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Prevalence of Antimicrobial Resistance in Enterococci and *Escherichia coli* in Meat Chicken Flocks During a Production Cycle and Egg Layer Pullets During Rearing

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Abstract: Avian Pathogenic *Escherichia coli* (APEC) and some enterococci are important zoonotic pathogens associated with poultry and some human illnesses. This study was conducted to evaluate the phenotypic antibiotic resistance and resistance genes during the production cycle of healthy free-range meat chickens and egg layer pullets raised in two different controlled farms in South Australia, in particular to determine at which point in the production cycle birds become colonized with antibiotic resistant bacteria. Antibiotic resistance was investigated in faecal *E. coli* (206) and enterococcal (252) isolates by agar dilution and polymerase chain reaction. Southern hybridization was carried on plasmids from selected multi-resistant *E. coli* isolates to determine the location of resistance genes. Our results revealed that birds are colonized with resistant bacteria encoding various resistance genes from a very early age. Resistance to ampicillin and tetracycline (and associated resistance genes) were the most frequently detected resistances in *E. coli* isolates from egg layer pullets and free-range meat chickens. Phenotypically resistant enterococcal isolates from 3-5 days old chickens carried genetic determinants for resistance to tetracycline, bacitracin and tylosin. Whilst statistical analysis revealed there was no significant differences ($p<0.05$) with the phenotypic resistance observed in the *E. coli* and enterococcal isolates from meat chickens and egg layer pullets, a significant difference was observed in resistant *E. coli* isolates containing bla_{TEM} and *tet* genes. This study demonstrates that newly hatched chicks are already colonized with resistant bacteria which persist through the production cycle and can potentially contaminate eggs and chicken carcasses. This study also confirms that poultry are a potential source of pathogenic *E. coli* strains.

Key words: *E. coli*, enterococci, resistance genes, shed raised egg layer pullets, free-range meat chickens, enterococcal isolates

INTRODUCTION

Avian Pathogenic *Escherichia coli* (APEC) and some enterococci can cause infections in poultry (Maturana *et al*., 2011; Xia *et al*., 2011). Regardless of any history of antibiotic usage in poultry, *E. coli* and eterococci are prevalent in intestines of poultry at different stages of production cycle (Silva *et al*., 2012). In addition, birds can become colonized with bacteria from faecal contamination of eggs or from breeder birds (Fertner *et al*., 2011; Ginns *et al*., 1996). The use of antibiotics for either prophylactic or therapeutic purposes in the poultry industry possess potential public health risks with regard to emergence of resistant food borne enteric bacteria of poultry origin that can infect humans (Hammerum and Heuer, 2009). Furthermore, improper use of disinfectants in intensive farming for cleaning can also select resistant bacteria (EUROPA, 2009). Therefore, there is a need for continuous monitoring of antibiotic resistance in enteric bacteria of poultry origin. The last two decades have revealed an increase in the demand for free-range and organically raised poultry products in some countries (Oberholtzer *et al*., 2006). One of the influencing drivers for such increase is the consumers' belief that free-range birds are less exposed to chemicals and antibiotics (Crandall *et al*., 2009; Martinez Michel *et al*., 2011; Sapkota *et al*., 2011). However to date, studies regarding the emergence of antibiotic resistance and associated resistance genes in enterococci and *E. coli* in either free-range or fully housed chickens over an entire production cycle are very limited.

A few studies have reported antibiotic resistance in bacteria from free-range and intensively raised chickens tracked during production cycles. Ozaki *et al*. (2011) reported resistance in *E. coli* isolates from different broiler farms in Japan that were tracked for 48 days. In Australia, a study with breeder birds (tracked for 32 weeks) and their progeny (tracked for 10 weeks) revealed colonization of progeny birds by multi-resistant *E. coli* occurred from the first week of production (Ginns

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et al., 1996). Although various studies have reported varying levels of antibiotic resistance in *E. coli* and enterococci from free-range and intensively raised chickens (Harisberger *et al*., 2011; Sapkota *et al*., 2011; Van Hoorebeke *et al*., 2012), there is currently lack of knowledge on when chickens acquire resistant organisms and their associated antimicrobial resistance genes.

In Australia, the poultry industry is broadly structured into meat chickens and egg layers (ACMF, 2010). The meat chickens can be reared as free-range or fully housed chickens but the pullet layers are normally reared within sheds. They are both raised under similar management regimes initially with controlled environmental conditions. However, free-range meat chickens are released to a confined open space from the 3rd week of production for a limited time period daily (except in inclement weather), whereas the layer pullets are kept confined within the shed on deep litter. In addition, both farms vaccinate their birds against viral diseases and employ good farm hygiene and biosecurity to keep the birds healthy. In Australia, there is a much more restricted range of antibiotics registered for use in layer birds compared to those available for use in meat chickens (Barton, 2004). With the exception of flavophospholipol, bacitracin and ionophores which are available without prescription, no other antibiotic are available without prescription. Only neomycin, lincomycin plus spectinomycin and chlortetracycline can be used in layer birds in lay, plus tylosin in replacement pullets and apramycin in non-laying birds; in addition avilamycin, erythromycin, tylosin, amoxicillin, tiamulin, virginiamycin and oxytetracycline can be used in meat chickens (AVPMA on-line services portal).

This study aims to address these specific objectives; the occurrence of antibiotic resistance encoded by resistance genes in *E. coli* and enterococci in fully housed egg laying pullets during rearing and in free-range meat chickens over an entire production cycle in Australia.

MATERIALS AND METHODS

Collection of faecal sample: Faecal samples were collected from birds of ages 3 to 39 days and 5 to 115 days from free-range meat chickens and intensively raised egg layer pullets from two different farms. As large numbers (varying from 10 000 to 15 000) of birds were raised on each farm, it was not feasible to find the same bird during each sampling time. Hence, freshly dropped faecal matters were collected in each of the poultry houses at two to three week time intervals from the first week to the end of production or rearing cycles. In addition, some environmental samples (feed, water, swabs from drinking water nipple and litter samples) were randomly collected from the farms. Faecal droppings and environmental samples from the various farms were collected into sterile containers and transported in chilled containers to the laboratory within 2 h. The farmers advised that no antibiotics were used on their farms over the time of the study.

Bacterial isolates and phenotypic tests: *E. coli* was isolated by inoculating 1 g of each faecal sample into 10 mL Gram Negative Broth (Oxoid) and incubating the broth aerobically at 37°C for 18-24 h. A loop full of the broth was subcultured onto MacConkey agar (Oxoid) and incubated aerobically for 18-24 h at 37°C. From each plate, a single colony of typical morphology was picked and subcultured onto 5% horse blood agar (HBA) for purity and biochemical testing. Isolates from pure cultures that were urease negative and indole positive by Urea Motility Indole (UMI) medium, oxidase negative and positive reaction by the production of an acid reaction (yellow colour change) in the slope and butt with/without gas formation in a test tube containing Triple Sugar Iron (TSI) agar were identified as *E. coli*. The strains were then stored snap frozen in glycerol broth at -70°C for subsequent phylogenetic grouping, virulence factors (VFs) determination, antimicrobial susceptibility testing and resistance genes identification.

For enterococcal isolates, faecal samples were inoculated into 10 mL of brain heart infusion broth for 24 h before subculture onto BD^{TM} Enterococcosel^{TM} agar (Becton Dickinson Company, Franklin Lakes, NJ, USA). A single colony with a black halo due to hydrolysis of aesculin on Enterococcosel^{TM} agar plates was selected. The selected colony was subcultured onto a 5% HBA plate and identified using catalase, oxidase, growth on 6.5% NaCl agar (salt agar), growth at 42°C and production of pyrrolidonyl arylamidase. Isolates were stored in glycerol broth at -70°C for further identification, antibiotic susceptibility testing and resistance genes determination.

Speciation of *Enterococcus* spp: In this study, enterococcal isolates were speciated by polymerase chain reactions (PCR) using primers listed in Table 1. DNA was extracted by picking a single colony on a 5% HBA plate and resuspending the colony in 200 µL of sterile MilliQ water before heating at 98°C for 10 min. The tubes were subsequently centrifuged at 17 000 x g for 5 min and 2 µL of the recovered supernatant was used as a template in the various PCR examinations. A multiplex PCR for the speciation of the enterococcal isolates was carried out with ATCC enterococcal control cultures (*E. faecalis* ATCC 29212, *E. faecium* ATCC 19434, *E. gallinarum* ATCC 49573 and *E. casseliflavus* ATCC 8293) using this amplification protocol: 4 min initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min. The multiplex PCR carried out consisted of 4 mM $MgCl_2$, 1 µL of 25

Table 1: Primers used for polymerase chain reactions

	---------- 5'-3' ----------				
Target (s) genes	Forward	Reverse	Amplicon size (bp)	Annealing Temperatures	References
***E. coli* resistance genes**					
tet (A)	gtgaaacccaacatacccc	gaaggcaagcaggatgtag	888	60°C	Maynard *et al.* (2004)
tet (B)	ccttatcatgccagtcttgc	actgccgtttttcgcc	774	60°C	Maynard *et al.* (2004)
tet (C)	acttggagccactatcgac	ctacaatccatgccaaccc	881	60°C	Maynard *et al.* (2004)
dhfrV	ctgcaaaagcgaaaaacgg	agcaatagttaatgtttgagctaaag	432	60°C	Maynard *et al.* (2004)
dhfrXIII	caggtgagcagaagattttt	cctcaaaggtttgatgtacc	294	60°C	Maynard *et al.* (2004)
sulI	ttcggcattctgaatctcac	atgatctaaccctcggtctc	822	60°C	Maynard *et al.* (2004)
sulII	cggcatcgtcaacataacc	gtgtgcggatgaagtcag	722	60°C	Maynard *et al.* (2004)
aph (3')-Ia (*aphA1*)	atgggctcgcgataatgtc	ctcaccgaggcagttccat	600	60°C	Maynard *et al.* (2004)
Bla_{SHV}	cactcaaggatgtattgtg	ttagcgttgccagtgctcg	885	60°C	Pitout *et al.*, (1998)
Bla_{OXA}	gtctttcgagtacggcatta	attttcttagcggcaacttac	755	60°C	Vahaboglu *et al.* (1998)
Bla_{TEM}	ttcttgaagacgaaagggc	acgctcagtggaacgaaaac	1207	60°C	Ho *et al.* (2005)
Enterococci speciation					
Enterococcus genus	tcaaccggggagggt	attactagcgattccgg	723	56°C	Deasy *et al.* (2000)
E. faecalis	acttatgtgactaacttaacc	taatggtgaatcttggtttgg	360	56°C	Jackson *et al.* (2004)
E. faecium	gaaaaaacaatagaagaattat	tgctttttgaattcttcttta	215	56°C	Jackson *et al.* (2004)
E. casseliflavus	tcctgaattaggtgaaaaaac	gctagtttaccgtctttaacg	288	56°C	Jackson *et al.* (2004)
E. gallinarium	ttacttgctgattttgattcg	tgaattcttctttgaaatcag	173	56°C	Jackson *et al.* (2004)
Enterococci resistance					
tet (O)	acggaragtttattgtatacc	tggcgtatctataatgttgac	171	55°C	Aminov *et al.* (2001)
tet (M)	gttaaatagtgttcttggag	ctaagatatggctctaacaa	657	54°C	Aarestrup *et al.* (2000)
tet (S)	gacgacgtggttggaaag	atcttagcacatgttgatg	1800	54°C	This study (Source X92946)
tet (L)	catttggtcttattggatcg	attacacttccgatttcgg	488	54°C	Aarestrup *et al.* (2000)
erm (B)	catttaacgacgaaactggc	ggaacatctgtggtatggcg	400	59°C	Thibodeau *et al.* (2008)
bcr (R)	acctaccgtcacaatgctga	gcatatcggaaagacgga	880	54°C	This study (Source AY 496968.10)

Table 2: Guidelines for interpreting antimicrobial susceptibility results for enterococci and *Escherichia coli* isolates in µg/mL

	--- Enterococci ---		-- *Escherichia coli* --	
Antibiotic	S	R	S	R
Ampicillin*	≤8	≥16	≤8	≥32
Bacitracin*##	<0.25	>32	-	-
Ciprofloxacin	-	-	<1	>4
Erythromycin*	<0.5	≥8	-	-
Florfenicol***	-	-	<1	>16
Gentamicin*	≤4	≥16	≤4	≥16
Neomycin***	-	-	<1	> 8
Lincomycin #	≤8	≥32	-	-
Spectinomycin***	-	-	<8	>64
Streptomycin***	-	-	<4	>16
Tetracycline*	≤4	≥16	≤4	≥16
Tylosin**	≤4	≥16	-	-
TMP/SU	-	-	≤2/38	≥4/76
Vancomycin*	≤4	>32	-	-
Virginiamycin***	<0.25	>32	-	-

S: Susceptible, R: Resistance

Keys, *(CLSI, 2008), **(ROSCO, 2007), ***(DANMAP, 2008), #(CIPARS, 2005) and ##(SVARM, 2008)

[a]MIC of bacitracin was expressed in international units per millilitre

pmol of each primer (Table 1), 2 µL of 2 mM dNTPs and 4 µL of 5 X PCR reaction buffer in a total reaction volume of 25 µL, including 2 µL DNA template and 1U Taq (Mango TAQ polymerase). PCR products were then electrophoresed on a 1% agarose gel containing ethidium bromide in 1 x TAE buffer. The sizes of the various amplicons were determined by comparing them with a 100 bp ladder (New England Biolabs, Bruningstrasse, Germany). The specific primers for the different enterococcal isolates (*E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarium*) were selected because a prior study (Obeng *et al.*, 2012b) identified these isolates to be prevalent in South Australian poultry.

Antibiotic susceptibility testing: Minimum inhibitory concentrations (MIC) were determined for all the isolates using an agar dilution method on Mueller-Hinton agar (Oxoid), based on the Clinical and Laboratory Standard Institute standards (CLSI, 2008). The bacteria were subcultured onto 5% HBA plates (37°C, 18 h) and then suspended in saline to a concentration equivalent to 0.5 McFarland units. The suspensions were diluted to 1/10 in 0.85% saline to give 1 X 10^7 colony forming units and this was inoculated with a multipoint inoculator onto Mueller-Hinton plates containing different concentrations of the antibiotics. Plates were incubated aerobically at 37°C for 18 h. The MIC values were defined as the lowest concentrations producing no visible growth. Antimicrobial-free agar plates were included as a control for normal growth. Due to low numbers in the different enterococcal species isolated, all the isolates were grouped together to aid analysis and discussion of the results produced.

Susceptibility break points used were as established in CLSI standards (CLSI, 2008), while additional break points not covered were sourced from the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (Danmap, 2008), ROSCO MIC for veterinary isolates (ROSCO, 2007), Canadian Integrated Programme for Antimicrobial Resistance Surveillance (CIPARS, 2005) and Swedish Veterinary Antimicrobial monitoring (Swedres, 2008) (Table 2). The control strains used for the determination of MIC values were *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.

The antimicrobial agents tested against *E. coli* were ampicillin, ciprofloxacin, florfenicol, gentamicin, neomycin, trimethoprim sulfamethoxazole, spectinomycin, streptomycin and tetracycline. Enterococcal isolates were tested for resistance to ampicillin, bacitracin, erythromycin, lincomycin, tylosin, tetracycline, vancomycin and virginiamycin.

Virulence factors, phylogenetic grouping, antibiotic resistance genes and plasmid profiling of *E. coli* isolates: DNA extraction, Virulence factors (VFs) and phylogenetic grouping of the *E. coli* isolates was carried out as previously described (Obeng *et al*., 2012a). Briefly a single colony of a fresh bacterial culture from 5% HBA was picked and resuspended in 200 ml of sterile milliQ water. Tubes were heated at 98°C for 10 min and subsequently centrifuged at 17 900 x g for 5 min. Eight VFs for all the *E. coli* isolates were analyzed using the primers by Johnson and Stell (2000). The virulence score was calculated using the total number of VF genes. Each reaction consisted of 4 mM $MgCl_2$, 1 µL of 25 pmol of each primer (*pap*AH, *pap*C, *afa*/*dra*BC, *sfa*/*foc*DE, *sfa*S, *aer*J, *kps*MT II, *foc*G), 2 µL of 2 mM dNTPs and 4 µL of 5 x PCR buffer, 1 U of Taq DNA polymerase (Bioline, Australia) in a total reaction volume of 25 µL, including 2 µL DNA template. The cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of denaturation (94°C, 30 s), annealing (64°C, 30 s), extension (68°C, 3 min) and final extension (72°C, 10 min). *E. coli* isolates were then phylogenetically grouped into A, B1, B2 or D using the triplex PCR reaction by Clermont *et al*. (2000). The reaction mixture consisted of 4 mM $MgCl_2$, 1 µL of 25 pmol of each primer (TSPE4.C2, chuA and yjaA), 2 µL of 2 mM dNTPs and 5 µL of 5 x PCR buffer, 1U of Taq DNA polymerase (Bioline, Australia), in a total reaction volume of 25 µL, including 2 µL DNA template. The cycling conditions for the reaction was 95°C for 10 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (59°C, 30 s), extension (72°C, 30 s) and final extension (72°C, 10 min). All the PCR products were then electrophoresed on 1.5% agarose gel containing ethidium bromide. The sizes of the amplicon were determined by comparing them with a 100 bp DNA ladder. Any *E. coli* strain failing to yield any of the three amplicons sizes (for TSPE4.C2, *chu*A and *yja*A) were subjected to PCR amplification for the 365 bp amplicon for the *E. coli* beta-galactosidase gene (*lac*Z), to confirm that the isolate was *E. coli* (Higgins *et al*., 2007). Any strain showing a positive result for the *lac*Z gene and a negative result for the three amplicon fragments, via comparison with the positive control (ATCC 25922), was then assigned to the phylogenetic group A (Clermont *et al*., 2000).

Furthermore, PCR was carried out on all the phenotypic resistant *E. coli* isolates for the following antimicrobial resistance genes; ampicillin (bla_{TEM}), tetracycline [*tet* (A), *tet* (B), *tet* (C) and *tet* (D)], trimethoprim (*dhfr*V and *dhfr*XIII), sulphonamide (*sul*I and *sul*II), spectinomycin (*aad*A2), integrase gene I (*int*I) and integrase gene II (*int*II) using primers described in Table 1. Each PCR mixture consisted of 4 mM $MgCl_2$, 1 µL of 25 pmol of each primer, 2 µL of 2 mM dNTPs and 4 µL of 5 X PCR reaction buffer, 1U of Taq DNA polymerase (Bioline, Australia) in a total reaction volume of 25 µL, including 2 µL DNA template. DNA amplification was carried out by using 7 min initial denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at various temperatures for 30 s (Table 1) and extension at 72°C for 45 s. PCR products were then electrophoresised on a 1.5% agarose gel containing ethidium bromide and compared with 100 bp and 1 kb. A selection of resistant *E. coli* isolates (n = 125) from indoor chickens and egg layer pullets were tested for plasmids; these isolates were resistant to two or more antibiotics. Plasmid DNA was extracted from 125 multi-resistant (resistance to two or more antibiotics) isolates by the following procedure; isolates were grown overnight on HBA and a single colony was inoculated into Heart Infusion broth (HI) (Oxoid) and incubated for 24 h at 37°C. Five ml of the overnight broth cultures were pelleted by centrifugation at 5,444 x g for 15 min. The liquid media supernatant was decanted and the plasmid DNA was extracted from the cell pellets using Ultra Clean Standard Mini Plasmid Prep Kit (MO BIO Laboratories, Inc) following the manufactures instructions. The plasmid extracts were then electrophoresed in a 1% agarose gel for 2 h at 150 volts for plasmid profiling.

Southern hybridization analysis: *E. coli* plasmid DNA were hybridized with prepared *tet*, *sul*1, *sul*2, *dhfr*V, *dhfr*XIII, bla_{TEM}, bla_{SHV} and bla_{OXA} resistant gene probes. An isolate was considered to harbour a gene in the plasmid DNA if the plasmid hybridized with the corresponding gene probes.

Briefly, southern hybridization analysis was carried out by aliquoting 10 µL volume of *Eco*R1 digested plasmid DNA samples and Hyper ladder III (BioRad, Australia) ladder separating them on a 1% (w/v) agarose gel. The agarose gel was then stained in ethidium bromide and photographed with Image Quant LAS 4000 to confirm the digestion of the plasmid. The gel was soaked in 250 mM HCl for 15 min, rinsed twice with sterile distilled water and washed twice for 30 min in 100 mL of denaturing solution (2.5 mM NaCl, 0.5 mM NaOH) with constant agitation. The gel was rinsed again in sterile distilled water and the plasmid DNA was then neutralized in neutralizing solution (1 M TrisCl, 1.5 M NaCl, pH 8.0) for 30 min with constant agitation. The DNA bands from the gel were allowed to transfer by capillary action onto a Hybond N^+ filter membrane (Amersham GE Healthcare, Australia) for 18 h at room temperature in 10 x SSC buffer (1 x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0).

PCR products of *tet*, *sul*I, *sul*II, *dhfr*V, *dhfr*XIII, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ were purified using an ultra clean PCR purification Kit (MO BIO Laboratories, Inc) according to the manufacturer's instructions and eluted in 50 µL of elution buffer prior to labeling. Labeling of the DNA fragments with digoxigenin-dUTP was carried out according to the manufacturer's instructions. After 18 h, the blot apparatus was dismantled and the nylon filter membrane was washed in 6 x SSC buffer. Excess fluid was drained and the membrane was allowed to dry on a filter paper in a laminar flow unit after which it was subjected to UV cross-linking for 5 min. The bound membrane was pre-hybridized in 10 ml of hybridization buffer (50% v/v formamide, 7% SDS, 1% skim milk powder, 5 x SSPE [1 x SSPE is 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA] and 2.5 mg/mL salmon sperm DNA) and incubated at 42°C for 30 min in an incubator.

Seven micro-litres of the labeled DNA were denatured just prior to use by adding 850 µL of hybridization buffer liquid (50% v/v formamide, 7% SDS, 1% skim milk powder, 5 x SSPE [1 x SSPE is 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA] and 2.5 mg/mL salmon sperm DNA) by heating at 70°C for 10 min. This was then poured into the plastic pouch containing the bound membrane. The plastic bag was massaged gently and hybridization was allowed to continue for 24 h at 42°C in an incubator. After hybridization, the membrane was washed for 10 min at room temperature with a low-stringency wash solution (2 x SSC containing 0.1% [w/v] sodium dodecyl sulphate). This was followed by one wash at 68°C for 20 min in a water bath with a high-stringency wash solution (1 x SSC containing 0.1% [w/v] sodium dodecyl sulphate). The membrane was briefly washed in washing buffer (0.1% maleic acid, 0.15% NaCl; pH 7.5, 0.3% [v/v] Tween 20) for 2 min at room temperature and blocked with blocking reagent supplied in the digoxigenin-labeling nucleic detection kit as described by the manufacturer.

The membrane was incubated in anti-digoxigenin alkaline phosphatise antibody conjugate for 30 min at room temperature, followed by 2 x 15 min washes in washing buffer to remove unbound conjugate. The colour substrate (4-nitro blue tetrazolium chloride plus 5-bromo-4-chloro-3-indolylphosphate [4-toluidene salt] in detection buffer (consisting of 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) was then added and the membrane developed in the dark for 24 h. The reaction was stopped by adding TE buffer.

Antibiotic resistance genes determination in enterococci: The enterococcal isolates were tested for tetracycline (*tet* (M), *tet* (L), *tet* (O), *tet* (S)), bacitracin (*bcr*R) and macrolide (*erm*B) resistance genes (Obeng *et al*., 2012b). The following primer amplification protocols used were as follows: 10 min initial denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at various temperatures for 45 s (Table 1) and extension at 72°C for 1 min. Each reaction consisted of 4 mM $MgCl_2$, 1 µL of 25 pmol of each primer, 2 µL of 2 mM dNTPs and 4 µL of 4 x PCR reaction buffer, 1 U of *Taq* DNA polymerase (Bioline, Australia), in a total reaction volume of 25 µL, including 2 µL DNA template. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide in 1 x TAE buffer. The sizes of the various amplicons were determined by comparing them with 100 bp and 1 Kb ladders (New England Biolabs, Bruningstrasse, Germany).

DNA sequencing of resistance genes and nucleotides accession numbers: PCR products from the representative isolates were purified and sequenced to confirm the identification of resistance genes prior to being used as positive controls in the various PCR analyses. A representative positive amplicon generated with each primer was sequenced at the SouthPath Sequencing Facility (Flinders Medical Centre, South Australia), after PCR products were purified using Ultra clean-up DNA purification kit (MO BIO, Carlsband) following the manufacturer's instructions.

The accession numbers of the enterococci sequences submitted to NCBI GenBank are *erm*B (JN003405), *bcr*R (JN796408), *tet* (L) (JN003407), *tet* (O) (JN003408), *tet* (M) (JN003411) and *tet* (S) (JN796409). With the exception of the following *E. coli* sequences; *bla*$_{OXA}$ (JQ342831), *bla*$_{TEM}$ (JN037848) and *bla*$_{SHV}$ (JQ342835), all the other sequences for the remaining resistance genes have been previously reported (Obeng *et al*., 2012a).

Statistical analysis: Comparisons of association between the different isolates from the two groups of poultry (free-range meat chickens and indoor raised egg layer pullets) from two different farms were performed separately using GraphPad Prism software, version 5.01. Group means were compared pairwise using t-tests and the different groups of chickens were compared using the Bonferroni test. Statistical significance was set at a $p<0.05$.

RESULTS

Phenotypic results on sensitivity testing

eterococci: From a total of 120 samples (84 faecal samples and 36 environmental samples) and 182 samples (137 faecal sample and 45 environmental samples) from free-range meat chickens and indoor raised egg layer pullets 79 enterococcal isolates (*E. faecalis* (64) and *E. faecium* (27)) and 145 enterococcal isolates (*E. faecalis* (70) and *E. faecium* (91)) were isolated from the two different farms. The dominant species identified from both farms were *E. faecalis* and

Table 3: Prevalence of antibiotic resistance in enterococcal isolates from free-range meat chickens and indoor raised egg layer pullets tracked during a production cycle

Age in days	No. of positive isolates /no of samples tested				No. of resistant isolates /no. of isolates tested		
(Wks)	Faeces	Env.	Total (%)	Antibiotic	Faeces	Env.	Total (%)
Prevalence of antibiotic resistance in enterococcal isolates from free-range meat chickens tracked for 7 weeks (n = 91)							
3 days (1st)	17/21	7/9	24/30 (80)	Ampicillin	0/17	0/7	0
				Bacitracin	14/17	7/7	21/24 (88)
				Erythromycin	15/17	6/7	21/24 (88)
				Lincomycin	17/17	7/7	24/24 (100)
				Tylosin	15/17	6/7	21/24 (88)
				Tetracycline	14/17	6/7	21/24 (88)
15 days (3rd)	19/21	6/9	25/30 (83)	Ampicillin	1/19	2/6	3/25 (12)
				Bacitracin	14/19	5/6	19/25 (76)
				Erythromycin	15/19	6/6	21/25 (84)
				Lincomycin	19/19	6/6	25/25 (100)
				Tylosin	17/19	6/6	23/25 (92)
				Tetracycline	12/19	6/6	18/25 (72)
30 days (5th)	19/22	3/8	22/30 (73)	Ampicillin	0/19	0/3	0
				Bacitracin	13/19	3/3	16/22 (73)
				Erythromycin	14/19	3/3	17/22 (77)
				Lincomycin	19/19	2/3	21/22 (95)
				Tylosin	14/19	2/3	16/22 (73)
				Tetracycline	15/19	2/3	17/22 (77)
39 days (7th)	14/20	6/10	20/30 (67)	Ampicillin	0/14	0/6	0
				Bacitracin	10/14	2/6	12/20 (60)
				Erythromycin	12/14	6/6	18/20 (90)
				Lincomycin	14/14	2/6	16/20 (80)
				Tylosin	11/14	2/6	13/20 (65)
				Tetracycline	12/14	2/6	14/20 (70)
Prevalence of antibiotic resistance in enterococcal isolates from indoor raised egg layer pullets tracked for 16 weeks (n = 161)							
5 days (1st)	14/23	6/7	20/30 (67)	Ampicillin	0/14	0/6	0
				Bacitracin	13/14	4/6	17/20(85)
				Erythromycin	14/14	4/6	18/20 (90)
				Lincomycin	14/14	6/6	20/20 (100)
				Tylosin	14/14	4/6	18/20 (90)
				Tetracycline	14/14	6/6	20/20 (100)
20 days (3rd)	23/24	4/6	27/30 (100)	Ampicillin	0/23	0/4	0
				Bacitracin	11/23	1/4	12/27 (44)
				Erythromycin	23/23	3/4	26/27 (96)
				Lincomycin	23/23	4/4	27/27 (100)
				Tylosin	22/23	3/4	25/27 (93)
				Tetracycline	23/23	3/4	26/27 (96)
40 days (6th)	24/24	6/6	30/30 (100)	Ampicillin	8/24	0/6	8/30 (27)
				Bacitracin	20/24	5/6	25/30 (83)
				Erythromycin	23/24	6/6	29/30 (97)
				Lincomycin	24/24	6/6	30/30 (100)
				Tylosin	22/24	6/6	28/30 (93)
				Tetracycline	24/24	6/6	30/30 (100)
70 days (10th)	22/22	8/8	30/30 (100)	Ampicillin	4/22	2/8	6/30 (20)
				Bacitracin	20/22	3/8	23/30 (77)
				Erythromycin	22/22	4/8	26/30 (87)
				Lincomycin	22/22	4/8	26/30 (87)
				Tylosin	22/22	4/8	26/30 (87)
				Tetracycline	22/22	4/8	26/30 (87)
100 days (13th)	24/24	6/6	30/30 (100)	Ampicillin	12/34	4/6	16/30 (53)
				Bacitracin	24/24	6/6	30/30 (100)
				Erythromycin	24/24	5/6	29/30 (97)
				Lincomycin	24/24	6/6	30/30 (100)
				Tylosin	24/24	6/6	30/30 (100)
				Tetracycline	24/24	6/6	30/30 (100)
115 days (16th)	12/20	12/12	24/32 (75)	Ampicillin	2/12	0/12	2/24 (8)
				Bacitracin	8/12	2/12	10/24 (42)
				Erythromycin	12/12	6/12	18/24 (75)
				Lincomycin	12/12	2/12	14/24 (58)
				Tylosin	12/12	2/12	14/24 (58)
				Tetracycline	12/12	2/12	14/24 (58)

E. faecium, no *E. gallinarium* or *E. casseliflavus* was identified in this study. However, due to low numbers in the different species isolated, all the enterococcal isolates were grouped together to aid analysis and discussion of the results produced. Enterococcal isolates from the free-range meat chickens and indoor-raised egg layer pullet were found to show varying levels of phenotypic resistance (Table 3). Resistance to bacitracin, erythromycin, lincomycin, tylosin and tetracycline was found to be prevalent from the 1st week of production in each class of chickens. In the free-range meat chickens, the percentage of resistance to bacitracin was found to be 88% in the 1st week of production (Table 3). This prevalence was found to decrease to 76-73% in the 3rd and 5th weeks and then reduced to 60% in the final week of production. Patterns of resistance to tylosin and erythromycin were found to be quite similar with an increase to 92 and 84% in the 3rd week of production and a reduction of 73 and 77% in the 5th week, respectively. All the isolates from 1st and 3rd weeks of production were resistant to lincomycin (100%), however the prevalence was found to decrease slightly in the 5th week (95%) and 7th week (80%). High resistance to tetracycline was detected in the 1st week (88%); this was found to decrease to 72 and 77% in the 3rd and 5th weeks.

In contrast, resistance to tetracycline and lincomycin in enterococcal isolates from indoor raised egg layer pullets was found to be high in the 1st, 3rd and 6th weeks (100%) (Table 3). Whilst resistance to tylosin was found to be high (93-87%) in the 3rd to the 10th weeks of production, a lower prevalence of 58% was observed in the final week of production. Erythromycin resistance was found to range from 87 to 97% during the production cycle. However, this prevalence was found to decrease to 75% in the final week of the rearing cycle. Although no resistance to ampicillin was observed in the 1st and 3rd weeks, a 27% resistance was detected in the 6th week.

Comparing enterococcal isolates from free-range meat chickens and indoor raised egg layer pullets a similar prevalence of resistance was found to lincomycin (100 - 85%) and erythromycin (100 - 77%) in isolates. However, a slight difference in resistance to tetracycline was detected between isolates from meat chickens (88 - 70%) and egg layer pullets (100 - 58%). Resistance to ampicillin was often detected in enterococcal isolates from egg layers (Table 3). Furthermore, no resistance was observed to vancomycin and virginiamycin in any of the enterococcal isolates. No significant difference was found when the total numbers of resistant isolates from the different groups of birds were compared ($p<0.05$).

***E. coli*:** In the different classes of birds (egg layer pullets and meat chickens), *E. coli* isolates were found to be resistant to tetracycline, ampicillin and trimethoprim-sulfamethoxazole (TMP/SU) (Table 4). In *E. coli* isolates from the free-range meat chickens, resistance to tetracycline was found to be lower (30%) in the 1st week of production; however it declined to 17% and 15% in the 5th and 7th weeks of production (Table 4). In contrast, resistance to ampicillin was found to increase from 40% in the 1st week of production to 100% in the final week of production. Whilst no resistance to trimethoprim-sulfamethoxazole was observed in the 1st week of production, a prevalence of 11.5-8.7% was observed in the 3rd and 5th week of production.

In contrast to the findings from free-range meat chickens, resistance to tetracycline was found to be moderately high (68.8%) in the 1st week in *E. coli* isolates from indoor raised egg layer pullets (Table 4). This prevalence was found to increase in the 3rd week to 72.2% and decreased to 55-50% in 6 and 10th week of production. All the isolates from the 1st and 3rd weeks of production were found to be resistant to ampicillin (100%). This prevalence declined to 93% in the 6th week of rearing cycle but later increased to 100% in the 10th, 13 and 16th weeks low in isolates from egg layers. Low resistance of 33 and 22% to streptomycin and florfenicol was detected in the 3rd week, a low prevalence of 7.4-3.8% was detected for spectinomycin. Furthermore, trimethoprim-sulfamethoxazole resistance was found in the 3rd (5.6%), 6th (3.4%), 10th (3.6%) and 13th (7.4%) weeks, although all the isolates from 1st and 16th weeks of production were susceptible.

Comparing the different groups of poultry, the total number of isolates resistant to ampicillin and tetracycline was higher in egg layer pullets (99 and 60%) than meat chickens (73 and 24%), respectively (Table 4). Lower levels of resistance to trimethoprim-sulfamethoxazole (7-3%) was observed in both groups of chickens but resistance to spectinomycin was observed in only four (2%) isolates from intensively raised egg layers. In addition, no resistance to neomycin, gentamicin and ciprofloxacin was detected in any of the *E. coli* isolates from intensively raised egg layers. Statistical analysis revealed no significant differences between different groups of chickens with regards to different tested antibiotics ($p<0.05$).

Resistance genes

enterococci: In this study emphasis were placed on tetracycline, bacitracin and macrolide resistance genes because most of the enterococcal isolates were phenotypically resistant to these antibiotics. Resistance to tetracycline, bacitracin, tylosin and erythromycin were found to be associated with *tet*, *bcr*R and *erm*B resistance genes, respectively, in the different groups of poultry isolates tested (Fig. 1).

Of the 21 phenotypically tetracycline resistant enterococcal isolates from the 1st week of production

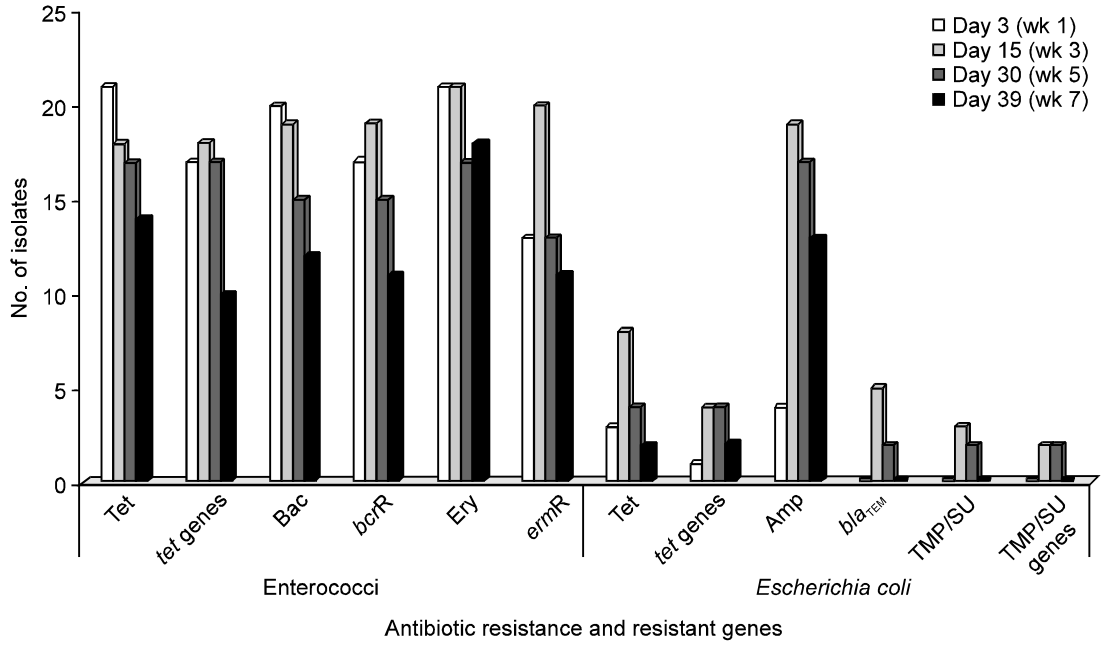

Fig. 1: Prevalence of antibiotic resistance and resistance genes in enterococci and *E. coli* isolates from free range meat chickens tracked over 39 days (7 weeks) of a production cycle. Tet: Tetracycline, Bac: Bacitracin, Ery: Erythromycin, TMP/SU: Trimethoprim-sulfamethoxazole and Amp: Ampicillin

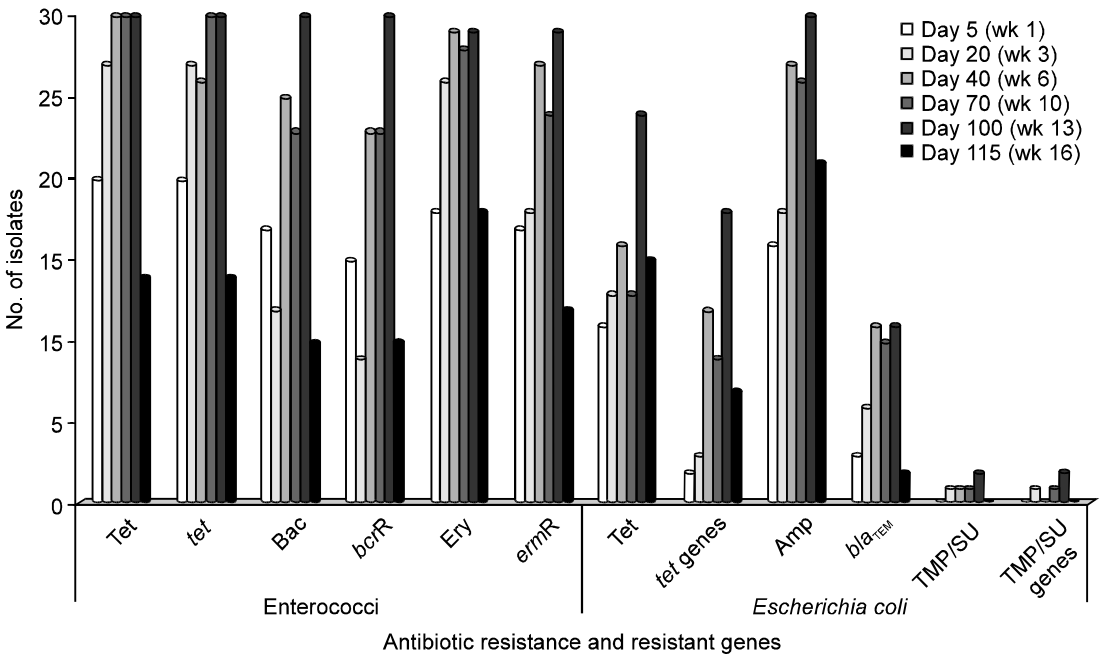

Fig. 2: Prevalence of common antibiotic resistance and resistance genes in enterococci and *E. coli* isolates from indoor raised egg layers pullets tracked over 115 days (16 weeks). Tet: Tetracycline, Bac: Bacitracin, Ery: Erythromycin, TMP/SU: Trimethoprim-sulfamethoxazole and Amp: Ampicillin

cycle from free-range meat chickens, 17 were found to carry the *tet* genes (Fig. 1). However, in the 3rd and 5th week of production, all the tetracycline resistant isolates were found to carry identified *tet* genes. A similar pattern was found for bacitracin resistant isolates carrying the bacitracin resistant gene (*bcr*R). Out of the 21 erythromycin and tylosin resistant isolates from the 1st week of production, 13 were found to carry the *erm*B gene, however, by the 3rd week, 20 isolates were found to carry the *erm*B gene.

Table 4: Prevalence of antibiotic resistance in *E. coli* isolates from free-range meat chickens and indoor raised egg layer pullets tracked during a production cycle

Age in days	No. of samples positive for *E. coli* /no. of isolates tested			No. of antibiotic resistant isolates /no. of samples tested			
(Wks)	Faeces	Env.	Total (%)	Antibiotic	Faeces	Env.	Total (%)
Prevalence of antibiotic resistance in *E. coli* isolates from free-range meat chickens (n = 72) tracked for 7 weeks (39 days)							
3 days (1st)	9/21	1/9	10/30 (33)	Ampicillin	4/9	0/1	4/10 (40)
				Tetracycline	3/9	0/1	3/10 (30)
				TMP/SU	0	0	0
15 days (3rd)	19/21	7/9	26/30 (86.7)	Ampicillin	15/19	4/7	19/26 (73.1)
				Tetracycline	4/19	4/7	8/26 (30.8)
				TMP/SU	1/19	2/7	3/26 (11.5)
30 days (5th)	18/22	5/8	23/30 (77)	Ampicillin	12/18	5/5	17/23 (73.9)
				Tetracycline	3/18	1/5	4/23 (17.4)
				TMP/SU	2/18	0/5	2/23 (8.7)
39 days (7th)	13/20	-	13/20 (65)	Ampicillin	13/13		13/13 (100)
				Tetracycline	2/13		2/13 (15.4)
				TMP/SU	0		0
Prevalence of antibiotic resistance in *E. coli* isolates from indoor raised egg layer pullets (n = 134) tracked for 16 weeks (115 days)							
5 days (1st)	12/23	4/7	16/30 (53)	Ampicillin	12/12	4/4	16/16 (100)
				Tetracycline	7/12	4/4	11/16 (68.8)
				Florfenicol	1/12	0/4	1/12 (8.3)
				TMP/SU	0/12	0/4	0
20 days (3rd)	14/24	4/6	18/30 (60)	Ampicillin	14/14	4/4	18/18 (100)
				Tetracycline	10/14	34	13/18 (72.2)
				Streptomycin	5/14	1/4	6/18 (33.3)
				Spectinomycin	1/14	0/4	1/18 (5.6)
				Florfenicol	3/14	1/4	4/18 (22.2)
				TMP/SU	1/14	0/4	1/18 (5.6)
40 days (6th)	23/24	6/6	29/30(97)	Ampicillin	21/23	6/6	27/29 (93.1)
				Tetracycline	12/23	4/6	16/29 (55.2)
				Florfenicol	1/23	1/6	2/29 (6.9)
				TMP/SU	0/23	1/6	1/29 (3.4)
70 days (10th)	22/22	4/8	26/30 (87)	Ampicillin	22/24	4/4	26/26 (100)
				Tetracycline	11/24	2/4	13/26 (50)
				Streptomycin	1/24	0/4	1/26 (3.8)
				Spectinomycin	1/24	0/4	1/26 (3.8)
				Florfenicol	4/24	0/4	4/26 (15.4)
				TMP/SU	1/24	0/4	1/28 (3.6)
100 days (13th)	24/24	3/6	27/30 (90)	Ampicillin	24/24	3/3	27/27 (100)
				Tetracycline	21/24	3/3	24/27 (89)
				Spectinomycin	2/34	0/3	2/27 (7.4)
				TMP/SU	2/34	0/3	2/27 (7.4)
115 days (16th)	18/20	-	18/20 (90)	Ampicillin	18/18		18/18 (100)
				Tetracycline	15/18		15/18 (83.3)
				Florfenicol	0/18		0
				TMP/SU	0/18		0

In contrast, enterococcal isolates from egg layer pullets frequently carried *tet*, *bcr*R and *erm*B resistance genes (Fig. 2). *tet* genes were found to be present in enterococci isolates from the 1st, 3rd and last week of rearing cycle. Whilst bacitracin resistant isolates carrying *bcr*R gene were found in 15 out of the 17 resistant isolates in the 1st week of production; by the 10th week of rearing cycle all the resistant isolates were positive for *bcr*R. *erm*B was present in most of the erythromycin and tylosin resistant isolates, however the highest prevalence was observed in the 1st week (94%) of production. No significant difference was found between the resistant strains encoding different resistance genes from different weeks of production cycle ($p<0.05$).

***E. coli*:** *E. coli* isolates from free-range meat chickens and indoor raised egg layer pullets were found to possess tetracycline, ampicillin and trimethoprim resistance genes to variable degrees (Fig. 1 and 2). Ampicillin resistant *E. coli* isolates from free-range meat chickens was found to carry the bla_{TEM} gene in the 3rd and 5th weeks of production (Fig. 1). Similarly the trimethoprim-sulfamethoxazole resistance genes were only detected in the 3rd and 5th weeks. No bla_{TEM} was detected in the 1st and 7th weeks of production cycle and tetracycline resistant strains from meat chickens were found to possess *tet* genes (Fig. 1).

In contrast, association of investigated *tet* resistance genes with tetracycline resistant strains was very low in indoor raised egg layer pullets in the 1st and 3rd weeks of the rearing cycle (Fig. 2). However, the prevalence of the tested *tet* genes was found to increase in the 6th and 13th weeks. Similarly the presence of the bla_{TEM} resistant gene was found to be low in ampicillin isolates in the 1st

Table 5: Prevalence of phylogenetic groups, virulence factors and resistance genes in *E. coli* isolates from free-range meat chicken

Age of birds (Wks)	Phylogenetic groups	Virulence factors (VFs)	Distribution of resistance genes	No. of isolates
3 days (1st)	A	*papC*	*tet* (A)	1
	A	*iut*A	No genes	1
15 days (3rd)	A	*papC*	bla_{TEM}	1
	A	*papC*	*tet* (A) + bla_{TEM}	1
	A	*iut*A+*papC*	bla_{TEM}	1
	B2	*iut*A	*int*I	1
	D	*papC*	*tet* (A) + bla_{TEM} + *dhfr*V + *sul*II	1
	D	*iut*A	*int*I	1
	D	*iut*A	*tet* (B) + *int*I + *dhfr*V + *sul*I + *sul*II	1
30 days (5th)	A	papC	bla_{TEM}	1
	A	*papC*	*tet* (A) + bla_{TEM} + *int*I + *dhfr*V + *sul*I	1
	B2	*iut*A	*tet* (C) + *int*I+ *dhfr*V + *sul*I	1
	D	*iut*A	*tet* (B) + *int*I+ *dhfr*V + *sul*II	1
39 days (7th)	B1	*iut*A	*tet* (C)	1
	D	*iut*A + *kpsM*TII	*tet* (B)	1

week but this prevalence was found to increase in the 3rd and 6th weeks. In contrast to the enterococcal isolates, statistical analysis revealed a significant difference between *E. coli* isolates that were resistant to ampicillin and tetracycline, and contained *tet* and bla_{TEM} genes ($p<0.05$) in both isolates from indoor egg layer pullets and free range meat chickens.

In both groups of birds (egg layers and meat chickens), the few trimethoprim-sulfamethoxazole resistant isolates were found to possess trimethoprim (*dhfr*V and *dhfr*XIII) and sulphonamide (*sul*I or *sul*II) resistance genes. The prevalence of resistant strains carrying resistance genes was found to be non-significant in the different groups of birds ($p<0.05$).

Phylogenetic grouping, virulence factors and antibiotic resistance genes in *E. coli*: In the free-range meat chickens, phylogenetic grouping of the *E. coli* isolates revealed 37 and 19% belonged to commensal group A and B1, respectively. The pathogenic groups B2 and D constituted 15 and 29%, respectively of the total isolates (Table 5). The virulence factors *iut*A and *papC* for aerobactin receptor and P fimbriae assembly were carried by most resistant isolates that possessed resistance genes (Table 5). One extra-intestinal pathogenic *E. coli* (ExPEC) isolate belonging to the phylogenetic group D and encoding more than one virulence factors was detected on the 39th day of sampling. This isolate was found to possess *tet* (B) resistant gene only.

Isolates from egg layer pullets were found to have similar prevalence of phylogenetic groups B2 (16%) and D (20%), however a higher number of the isolates were found to belong to commensal group A (42%) (Table 6). In this class of birds the S and F1C fimbriae subunits (*sfa*) and group 2 capsular polysaccharide units (*kps*MTII) were detected in isolates in groups B2 and D (Table 6). ExPEC isolates were detected on the 40th (3 isolates), 70th (3 isolates) and the 100th (3 isolates) day of sampling (Table 6). These isolates were found to carry bla_{TEM}, *tet* (A) or integrase gene I and II.

Plasmid Profiling and Southern blot hybridization of *E. coli* isolates: Plasmid extractions from the 125 multi-resistant *E. coli* isolates revealed varying numbers of plasmids in 88 (70%) of these strains. However 37 (29.6%) out the 125 *E. coli* isolates did not carry any plasmids. We found 29 (23.2%) of the plasmid positive isolates had similar plasmid profiles that were characterized by plasmids of sizes~2-12 kb and the remaining 59 (47.2%) isolates were found to contain plasmids of sizes~5-8 kb.

The detected genes (*tet*, *sul*I, *sul*II, *dhfr*V, *dhfr*XIII, bla_{TEM}, bla_{SHV} and bla_{OXA}) that were DIG-labeled and used as probes to confirm the involvement of plasmids in the observed resistance revealed the following; plasmids from 11 isolates hybridized with *tet* (A) and plasmids from 6 isolates hybridized with bla_{TEM}. Example of plasmids digested with *Eco*R1, separated on a 1% agarose gel and with a picture of the southern blot of the same gel after hybridization with bla_{TEM} or *tet* (A) probe is shown in Fig. 3 and 4.

Since most of the plasmids were negative for the genes used as probes above, the integrase genes (*int*I and *int*II) from integrase positive isolates were DIG-labeled and used as probes to ascertain the location of the integrons on the plasmids of the integrase positive isolates. There was no hybridization observed in any of the integrase positive isolates tested.

DISCUSSION

In this study we found resistant isolates of *E. coli* and enterococci carrying a variety of resistance genes were present in both meat chickens and egg layer pullets at 3 to 5 days of age. The small fluctuations in resistance profiles seen at the different sampling points may reflect the fact that it was not possible to sample the same birds at each visit; in addition the high density of chickens in both groups would have allowed for transfer of resistant organisms between birds. Both classes of chickens carried enterococci with varying high levels resistance to bacitracin, erythromycin, lincomycin, tylosin and tetracycline. Our findings are in contrast to those of

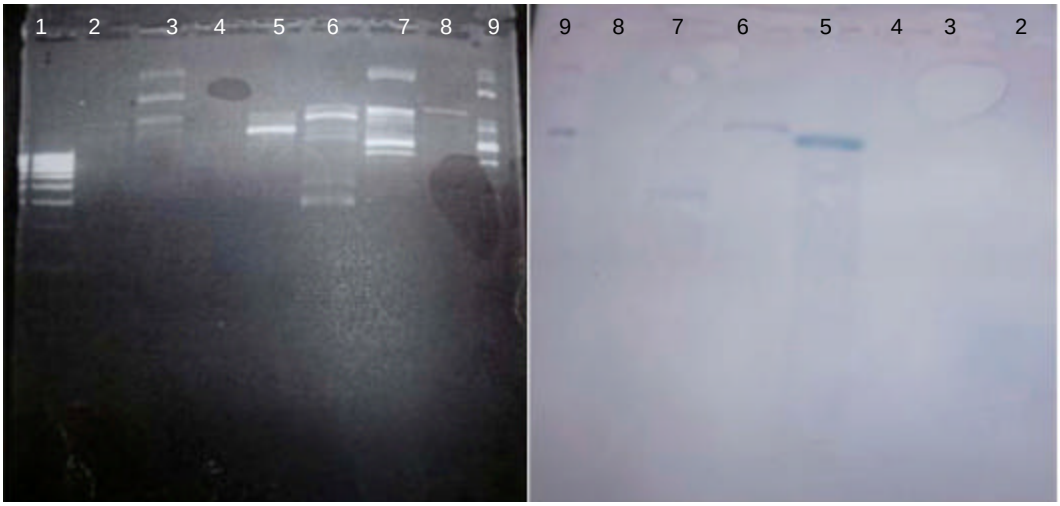

Fig. 3: Plasmid DNA restricted with *Eco*R1 to confirm hybridization with blaTEM gene. Left panel is a picture of an ethidium bromide stained gel containing *Eco*RI digested plasmid DNA from plasmid positive *E. coli* isolates and the right panel is a picture of the southern blot of the same gel after hybridization with blaTEM probe. Lane 1:1 kb ladder, lane 2, 3, 5, 6, 7, 8 and 9 are plasmid positive isolates on the left panel. Hybridization occurred on plasmids on lane 5, 6, 7 and 9

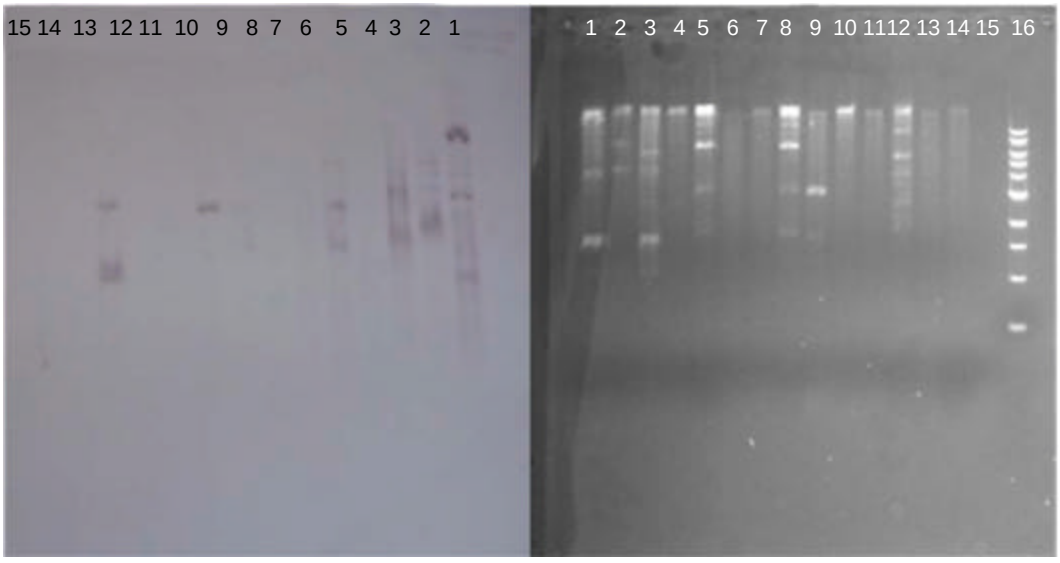

Fig. 4: Plasmid DNA restricted with *Eco*R1 to confirm hybridization with tet (A) gene. Right panel is a picture of an ethidium bromide stained gel containing *Eco*R1 digested plasmid DNA from plasmid positive *E. coli* isolates and the left panel is a picture of the southern blot of the same gel after hybridization with tet (A) probe. On the right panel, lane 16 is a 1 kb ladder; lanes 1 to 14 are plasmid positive *E. coli* isolates. Hybridization occurred on plasmids on lanes 1, 2, 3, 5, 10 and 12 on the left panel

Van Hoorebeke *et al.* (2011) and Schwaiger *et al.* (2010), who found low levels of resistance in enterococci isolates from egg layers. The study also revealed that isolates from egg layers raised under indoor (covered) settings were more resistant than isolates from birds raised under free-range settings. The failure to detect any statistical significant differences between the enterococci isolates from free-range meat chickens and indoor egg layer pullets in this study were surprising because meat chickens and egg layers are different

Table 6: Prevalence of phylogenetic groups, virulence factors and resistance genes in *E. coli* isolates from indoor-raised egg layer pullets

Age of birds (Wks)	Phylogenetic grouping	Virulence factors (Vfs)	Distribution of resistance genes	No. of isolates
5 days (1st)	A	*papC*	bla_{TEM}	1
	A	*sfa*+*papC*	bla_{TEM}	1
	A	*sfa*+*papC*	*tet* (A) + bla_{TEM}	1
20 days (3rd)	A	*papC*	bla_{TEM}	1
	D	*papC*	*tet* (A)	1
40 days (6th)	A	*iut*A	bla_{TEM}	1
	A	*focG*+*kpsM*TII	*tet* (C)	1
	A	*kpsM*TII+*papC*	*tet* (A)	1
	A	*iut*A	*tet* (B) + bla_{TEM} + *int*I	1
	B1	*sfa*	*tet* (A)	1
	B2	*Sfa*	*tet* (C)	1
	B2	*focG*+*kpsM*TII+*papC*	*tet* (A)+bla_{TEM}	1
	D	*papC*	bla_{TEM}	1
70 days (10th)	A	*kpsM*TII	*tet* (A)	1
	A	*sfa*	*tet* (C)	1
	A	*iut*A+*kpsM*TII	*tet* (B)	1
	A	*sfa*	*tet* (A)	1
	A	*papC*	*tet* (A) + bla_{TEM}	1
	D	*papC*	bla_{TEM}	1
	D	*kpsM*TII	bla_{TEM}	2
	D	*kpsM*TII+*papC*	bla_{TEM}	1
	D	*sfa*+*papC*	bla_{TEM} + *int*II	1
	D	*iut*A+*kpsM*TII	bla_{TEM} + *int*I	1
100 days (13th)	A	*papC*	*tet* (B)	1
	A	*papC*	*tet* (C)	1
	A	*papC*	*tet* (B) + *int*I	1
	A	*iut*A	*tet* (A) + bla_{TEM}	1
	A	*kpsM*TII	*tet* (A) + *sul*I+*int*I	1
	A	*iut*A+*KpsM*TII	*tet* (A) + *tet* (C) + bla_{TEM}	1
	A	*KpsM*TII+*papC*	*tet* (A) + bla_{TEM}	1
	B1	*iut*A	bla_{TEM}	1
	B1	*papC*	*tet* (A) + *int*II	1
	B1	*sfaS*+*papC*	bla_{TEM} + *int*I	1
	B1	*iut*A+*sfaS*+*papC*	*tet* (A) + *tet* (C) + *int*II	1
	B2	*papC*	*tet* (A)	2
	D	*kpsM*TII	bla_{TEM}	1
115 days (16th)	A	*kpsM*TII	*tet* (A)	2
	A	*kpsM*TII	*tet* (B) + *tet*(C)	1
	D	*kpsM*TII	*int*1	1
	D	*papC*	*tet* (A) + bla_{TEM} + *int*I	1
	D	*kpsM*TII	*tet* (A) + bla_{TEM} + *int*I	6

strains of poultry and thus the chicks were obtained from quite distinct hatcheries. Despite the differences in strains, managerial practices and housing systems employed by the various farmers to raise the birds, we found that the enterococcal isolates had no significant difference in resistance phenotypes.

In the Netherlands and Denmark, some studies have revealed poultry as a potential source of pathogenic *E. coli* strains that are capable of causing extraintestinal infection (Jakobsen *et al*., 2011; Xia *et al*., 2011). The phylogenetic grouping of the *E. coli* isolates from the different farms revealed indoor-raised egg layer pullets were more likely to be carriers of potential ExPEC isolates than free-range meat chickens. Furthermore, a difference in antibiotic resistance profiles in the *E. coli* isolates was observed in the free-range meat chickens and indoor-raised egg layers. Although none of the isolates were resistant to neomycin or gentamicin, we found a few isolates from egg layer pullets were resistant to florfenicol, spectinomycin and streptomycin; however, none of the isolates from the meat chickens were resistant to these antibiotics.

E. coli isolates from both classes of birds expressed resistance to tetracycline and ampicillin. Resistance to tetracycline and ampicillin were detected from the 1st week of production in both groups of birds. The resistance patterns in *E. coli* detected in this study are consistent with previous reports by Ozaki *et al*. (2011) and Ginns *et al*. (1996). Tetracycline resistance has been reported in organic, free-range and intensive poultry (Ginns *et al*., 1996; Ozaki *et al*., 2011; van den Bogaard *et al*., 2001; Van Hoorebeke *et al*., 2012), however, most of these studies did not investigate the prevalence of *tet* genes in the resistant *E. coli* isolates. In contrast to Maynard *et al*. (2004) study which reported *tet* (A), *tet* (B), *tet* (C), *tet* (D) and *tet* (E), the commonly found genes in this study were *tet* (A), *tet* (B) and *tet* (C). Resistance to tetracycline and ampicillin encoded by *tet* and *bla* genes was found to be higher in isolates from indoor-raised egg layer pullets.

In addition, we found all the trimethoprim-sulfamethoxazole resistant *E. coli* isolates contained *sul*I, *sul*II, *dhfr*V and/ *dhfr*XIII genes. Since the presence of these genes indicates the presence of resistance encoding integrons (Lapierre *et al*., 2008), we tested all the *E. coli* isolates for integrase genes I and II and amplified the conserved regions of integrase positive isolates as described previously (Obeng *et al*., 2012a). We found all the resistant trimethoprim-sulfamethoxazole and spectinomycin isolates were positive for integrase genes 1 and II (Data not shown). The amplification and sequencing of the conserved regions of the integrase positive isolates revealed the presence of *sul*I, *sul*II, *dhfr*V and or *dhfr*XIII genes as previously reported (Obeng *et al*., 2012a). Southern blot characterisation of the plasmids for the integrons was found to be negative. Thus, suggesting that the integrons may be located on the chromosome or transposons of the bacteria (Fluit and Schmitz, 2004).

Furthermore, southern blot characterisation of the plasmids extracted from selected multi-resistant *E. coli* isolates revealed the presence of *bla*$_{TEM}$ and *tet* (A) in a few *E. coli* isolates. Our findings are in conformity to Bergenholtz *et al*. (2009) and Lapierre *et al*. (2008) studies from Denmark and Santiago, where they found *bla*$_{TEM}$ and *tet* (A) genes located on plasmids of some resistant *E. coli* isolates from poultry. Although this study found none of the plasmid positive isolates carried *tet* (B), *tet* (C), *sul*I, *sul*II, *dhfr*V, *dhfr*XIII, *bla*$_{SHV}$ and *bla*$_{oxa}$, further studies are required to determine the involvement of other genetic elements (such as transposons and chromosomes) that may be associated with resistance genes detected in this study.

There are several possible explanations for our findings. Firstly the fact that the chickens carried resistant strains from such a young age strongly suggests that there is transfer of resistant bacteria from the breeder flocks. The presence of ampicillin resistant *E. coli* possessing *bla*$_{TEM}$ genes provides further evidence for the possibility of vertical transfer of resistant strains from breeder birds to progeny since amoxicillin is not permitted in meat chickens, egg layers or in birds which will produce eggs for human consumption in Australia (Barton, 2004). Since antibiotic use in meat chickens and egg layer pullets is not common in Australia, it is more likely that the resistant isolates in the chickens were acquired from breeder birds by para-vertical transmission (on the outside of the egg shells of hatching eggs). This is in line with the findings of Fertner *et al*. (2011) in regard to enterococci in layer hens and Ginns *et al*. (1996) with regard to *E coli* in meat chickens. Secondly we suspect cross contamination of feeds in the feed mills producing feed for these birds because the same feed mills are known to produce antibiotic-containing feed for pigs. Thirdly there is the possibility of environmental contamination but this is unlikely to result in such similar resistance profiles as found in the isolates in this study. The two farms are 115 km apart; hence it is unlikely that cross-contamination could account for the phenotypic resistance patterns observed on the different farms. The spread of resistant isolates between the birds may have also been facilitated by contaminated litter, farm equipment and transport crates. In addition, no antibiotic was administered to the birds on both farms during this study as reported by the farmers. Both farms employed good farm hygiene practices and high standards of biosecurity to ensure the health of the birds.

Conclusion: In summary, this small study supports the view that antibiotic resistant bacteria in both meat and egg layer chickens can be acquired from para-vertical transmission on egg shells to chicks as they hatch, or from cross-contamination of feed at feed mills producing medicated feeds for other food animals. Furthermore, the use of disinfectants during various cleaning processes in the hatcheries or farms may be enabling the selection and the spread of resistant bacteria.

The differences observed in this study could have arisen because of differing population genetics between meat chickens and egg layers pullet flocks. Although, it was certain that once the birds became colonized with resistant bacteria, the resistant strains persisted throughout the production or rearing cycle. This study was not able to explicitly determine the source of resistance genes in the enteric bacteria of birds that were colonized from the first week of production cycle. Findings from this study call into question the management practices on the various breeder farms producing the chickens. Further studies are required to determine the prevalence of antibiotic resistance in breeder flocks and the extent of contamination of feeds in feed mills in order to address the source of these resistance genes.

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