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Vol. 10(34), pp. 1422-1429, 14 September, 2016
DOI: 10.5897/AJMR2016.8251
Article Number: 14ED3AF60517
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparison of the prevalence of antibiotic-resistant *Escherichia coli* isolates from commercial-layer and free-range chickens in Arusha district, Tanzania

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Received 4 August 2016; Accepted 31 August, 2016

The antibiotic susceptibility of fecal *Escherichia coli* isolates from commercial-layer and free-range chickens in Arusha district, Tanzania were compared. All the chickens were raised by individual households, but commercial-layer chickens were purchased from commercial vendors, whereas no systematic breeding system was used to produce free-range chickens. A total of 1,800 *E. coli* isolates (1,200 from commercial-layer chickens and 600 from free-range chickens) were tested for susceptibility to 11 antibiotics by breakpoint assays. All *E. coli* isolates were susceptible to gentamicin, ceftazidime and cefotaxime. Isolates from commercial-layer chickens had a high prevalence of resistance (32.4-74.5%) for amoxicillin, ampicillin, ciprofloxacin, tetracycline, streptomycin, trimethoprim and sulfamethoxazole, while the prevalence of resistance to these antibiotics was lower (7-31.5%) for free-range chickens (*P*<0.05). Both groups had a similar prevalence of resistance to chloramphenicol (1.17-1.5%; *P*>0.05). For antibiotic resistant strains, 64.1 and 91.5% of free-range and commercial-layer isolates, respectively, were resistant to ≥ 2 antibiotics. Commercial-layer chickens harbored significantly more resistant *E. coli* isolates (*P*<0.001) than free-range chickens, consistent with more exposure to antibiotics when compared with free-range chickens. Efforts should be directed towards motivating household owners to limit the use of antibiotics when they are investing in these breeds.

Key words: Antibiotic resistance, free-range, commercial-layer, *Escherichia coli*, Arusha, Tanzania.

INTRODUCTION

Escherichia coli is a commensal bacterium in the gastrointestinal tract of humans and animals. Although most *E. coli* strains are harmless, there are pathogenic strains capable of causing infectious disease including

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diarrhea, neonatal meningitis, blood stream infections and urinary tract infections (UTIs) (Nakazato et al., 2009; Jakobsen et al., 2010). Avian pathogenic *E. coli* causes yolk-sac infections, respiratory-tract infections, bloodstream infections and colibacillosis (Yang et al., 2004; Horn et al., 2012). Transmission of these diseases accounts for significant losses for poultry producers (Ewers et al., 2009).

Antibiotics are used in poultry production particularly for commercial production to treat and prevent diseases. The demand for antibiotics is generally correlated with increased flock size both due to the number of animals and associated increases in the incidence of disease (Mathew et al., 2007). Antibiotics are also used as growth promoters. For example, tetracyclines are used in low concentrations as feed additives to enhance growth whereas higher concentrations are used to prevent or treat disease (Stead et al., 2007). In developing countries, there is an increased demand for chicken and chicken products as a result of population increase, urbanization and improved economic status. Responding to this increased demand, chicken farmers tend to shift to increasingly intensive production systems and antibiotics are often used to manage diseases in these operations (Hao et al., 2014). At a household level, free-range chickens likely forage for their food rather than receive commercially prepared feeds. Furthermore they may be more resistant to diseases and may be exposed to fewer diseases simply due to lower population densities (Hamisi et al., 2014).

Resistant bacteria from food animals can spread to humans directly or indirectly (Adenipekun et al., 2015). Direct transmission involves contact with reservoir animals, their feces or consumption of contaminated animal food products such as meat and eggs. Indirect transmission can be through contaminated water, food and environments. This is a significant public health concern when animal husbandry practices promote resistance to medically important antibiotics (Anderson et al., 2003). If antibiotics are being used in commercial-source poultry production, then *E. coli* from these sources should exhibit a significantly greater prevalence of resistance as compared to free-range chickens that are not likely to be exposed to antibiotics. To test this hypothesis, *E. coli* collected from poultry in the Arusha district of Tanzania were evaluated.

MATERIALS AND METHODS

Sampling details

In Arusha, commercial-layer chickens are purchased as day-old chicks from different commercial producers and are raised by farmers adjacent to their houses (typically up to 200 birds). These chickens are raised in enclosed structures and are given feed and water that may contain antibiotics (tetracycline for growth promotion and enrofloxacin and sulfa-trimethoprim for prophylaxis and treatment) and vaccines recommended by the commercial vendors. In addition to commercial layers, these farmers and others in the same area also raise free-range, indigenous chickens (usually in small numbers up to 20 chickens) that are used for egg and meat production. The free-range chickens used in this study were those that were neither treated with antibiotics nor fed commercial feeds but instead scavenge freely without strict physical constraints.

Fecal samples from commercial-layer and free-range chickens were obtained through convenience sampling method between April and July 2015. Briefly, five wards in Arusha (Mifugo, Nambala, Njiro, Sakina and Sansi) were selected for sampling. From each ward, one household that exclusively raised commercial-layer and one that exclusively raised free-range chickens were identified and distinct, spatially discreet fecal samples (n = 5 or 10 for free-range or commercial layers, respectively) were collected.

Sample collection and preparation

A total of 50 commercial-layer and 25 free-range chicken fecal samples were collected in individual sterile plastic bags and were transported to the laboratory at NM-AIST (the Nelson Mandela African Institution of Science and Technology) at ice cold temperature. In the laboratory, samples were mixed with sterile distilled water (approximately 1:9 ratio, feces: water) to make suspensions. An aliquot of 1 ml of each fecal suspension was added with glycerol (15% final concentration) and stored at -80°C for long-term preservation of original samples. The fecal suspensions were further diluted (1:10) with sterile distilled water. Sterile glass beads were then used to spread 30 uL of diluted fecal suspension onto 100 mm diameter MacConkey (MAC; Becton, Dickinson Company, Sparks, MD) agar plates that were then incubated overnight at 37°C.

E. coli isolation

After incubation, the plates were examined for the growth of morphologically distinct *E. coli* colonies (pink to reddish, lactose-fermenting colonies surrounded by bile salt precipitate). If the growth was numerous and individual isolates unavailable, the frozen fecal suspensions were thawed and serially diluted (10-fold) and higher dilutions were plated to obtain distinct *E. coli* colonies. Presumptive, *E. coli* colonies (n=24) were picked for each sample from the MAC agar plates and inoculated into wells containing 150 µl of LB broth1 (Luria-Bertani broth) in 96-well micro-titre plates using autoclaved tooth picks. After inoculating, 96 colonies (4 fecal samples per plate), the 96-well plates were wrapped in cling-wrap to minimize evaporation and were incubated overnight (16 to 18 h) at 37°C. Isolation of *E. coli* based on colony morphology alone yields >95% accurate identification in our hands (Liu et al., 2016), but for the current study, we further confirmed *E. coli* identification by re-growing isolates on HiChrome coliform agar (SIGMA-ALDRICH Co., St. Luis, MO). This media contains two chromogenic substrates that allow simple differentiation of *E. coli* (dark blue to violet colored colonies). Only presumptive *E. coli* from both agar media were analyzed for this study. All strains where stored at -80°C in sterile phosphate-buffered glycerol (15% final concentration).

*Determin*ation of *the antibiotic resistance profile*

To determine the prevalence of antibiotic resistance, *E. coli* isolates were tested against a panel of 11 antibiotics that belonged to seven different classes (β-lactams, cephalosporins, amphenicols, tetracyclines, sulfonamides, aminoglycosides and fluoroquinolones) by using a breakpoint assay (Subbiah et al., 2011). Briefly, MAC agar plates (150 mm, diameter) were prepared with each antibiotic at a fixed concentration (given below) that was guided by the Clinical Laboratory Standard Institute (CLSI) recommended

minimum inhibitory concentration for *E. coli* (NCCLS, 2007). The 96-well plates containing *E. coli* cultures were thawed at room temperature and stamped simultaneously on MAC agar plates containing antibiotics using a sterile 96-pin replicator. After stamping, the plates were left open at room temperature for a few minutes until the cultures were dried and then incubated overnight at 37°C. On every culture-stamped MAC agar plate a susceptible (*E. coli* K-12) and two resistant (*E. coli* NM-1 and E. coli NM-2) strains were used as negative and positive controls, respectively. The NM-1 strain was resistant to ampicillin, ciprofloxacin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. The NM-2 strain was resistant to amoxicillin, ceftazidime, cefotaxime and gentamicin. After incubation, the MAC agar plates were examined for the growth of resistant isolates and the antibiotic resistant patterns for each isolate were recorded.

The concentration for antibiotics and vendor information were as follows: ampicillin VWR International LLC, Sanborn, NY (Amp, 32 µg/ml), cefotaxime Chem-Impex International Inc, Wood Dale, IL (Ctx, 8 µg/ml), chloramphenicol Mediatech Inc., Manassas, VA (Chm, 32 µg/ml), tetracycline MP Biomedicals, LLC, Solon, OH (Tet, 16 µg/ml), trimethoprim (Tri, 8 µg/ml), ceftazidime (Cfd,8 µg/ml), sulfamethoxazole (Sul, 512 µg/ml), streptomycin Amresco Inc., Solon, OH (Str, 16 µg/ml), ciprofloxacin Enzo Life Sciences Inc., Farming Dale, NY (Cip, 4 µg/ml), amoxicillin (Amx, 32 µg/ml) and gentamicin above (Gen, 64 µg/ml).

Data analysis

Antibiotic resistance data for each isolate (coded '1' or '0' if resistant or susceptible, respectively) was managed using Microsoft Excel and Microsoft Access (ver. 2007) for descriptive analysis. A Bartlett test was used to compare variances across data and a logit transformation ($y = \ln[x/(1-x)]$) was used to meet the homogeneity of variance assumption when comparing proportions between commercial-layer and free-range chickens. Analysis of variance (ANOVA) and a Tukey-Kramer post-comparison test were used to evaluate differences between the prevalence of resistant *E. coli* isolates and identify which antibiotic resistance phenotypes differed between commercial-layer and free-range chickens. A Wilcoxon rank-sum test was used to compare the number of *E. coli* isolates resistant to at least two or more antibiotics between commercial layer and free-range chickens. Values of $P < 0.05$ were considered statistically significant.

RESULTS

A total of 1,800 *E. coli* isolates (n=600 free-range and n=1,200 commercial chickens) were collected from fecal samples. *E. coli* resistant to >1 antibiotic accounted for 47.5 and 90.7% of the *E. coli* isolates collected from free-range chickens and commercial-layer chickens, respectively. For free-range chickens the most common resistance phenotypes included sulfamethoxazole (31.5%) and trimethoprim (28.17%). The rank order of resistance was Sul, Tri, Str, Tet, Amx, Amp, Cip and Chm (Table 1). A very similar pattern was found for *E. coli* isolates from commercial-layer chickens where resistance was most prevalent for sulfamethoxazole (74.56%) and trimethoprim (68.83%). In fact, the rank order of prevalence was remarkably similar to free-range chickens with the exception that Amx and Amp were reversed (Table 1). Furthermore, the ratio of resistance to sulfamethoxazole, trimethoprim, streptomycin and tetracycline for commercial source vs. free-range isolates was remarkably constant (0.38 to 0.42), which is consistent with the presence of one or more similar populations of multidrug resistant isolates in both poultry populations. Importantly, no resistance was detected for ceftazidime, cefotaxime and gentamycin.

There were differences in the proportion of resistant isolates based on chicken type (greater for commercial-layer vs. free-range; $P<0.001$), and antibiotic type ($P<0.001$). There was also a significant interaction between the proportion of resistant isolates and antibiotic type ($P<0.01$). A plot of the interaction effect demonstrated that this was caused by the rank-order change for Amp and Amx between commercial-layer and free-range chickens. Among the antibiotic resistant free-range chicken isolates, 64.1 and 39% were resistant to ≥2 and ≥3 antibiotics (Table 2). For commercial-layer chicken isolates, 91.5 and 73.4% were resistant to ≥2 and ≥3 antibiotics, respectively. The frequency of multidrug resistance was significantly higher among *E. coli* isolates from commercial-layer chickens as compared to free-range chickens ($P<0.05$). Resistance phenotypes were diverse. For example, if we limit the analysis to seven antibiotics (excluding chloramphenicol) there were $2^7 = 128$ possible combinations of resistance phenotypes of which was observed as 111 (Table 2). The broadest resistance phenotypes were AmpChmStrSulTetTri 1 (0.5%) and AmpAmxStrSulTetTri 3 (1.5%) for free-range chickens and AmpAmxChmCipStrSulTetTri 3 (0.3%) for commercial-layer chickens.

DISCUSSION

In studies by Carraminana et al. (2004) in Spain and Kilonzo-Nthenge et al. (2008) in Cameron, all *E. coli* isolates from poultry were susceptible to cefotaxime and gentamicin. Comparable results including susceptibility to third-generation cephalosporins (ceftazidime and cefotaxime) were reported. Hamisi et al. (2014) also sampled free-range chickens in the Arusha area but found resistant strains of *E. coli* for cefotaxime and ceftazidime (29.9 and 6.5%, respectively). Hamisi et al. (2014) also reported higher resistance (54.5%) among *E. coli* isolates to a fluoroquinolone drug (ofloxacin) whereas relatively limited resistance to ciprofloxacin (3.5%) was found. This difference might be explained, in part, by published observations that ciprofloxacin is more active than ofloxacin for most bacteria (Lautzenhiser et al., 2001). Comparisons across studies, however, may be complicated when different methodologies and definitions of resistance and susceptibility are employed by the investigators.

Although, chloramphenicol is not used in Tanzanian food animals and it is not available in local veterinary

Table 1. Prevalence (%) of antibiotic resistant *E. coli* obtained from fecal samples of free-range chickens and commercial layer chickens from Arusha District, Tanzania.

Location	Amp	Amx	Chm	Cip	Str	Sul	Tet	Tri
Free-range chickens								
Mifugo	8.33	8.33	0.83	0.00	33.33	26.67	28.33	26.67
Nambala	12.50	13.33	0.83	0.00	8.33	17.50	5.00	16.67
Sakina	1.67	14.17	0.00	8.33	23.33	25.00	14.17	15.83
Sansi	11.67	6.67	0.83	0.83	9.17	42.50	23.33	40.83
Njiro	0.83	12.50	5.00	8.33	30.00	45.83	11.67	40.83
Mean (SE)	7.0 (2.45)	11.0 (1.48)	1.5 (0.89)	3.5 (1.98)	20.8 (5.19)	31.5 (5.42)	16.5 (4.17)	28.2 (5.51)
Commercial chickens								
Mifugo	39.17	36.67	1.25	17.92	50.42	83.33	31.25	75.42
Nambala	38.75	22.08	0.42	13.75	52.92	67.92	45.42	59.58
Sakina	37.92	28.33	0.42	13.75	53.75	75.00	25.42	67.50
Sansi	27.08	28.75	0.83	20.42	58.33	70.29	58.33	70.83
Njiro	47.50	46.25	2.92	26.25	50.00	76.25	57.92	70.83
Mean (SE)	38.1(3.3)	32.4(4.2)	1.2(0.5)	18.4(2.3)	53.1(1.5)	74.6(2.7)	43.7(6.7)	68.8(2.6)
P values from Tukey-Kramer post comparison test showing antibiotic effects on the prevalence of resistance between commercial-layer and free-range chickens.	<0.0001	0.15	0.99	0.0003	0.0381	0.0042	0.07	0.0097

Amp = Ampicillin (32 µg/ml), Amx = amoxicillin (32 µg/ml), Chm = chloramphenicol (32 µg/ml), Cip = ciprofloxacin (4 µg/ml), Str = streptomycin (16 µg/ml), Sul = sulfamethoxazole (512 µg/ml), Tet = tetracycline (16 µg/ml), and Tri = trimethoprim (8 µg/ml). No resistance was detected forCfd = ceftazidime (8 µg/ml), Ctx = cefotaxime (8 µg/ml) or Gen = gentamicin (64 µg/ml).

medicine outlets, low-level resistance was observed for *E. coli* isolates from both chicken populations. The presence of this trait might be explained by chance alone. It is also possible that unintentional chloramphenicol exposure occurs infrequently (Levy and Marshall, 2004). For example, Berendsen et al. (2010) reported natural occurrence of chloramphenicol in plants in Mongolia and the Netherlands. No farmer was found to use chloramphenicol for the current study.

In this study, *E. coli* isolates from free-range chickens exhibited antibiotic resistance although lower as compared to those from layer chickens. It is possible that these resistant strains spilled over from commercial-source flocks, or these animals might be exposed to selection pressure or populations of resistant bacteria in the environment (Finley et al., 2013; Wellington et al., 2013). Furthermore, environments often harbor non-pathogenic and opportunistic bacteria that are resistant to antibiotics (Wright, 2010). In this study, both chicken populations were obtained from the same wards and were located <1,000 m apart and therefore some exposure to bacteria is likely to happen between commercial-layer and free-range chickens. Regardless of chicken origin, the most frequent resistance phenotypes were for sulfonamide and trimethoprim followed by resistance to streptomycin and beta-lactams. Sulfonamide resistance genes have been linked with spread of multiple antibiotic resistance genes in *E. coli* (Bean et al., 2005). Streptomycin, trimethoprim and ampicillin resistance are the common resistances associated with sulfonamide resistance (Wu et al., 2010). Other studies assayed isolates using sulfamethoxazole in

Table 2. Prevalence (%) of antibiotic resistant phenotypes of *E. coli* isolates from fecal samples of free-range chickens and commercial-layer chickens from Arusha district, Tanzania.

Antibiotic resistance phenotypes[1]	Commercial-layer chickens (%)	Free-range chickens (%)
Susceptible	9.8	41.8
Amp	0.3	0.03
AmpAmx	0.3	-
AmpAmxChmCipStrSulTetTri	0.3	-
AmpAmxChmStrSulTetTri	0.3	-
AmpAmxChmStrSulTri	0.1	-
AmpAmxChmSul	-	0.2
AmpAmxChmStrTri	-	0.3
AmpAmxCipStrSul	0.1	-
AmpAmxCipStrSulTet	0.3	-
AmpAmxCipStrSulTetTri	5.2	-
AmpAmxCipStrSulTri	0.5	-
AmpAmxCipStrTet	0.1	0.2
AmpAmxCipSulTet	0.1	-
AmpAmxCipSulTetTri	0.8	-
AmpAmxCipSulTri	0.2	-
AmpAmxStrSul	0.6	-
AmpAmxStrSulTetTri	3.6	0.5
AmpAmxStrSulTri	5.1	1.5
AmpAmxStrTetTri	0.4	0.2
AmpAmxStrTri	0.2	0.2
AmpAmxSul	1.1	0.2
AmpAmxSulTet	0.2	0.2
AmpAmxSulTetTri	1.8	0.5
AmpAmxSulTri	3.0	0.2
AmpAmxTet	0.2	-
AmpAmxTetTri	0.2	-
AmpAmxTri	0.3	0.2
AmpChm	-	0.2
AmpChmStr	-	0.2
AmpChmStrSulTetTri	-	0.2
AmpChmStrTri	-	0.2
AmpCipStr	0.1	-
AmpCipStrSul	0.1	-
AmpCipStrSulTet	0.1	-
AmpCipStrSulTetTri	0.3	0.3
AmpCipStrSulTri	0.3	0.5
AmpCipSulTetTri	0.5	0.5
AmpCipSulTri	0.1	-
AmpCipTet	0.2	-
AmpCipTri	-	0.2
AmpStr	0.8	0.2
AmpStrSul	1.0	-
AmpStrSulTet	0.6	-
AmpStrSulTetTri	1.3	0.3
AmpStrSulTri	2.0	1.5
AmpStrTetTri	0.1	-
AmpStrTri	0.1	0.5
AmpSul	0.3	-
AmpSulTet	0.7	-

Table 2. Contd.

AmpSulTetTri	0.3	0.2
AmpSulTri	1.9	0.2
AmpTet	0.1	-
AmpTri	0.3	0.3
Amx	0.3	3.5
AmxChmStrSulTetTri	0.1	-
AmxChmSulTri	-	0.2
AmxCip	0.2	0.3
AmxCipStr	-	0.2
AmxCipStrSulTetTri	0.1	0.2
AmxCipStrSulTri	0.2	-
AmxCipSul	-	0.2
AmxCipSulTetTri	0.1	-
AmxStr	-	0.2
AmxStrSul	0.3	0.2
AmxStrSulTetTri	0.3	1.3
AmxStrSulTri	0.9	0.2
AmxStrTet	-	0.2
AmxStrTri	0.1	-
AmxSul	0.2	0.3
AmxSulTet	0.1	-
AmxSulTetTri	0.2	3.8
AmxSulTri	0.6	3.5
AmxTet	0.1	0.3
AmxTetTri	0.2	-
AmxTri	0.3	-
ChmCipStrSulTetTri	0.1	-
ChmCipSulTetTri	0.1	-
ChmStr	-	0.2
ChmStrSulTetTri	0.1	-
ChmStrSulTri	0.1	-
ChmSulTetTri	0.1	-
ChmSulTri	0.1	-
Cip	0.8	2.0
CipStr	-	0.3
CipStrSulTet	1.1	-
CipStrSulTetTri	2.2	0.2
CipStrSulTri	0.5	-
CipStrTet	0.6	-
CipStrTri	0.1	-
CipSul	0.2	-
CipSulTet	0.1	-
CipSulTetTri	0.8	-
CipSulTri	0.7	0.2
CipTet	0.3	-
CipTetTri	0.1	0.2
CipTri	0.6	-
Str	2.1	5.7
StrSul	1.8	1.3
StrSulTet	1.7	-
StrSulTetTri	7.9	1.3
StrSulTri	7.3	1.5

Table 2. Contd.

StrTet	0.5	0.3
StrTetTri	0.7	0.2
StrTri	1.6	0.8
Sul	1.8	4.0
SulTet	0.8	0.2
SulTetTri	4.0	2.5
SulTri	7.9	3.7
Tet	1.7	5.2
TetTri	1.2	0.5
Tri	1.5	3.3

[1]Amp = Ampicillin (32 µg/ml), Amx = amoxicillin (32 µg/ml), Chm = chloramphenicol (32 µg/ml), Cip = ciprofloxacin (4 µg/ml), Str = streptomycin (16 µg/ml), Sul = sulfamethoxazole (512 µg/ml), Tet = tetracycline (16 µg/ml), and Tri = trimethoprim (8 µg/ml).

combination with trimethoprim (Arslan and Eyi, 2010; Chiu et al., 2010). Adenipekun et al. (2015) reported lower resistance to sulfamethoxazole-trimethoprim (39.8%) in food producing animals in Nigeria. While resistance to these two antibiotics is conveyed by different genetic traits (Blahna et al., 2006; Hu et al., 2011), our data showed a strong correlation between these two resistance phenotypes (r = 0.99) that is consistent with closely-linked resistance traits.

Producers reported that they frequently treated commercial-layer chickens with antibiotics, including enrofloxacin, amoxicillin, oxytetracycline, chlortetracycline, sulfamethazine+trimethoprim and sulfadiazine. Farmers also reported using a coccidiostat called amprolium. Farmers specifically reported using antibiotics to treat Newcastle disease (a viral infection). We observed use of expired drugs in part because these commodities are purchased in large volumes and are simply used until gone. Farmers reported that when sick animals were observed, these were isolated and the entire flock was treated immediately to prevent a large disease outbreak. Farmers also reported using higher than recommended doses with hopes that this would lead to a shorter period of infection. We surmise that these antibiotic use practices drive the difference in prevalence of antibiotic resistant *E. coli* between the commercial-layer and free-range chickens. This also indicates that more investment is needed to help small-scale producers raise healthy animals through the use of better husbandry practices and vaccines. Such efforts are likely to help farmers reduce their reliance on antibiotics while increasing the success of their production efforts (Palmer and Call, 2013).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was funded in part by the Government of Tanzania through NM-AIST, and by the Paul G. Allen School for Global Animal Health and the National Science Foundation (DEB1216040).

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