. The variable regions of the class 2 integrons detected among the *int2* positive isolates showed three different sized PCR products. The product of one representative isolate (EC3, EC111 and EC113) for each type was sequenced. The partial sequences showed 88% identity with position 1057 to 436 of *E. coli* encoding a *sat1* (GenBank accession number X56815) for isolate EC111, 100% identity with position 2073 to 2608 of *E. coli* encoding a *dfrA1-sat2*(partial) (GenBank accession number HM439239) for EC113 and 100% identity with position 1995 to 2668 of *E. coli* encoding a *sat2* (GenBank accession number AY183453) for isolate EC3.

In addition to *sul1* carried as part of *int1, sul2* and *sul3* were detected in 16 (67%) and 4 (17%) of 24 sulphamethoxazole – resistant isolates. All the isolates positive for *sul3* were from poultry waste or farm soil from Ogbomoso. Three of the *int1*/*sul1* positive isolates were also positive for *sul2*. However, *sul2/sul3* and *sul1/sul3* combinations could not be detected in any of the isolates. Of 23 strains with phenotypic resistance to streptomycin, spectinomycin, or a combination of both antibiotics, *aadA, strA* and *strB* encoding resistance to spectinomycin / streptomycin were detected in 15 (65%) (including two isolates, EC123 and EC226 with intermediate resistance to streptomycin and spectinomycin), 16 (70%) and 14 (61%) isolates respectively. Five of these isolates were positive for the *aadA/strA/strB* combination while *strA* was found to occur together with *strB* in all 14 isolates positive for *strB*. Further, 12 (86%) of 14 isolates carrying the *strA/strB* combination were also positive for *sul2*.

The only tested ampicillin resistance gene *bla-TEM* was found in 11 (85%) of 13 ampicillin-resistant isolates in the present study. The highest prevalence (7 of 8 ampicillin resistant isolates) of this gene was found among isolates from Ogbomoso. *catA1* and *cmlA1* was detected in 2 (25%) and 1 (13%) of eight chloramphenicol resistant isolates respectively.

Table 2. Distribution of MICs and occurrence of resistance among the *E. coli* isolates from each source

Compound	% Resistance	Distribution of MICs																		
		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	mg/L
Chloramphenicol	PO[a]=27									5	6			2	2					
	SW[b]=0									3	4	1								
	SO[c]=33									1	4	1	1	1	1					
	WA[d]=25									2		1		1						
Florfenicol	PO=0									5	8	2								
	SW=0									3	5									
	SO=0									2	5	2								
	WA=0									2	2									
Trimethoprim	PO=53							7						8						
	SW=25							7						1						
	SO=56							4		1				4						
	WA=75							1						3						
Amoxicillin + Clavulanate	PO=0								3	5	5	2								
	SW=0								5	3										
	SO=0								1	6	1	1								
	WA=0									2	1	1								
Ceftiofur	PO=0						14	1												
	SW=0						8													
	SO=0						9													
	WA=0						4													
Nalidixic Acid	PO=33									5	2	3	1		4					
	SW=13									7					1					
	SO=11									5	2	1			1					
	WA=50									2			1		1					
Neomycin	PO=13								12	1		1		1						
	SW=0								8											
	SO=22								5	2				2						
	WA=25								3					1						
Spectinomycin	PO=47											8			2	3	2			
	SW=13											6	2				2			
	SO=44											2	2	1	3	1				
	WA=25											2	1			1				

Table 2. ***(continued)*** Distribution of MICs and occurrence of resistance among the *E. coli* isolates from each source

Streptomycin	**PO=67**									**4**	**1**	**1**		**1**	**8**	
	SW=50									**3**	**1**	**1**	**2**		**1**	
	SO=44									**4**	**1**		**1**		**3**	
	WA=50									**1**	**1**			**2**		
Tetracycline	**PO=87**							**1**	**1**				**13**			
	SW=75							**2**					**6**			
	SO=89							**2**					**7**			
	WA=75								**1**				**3**			
Sulphamethoxazole	**PO=73**												**4**			**11**
	SW=25												**6**			**2**
	SO=89														**1**	**8**
	WA=75												**1**			**3**
Colistin	**PO=0**						**15**									
	SW=0						**8**									
	SO=22						**9**									
	WA=0						**4**									
Ciprofloxacin	**PO=60**	**6**			**3**	**2**	**2**			**2**						
	SW=13	**7**			**1**											
	SO=33	**5**	**1**		**2**	**1**										
	WA=50	**1**	**1**	**1**						**1**						
Ampicillin	**PO=40**							**4**	**4**	**1**			**6**			
	SW=25						**1**	**2**	**3**				**2**			
	SO=22							**2**	**4**		**1**		**2**			
	WA=75								**1**				**3**			
Gentamicin	**PO=7**					**14**						**1**				
	SW=0					**7**	**1**									
	SO=22					**4**	**2**	**1**				**2**				
	WA=0					**4**										
Cefotaxime	**PO=0**			**14**	**1**											
	SW=0			**7**	**1**											
	SO=0			**9**												
	WA=0			**4**												
Apramycin	**PO=0**								**11**	**4**						
	SW=0								**7**	**1**						
	SO=0								**7**	**1**	**1**					
	WA=0								**4**							

-the frequency of resistance to each concentration of antibiotics tested in this study. Solid vertical lines indicate EUCAST epidemiological cut off values. Where only a solid line is shown, the cut off value and breakpoint are the same. Footnotes a: PO = Poultry waste (n = 15), b: SW = Swine waste (n = 8), c: SO = Farm Soil and fish pond sediments (n = 9), d: WA = well water (n = 4).

Table 3. Phenotypic pattern of resistance and resistance genes profiles of *E. coli* isolates included in this study

Isolates	Farm[a]	Source	Phenotypic resistance	Resistance genes profile
IBADAN				
EC3	IB2	Poultry droppings	chl, tmp, nal, neo, spe, str, smx, cip, amp	*int1/sul1*[b]*, int2, sul2, bla-$_{TEM,}$ aadA, strA, strB*
EC40	IB2	Poultry droppings	nal, str, tet, smx, cip, amp	*sul2, bla-$_{TEM}$, strA, strB*
EC42	IB2	Poultry droppings	chl, tet, cip	*qnrS*
EC54	IB1	Poultry droppings	tmp, str, tet, smx, cip, amp	*qnrS, sul2, bla-$_{TEM}$, strA, strB*
OGBOMOSO				
EC70	OG1	Poultry litter	tmp, spe, str, tet, smx, cip, amp	*qnrS, int2, sul2, bla-$_{TEM}$, aadA, strA, strB, tet(A)*
EC75	OG1	Poultry litter	tet	*tet(A)*
EC87	OG1	Poultry litter	chl, tmp, nal, spe, str, tet, smx, cip, amp	*sul3, bla-$_{TEM}$, aadA*
EC78	OG4	Poultry droppings	tmp, nal, tet, smx, cip	*sul2, tet(A)*
EC111	OG4	Poultry droppings	spe, str, tet	*int2, aadA*
EC114	OG2	Poultry droppings	tmp, neo, str, tet, smx, amp	*bla-$_{TEM,}$ strA, strB, tet(A)*
EC157	OG3	Poultry droppings	tmp, spe, str, tet, smx	*int2, sul2, aadA, strA, strB, tet(A)*
EC199	OG4	Poultry droppings	nal, str, tet, smx, cip	*qnrS, sul2, strA*
EC232	OG1	Poultry droppings	chl, tet, smx, gen	*sul3, aadA, cmlA*
EC146	OG4	Swine waste	Susceptible to all Antibiotics tested	
EC160	OG4	Swine waste	str, tet, smx, amp	*sul2, bla-$_{TEM}$, strA, strB*
EC170	OG4	Swine waste	str, tet	*strA, strB, tet(B)*
EC96	OG4	Fish pond sediment	nal, str, tet, smx, cip	*sul2, strA, strB, tet(A)*
EC233	OG4	Fish pond sediment	str, tet, smx, cip	*qnrS, sul2, strA, strB*
EC69	OG4	Manured soil	chl, spe, tet, smx, gen	*sul3, aadA*
EC113	OG2	Farm soil	chl, tmp, neo, spe, str, tet, smx, cip, amp	*qnrS, int2, sul2, bla-$_{TEM}$, catA1, aadA,strA, strB*
EC117	OG3	Farm soil	tmp, neo, spe, str, tet, smx, amp	*int1/sul1, sul2, aadA, strA, strB, tet(B)*
EC121	OG3	Farm soil	tmp, spe, tet, smx	*int2, sul2, aadA, tet(B)*
EC162	OG3	Farm soil	Susceptible to all Antibiotics tested	
EC1801	OG3	Farm soil	tmp, smx	*sul2*
EC226	OG4	Farm soil	chl, tet, smx, gen	*sul3, aadA*
EC116	OG3	Well water 2	Susceptible to all Antibiotics tested	
EC142	OG3	Well water 2	tmp, nal, str, tet, smx, cip, amp	*sul2, bla-$_{TEM,}$ strA, strB, tet(B)*
EC164	OG3	Well water 5	chl, tmp, nal, neo, str, tet, smx, cip, amp	*int1/sul1, sul2, bla-$_{TEM}$, catA1, strA, tet(B)*
OSOGBO				
EC143	OS1	Poultry droppings	tmp, tet, cip	*qnrS*
EC149	OS1	Poultry droppings	str, smx	*sul2, strA, strB*
EC122	OS1	Swine waste	tet	No resistance genes detected
EC123	OS1	Swine waste	tmp, nal, tet, smx, cip	*int1/sul1, aadA*
EC137	OS1	Swine waste	Susceptible to all Antibiotics tested	
EC138	OS1	Swine waste	tmp, str, tet, amp	*aadA*
EC236	OS1	Swine waste	spe, str, tet	*int2, aadA*
EC197	OS2	Well water	tmp, spe, tet, smx, amp	*int1/sul1, bla-$_{TEM}$, aadA*

chl; chloramphenicol, tmp; trimethoprim, nal; naldixic acid, neo; neomycin, str; streptomycin, spe; spectinomycin, smx; sulphamethoxazole, cip; ciprofloxacin, amp; ampicillin, gen; gentamicin, tet; tetracycline.

Footnote a: See Table 1 for farm codes.

Footnote b: *sul1* gene was detected as part of class 1 integrons in all *int1* positive isolates.

None of the eight chloramphenicol resistant isolates, including those that were negative for *catA1* and *cmlA*, were positive for *floR*. Majority of the tetracycline resistant isolates did not give positive amplification signals for most of the tetracycline resistant genes we tested for.

Even though all positive controls gave positive amplification signals, *tet*(C), *tet*(D), *tet*(E), *tet*(G) or *tet*(39) could not be detected among the 29 tetracycline resistant isolates. However, *tet*(A) and *tet*(B) were found in six (21%) and five (17%) isolates respectively. Compared to the present study, other studies reported higher frequencies of *tet*(A) and *tet*(B). For example, in a study of 317 *E. coli* isolated from pigs, cattle and poultry in Germany, Guerra *et al.* [15] found *tet*(A) and *tet*(B) in 66% and 42% of the isolates respectively. Similarly, Enne *et al* [26] found 56% of 103 tetracycline resistant *E. coli* from farm animals at slaughter to contain *tet*(B). *acc(3)-IV,* the only gentamicin resistance gene tested for in this study was not detected in any of the four gentamicin resistant isolates.

Seven out of eight isolates that were resistant to ciprofloxacin and had MIC values to nalidixic acid of ≤32 mg/L contained *qnrS.* Of these isolates, the PCR products of three isolates (EC54, EC143, EC199) were sequenced and the sequences showed 100% identity to *qnrS1* from *E. coli* (GenBank accession number GQ214053).

Discussion

Development of resistance to antimicrobial agents is a problem of global proportion which is increasingly frustrating efforts to treat infectious diseases. This has been attributed to human and agricultural use of antimicrobials. Thus, our study investigated the occurrence and molecular mechanisms of antibiotic resistance among commensal *E. coli* isolated from selected poultry farms in southwestern Nigeria, an area of the world where little is known on antibiotic use and its contribution to the development and dissemination of resistance. A serious limitation to this study was the difficulty encountered in sample collection which limited the number of isolates included in the study. It was difficult convincing farm owners to participate in the study and most of the volunteer farms restricted access to specified areas of the farms, and none allowed sample collection more than twice. Nonetheless, our findings demonstrated that the commensal *E. coli* included in this study were commonly resistant to antibiotics used in human and veterinary medicine. However, because our study did not include isolates from farms without antimicrobial use, it was difficult to draw a definite conclusion on the association between drug use on the study farms and the occurrence of resistance in the isolates.

The observed levels of resistance to ciprofloxacin (42%) and trimethoprim (47%) were however unexpected since only one of the eight farms admitted using the fluoroquinolone enrofloxacin and no farm used trimethoprim. Thus, it is quite possible that use of trimethoprim, a common component of poultry pre-mixes used widely in the farms of the present study was underreported. More importantly, our observation about the levels of resistance to ciprofloxacin and trimethoprim suggested that factors other than antibiotic use may be contributing to the selection of resistance among the present isolates.

Similar to the observed pattern of phenotypic resistance, genes conferring resistance to streptomycin/spectinomycin, sulphamethoxazole and ampicillin were also found to be common among the isolates. However, it seemed that local and geographical factors played a role in the occurrence of some resistance genes; all isolates positive for *sul3* and tetracycline resistance genes are from Ogbomoso. In a sharp contrast to this, other genes seem to have a widespread occurrence within the study farms. For example, isolates with *qnrS* originated from six different farms (including the farm admitting usage of fluoroquinolones) and from four different sources: poultry droppings, fish pond sediment and farm soil. The only isolate that did not contain any of the quinolone resistance genes screened for was from well water (EC142). The widespread occurrence of *qnrS* observed in this study may be a result of their co-selection and transfer with other resistance determinants on mobile genetic elements. *qnr*, a plasmid-mediated horizontally transferable gene encoding quinolone resistance was discovered for the first time in 1998 and has been in circulation for at least 20 years [27]. Qnr proteins are capable of protecting DNA gyrase from the action of quinolones and *qnr* are increasingly detected in isolates of clinical importance [28]. Our findings indicated that *qnrS* was widely spread among poultry and their production environment in Nigeria. *qnrS1* was recently found among *Salmonella* and *E. coli* from humans and animal sources in Nigeria [29,13].

Our study provided additional evidence that food animals production environments may be important reservoirs of clinically important antibiotic resistance genes such as plasmid-mediated quinolone resistance (PMQR) genes in this part of the world. This is of

concern as fluoroquinolones were listed by the World Health Organization as critically important antimicrobials for human health [30]. Therefore, it seems very important to pay more attention to the spread of resistance through such production systems in the tropical climate, and regulation of antimicrobial usage in production systems with close interaction between human and animal ecosystems such as this, prevalent in the developing world, seems crucial.

Even though we tested for the resistance genes most commonly found in *E. coli*, resistance in a number of isolates appeared to be conferred by mechanisms other than those tested in this study. Thus resistance genes investigated in this study could not be detected in two ampicillin-resistant, one sulphamethoxazole-resistant, five chloramphenicol-resistant and eighteen tetracycline-resistant isolates. These isolates may contain known mechanisms not tested for in this study or unknown mechanisms. The large fraction of tetracycline resistant isolates without any of the tested tetracycline resistance genes is unexpected and may be due to geographical differences between this and previous studies on tetracycline resistant *E. coli.* The results of this study underscore the importance of routine screening for antibiotic resistance in commensal bacteria from food animal production farms using both phenotypic and genotypic methods. This is very crucial, especially in countries such as Nigeria with limited studies on occurrence of resistance genes in food animal production systems.

In conclusion, data from the present study suggested that commensal *E. coli* from the study farms may act as reservoirs of antimicrobial drug resistance genes such as *qnrS* which may be mobilized into human populations. To the best of our knowledge, this is the first comprehensive study of molecular mechanisms of resistance in commensal *E. coli* associated with livestock farms in Nigeria. Results should however be interpreted with caution as a limited number of isolates were investigated and a comprehensive history of antibiotic use necessary to make informed decision on the contribution of antibiotic use to the observed resistance is not available. It however pointed attention to the critical need for the regulation of antimicrobial drug usage in livestock production and continuous monitoring of antibiotic resistance in developing countries for public health safety.

Acknowledgements

We would like to thank Jacob Dyring Jensen and Hanne Mordhorst for excellent technical assistance and the International Union of Microbiological Societies (IUMS), Society for General Microbiology (SGM), UK and UNESCO for a Short term Fellowship to the first author under the UNESCO-IUMS-SGM Short term fellowship.

References

1. Bauer – Garland J, Frye JG, Gray JT, Berrang ME, Harrison MA, Fedorka–Cray PJ (2006) Transmission of *Salmonella entrica* serotype Typhimurium in Poultry with or without Antimicrobial Selective Pressure. J Appl Microbiol, 100: 1301-1308.
2. Gosh S, LaPara, TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J 1: 191-203.
3. Marshall BM, Levy SB (2011) Food animals and antimicrobials: Impacts on human health. Clin Microbiol Rev 24: 718-733.
4. WHO (2011) Tackling antibiotic resistance from a food safety perspective in Europe. Available: http://www.euro.who.int/__data/assets/pdf_file/0005/136454/e94889.pdf. Accessed 12 December 2012.
5. Heuer H, Smalla, K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ Microbiol 9: 657-666.
6. Adelowo OO, Ojo FA, Fagade OE (2009) Prevalence of multiple antibiotic resistance among bacteria isolates from selected poultry wastedumps in Southwestern Nigeria. World J Microbiol Biotechnol 25: 713-719.
7. Kelley TR, Pancorbo OC, Merka WC, Barnhart HM (1998) Antibiotic resistance of bacterial litter isolates. Poult Sci 77: 243–247.
8. Khan AA, Nawaz MS, Summage WC, Khan SA, Lin J (2005) Isolation and molecular characterization of fluoroquinolone-resistant *Escherichia coli* from poultry litter. Poult Sci 84: 61–66.
9. Furtula V, Farrell EG, Diarrassouba F, Rempel H, Pritchard J, Diarra MS (2010) Veterinary pharmaceuticals and antibiotic resistance of *Escherichia coli* isolates in poultry litter from commercial farms and controlled feeding trials. Poult Sci 89: 180–188
10. Hayes JR, English LL, Carr LE, Wagner DD, Joseph SW (2004) Multiple-antibiotic resistance of *Enterococcus* spp. Isolated from commercial poultry production environments. Appl Environ Microbiol 70: 6005–6011.
11. Nilsson O, Greko C, Bengtsson B (2009) Environmental contamination by vancomycin resistant enterococci (VRE) in Swedish broiler production. Acta Veterinaria Scandinavica doi:10.1186/1751-0147-51-49.
12. Girlich D, Poirel L, Carattoli A, Kempf I, Lartigue MF, Bertini A, Nordman P, (2007) Extended-spectrum β-lactamase CTX-M-1 in *Escherichia coli* isolates from healthy poultry in France. Appl Environ Microbiol 73: 4681–4685.
13. Fortini D, Fashae K, Garcia-Fernandez A, Villa L, Carattoli A (2011) Plasmid mediated quinolone resistance and β-lactamases in *E. coli* from healthy animals from Nigeria. J Antimicrob Chemother 66: 1269-1272.

14. Rwego IB, Gillespie TR, Isabirye-Basuta G, Goldberg TL (2008) High Rates of *Escherichia coli* Transmission between Livestock and Humans in Rural Uganda. J Clin Microbiol 46: 3187-3191.
15. Guerra B, Junker E, Schroeter A, Malorny B, Lehmann S, Helmuth R (2003) Phenotypic and genotypic characterization of antimicrobial resistance in German Escherichia coli isolates from cattle, swine and poultry. J Antimicrob Chemother 52: 489-492.
16. Agersø Y, Sandvang D (2005) Class I integrons and tetracycline resistance genes in *Alcaligenes, Arthrobacter* and *Pseudomonas* spp. isolated from pigsties and manured soil. Appl Environ Microbiol 46: 7941-7947.
17. Ogunleye AO, Oyekunle MA, Sonibare AO (2008) Multidrug resistant *Escherichia coli* isolates of poultry origin in Abeokuta, South Western Nigeria. Vet. arhiv 78:501-509.
18. Adelowo OO, Akinlabi IA, Fagade OE (2012) Environmental impact assessment of Attenda abattoir, Ogbomoso southwestern Nigeria on surface and groundwater quality using geo-electrical imaging and microbiological analysis. Environ Monit Assess 184:4565-4574.
19. [CLSI]Clinical and Laboratory Standards Institute (2005) Performance Standards for Antimicrobial Susceptibility Testing: Fifteenth Informational Supplement M100-S15. CLSI, Wayne, PA, USA.
20. Peirano G, Agersø Y, Aarestrup FM, Falavina dos Reis FM, Rodirgues DP (2006) Occurrence of integrons and antimicrobial resistance genes among *Salmonella enterica* from Brazil. J Antimicrob Chemother 58: 305-309.
21. Peirano G, Agersø Y, Aarestrup FM, Rodirgues DP (2005) Occurrence of integrons and resistance genes among sulphonamide-resistant *Shigella* spp. from Brazil. J Antimicrob Chemother 55: 301-305.
22. Agersø Y, Guardabassi L (2005) Identification of Tet 39, a novel class of tetracycline resistance determinant in *Acinetobacter* spp. of environmental and clinical origin. J Antimicrob Chemother 55: 566-569.
23. Agersø Y, Bruun MS, Dalsgaard I, Larsen JL (2007) The tetracycline resistance gene *tet*(E) is frequently occurring and present on large horizontally transferable plasmids in *Aeromonas* spp. from fish farms. Aquaculture 266: 47-52
24. Cavaco LM, Hasman H, Xia S, Aarestrup FM (2009) *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. Antimicrob Agents Chemother 53: 603-608.
25. Wang M, Guo Q, Xu X, Wang X, Ye X, Wu S, Hooper DC, Wang M (2009) New plasmid-mediated quinolone resistance gene, qnrC, found in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother 53: 1892-1897.
26. Enne VI, Cassar C, Sprigings K, Woodward MJ, Bennett PM (2008) A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. FEMS Microbiol Lett 278: 193-199.
27. Martinez-Martinez L, Pascual A, Jacoby GA (1998) Quinolone resistance from a transferable plasmid. Lancet 351: 797–799.
28. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A (2009) Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat. Clin Microbial Rev 22: 664–689.
29. Fashae K, Ogunsola F, Aarestrup FM, Hendricksen RS (2010) Antimicrobial susceptibility and serovars of *Salmonella* from chickens and humans in Ibadan, Nigeria. J Infect Dev Ctries 4: 484-494. doi:10.3855/jidc.909.
30. Collignon P, Powers JH, Chiller TM, Aidara-Kane A, Aarestrup FM (2009) World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. Clin Infect Dis 49: 132-141.

Corresponding author

Yvonne Agersø
Department for Microbiology and Risk Assessment, National Food Institute,
Technical University of Denmark, Kemitorvet building 204st
DK-2800 Lyngby, Denmark
Phone +4535886273
Fax: +4535886341
Email: yvoa@food.dtu.dk

Conflict of interests: No conflict of interests is declared.