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Genotypic Analysis of *Escherichia coli* Strains from Poultry Carcasses and Their Susceptibilities to Antimicrobial Agents

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Plasmid profiling and amplified fragment length polymorphism (AFLP) analysis were used to genotype 50 *Escherichia coli* strains from poultry carcasses. Thirty different plasmid profiles were evident, and clustering of the AFLP data showed that they were a distinctly heterogeneous group of strains. Susceptibility testing against five antimicrobial agents used in the South African poultry industry showed all strains to be susceptible to danofloxacin and colistin, while the majority (96%) were resistant to two tetracyclines.

Escherichia coli forms part of the bacterial population of the chicken gastrointestinal tract. In poultry processing, *E. coli* is regarded as an indicator of fecal contamination (19). Levels of *E. coli* associated with poultry carcasses can increase or decrease during processing depending on factors such as levels of fecal contamination on live birds, length of time and temperature of scalding, efficiency of evisceration, bacterial load and temperature of the immersion chiller water, and hygienic practices in the abattoir (23). *E. coli* is also regarded as a major pathogen of worldwide importance in commercially produced poultry and can result in significant economic losses (20). Poultry-associated diseases caused by pathogenic *E. coli* strains include colibacilliosis and airsacculitis, which can cause high morbidity and mortality in poultry (20). To control and prevent poultry diseases, breeders are known to administer subtherapeutic and therapeutic levels of antimicrobial agents to chickens via feed and water (7). This practice also improves feed efficiency and accelerates weight gain (7). The administration of antimicrobial agents to poultry, however, has provided a selection pressure for antimicrobial resistance genes, and as a result, many bacteria associated with chickens and poultry meat are now resistant to antimicrobial agents (32, 36).

Several molecular typing techniques, including plasmid profiling, random amplified polymorphic DNA analysis, pulsed-field gel electrophoresis, and ribotyping have been used to characterize and determine epidemiological relationships of *E. coli* strains (1, 17, 26, 30, 34). Amplified fragment length polymorphism (AFLP) analysis, based on the principles of restriction fragment length polymorphism analysis and PCR amplification (25, 37), is a high-resolution typing method which has been used to differentiate between strains of *Campylobacter jejuni* and *Campylobacter coli* (9); *E. coli* O157:H7 (24), *Helicobacter pylori* (16), *Streptococcus pyogenes* (8), *Pseudomonas fluorescens*, and *Pseudomonas putida* (15); and *Lactobacillus plantarum* and *Leuconostoc mesenteroides* (27).

In this study, *E. coli* strains from poultry carcasses were analyzed to determine their susceptibilities to antimicrobial agents used in the South African poultry industry, and genetic relationships based on plasmid profiling and AFLP analysis.

Bacterial strains. The 50 strains examined (Table 1) were obtained from a microbiological survey of a poultry abattoir where bacterial counts and populations associated with the neck skins of carcasses at six processing stages were determined (13, 14). The API 20E system (bioMérieux, Marcy l'Etoile, France) was used to confirm the identity of the *E. coli* strains. O- and K-antigen serogrouping of the strains was performed by the Onderstepoort Veterinary Institute of the Agricultural Research Council (Onderstepoort, South Africa). Standard *E. coli* antisera were used, excluding the antisera against antigens K21, K64, K65, K77, K92, and K100 through K102 (31). All 50 strains were O rough and K minus.

Strains were stored at −70°C in tryptone soya broth (TSB) (Oxoid, Basingstoke, United Kingdom) supplemented with 15% (vol/vol) glycerol.

Antimicrobial susceptibility testing. MICs for the *E. coli* strains of the five antimicrobial agents used in the South African poultry industry were determined by the microdilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (29). Reference powders were kindly provided by Pfizer (Groton, Conn.) (danofloxacin) and Logos AgVet (Midrand, South Africa) (colistin sulfate, neomycin sulfate, chlortetracycline hydrochloride, and oxytetracycline base). Mueller-Hinton broth (Oxoid) was supplemented with cations, and concentrations of test strains were standardized to 5×10^5 CFU/ml (29). MICs were read after 18 h of incubation at 37°C. The MIC was interpreted as the lowest concentration that visibly inhibited growth. *E. coli* ATCC 25922 was used as the quality control reference strain (29).

MIC ranges and MICs at which 50 and 90% of the strains tested are inhibited (MIC_{50}s and MIC_{90}s, respectively) are shown in Table 2. MIC breakpoints for resistance and susceptibility have not been established by the NCCLS for any of the antimicrobial agents tested here. For purposes of this study, therefore, MIC breakpoints were assigned to each of the antimicrobial agents that were based on breakpoints established by the NCCLS for related antibiotics (29). The strains analyzed here were thus considered resistant when MICs were ≥4 μg/ml for danofloxacin and ≥16 μg/ml for neomycin, chlortetracy-

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TABLE 1. Summary of profiles of 50 *E. coli* strains from poultry carcasses

Processing stage	Strain	Antimicrobial resistance[a]	Plasmid profile	AFLP profile
After defeathering (n^b = 9)	181	CT, OT	P5	A15
	183	CT, OT	P9	A12
	184	CT, OT	P2	A9
	185	CT, OT	P21	A10
	186	NM	P28	A30
	187	CT, OT	P17	A8
	188	CT, OT	P2	A7
	189	NM, CT, OT	P16	A13
	192	CT, OT	P19	A13
Before evisceration (n = 9)	193	CT, OT	P14	A13
	195	CT, OT	P3	A13
	196	CT, OT	P27	A34
	197	CT, OT	P15	A4
	198	CT, OT	P9	A5
	199	NM, CT, OT	P7	A6
	201	CT, OT	P26	A14
	202	NM, CT, OT	P29	A3
	204	CT, OT	P2	A18
After evisceration (n = 12)	205	CT, OT	P2	A18
	206	CT, OT	P14	A17
	207	CT, OT	P2	A38
	208	NM, CT, OT	P23	A24
	209	CT, OT	P19	A9
	210	NM	P30	A32
	211	CT, OT	P2	A1
	212	CT, OT	P19	A2
	213	CT, OT	P19	A2
	214	NM, CT, OT	P6	A19
	215	CT, OT	P18	A16
	216	CT, OT	P19	A20
After spray washing (n = 11)	217	Susceptible	P27	A31
	218	CT, OT	P13	A21
	219	CT, OT	P13	A21
	220	CT, OT	P24	A36
	221	NM	P1	A37
	222	CT, OT	P10	A11
	224	NM, CT, OT	P22	A11
	225	CT, OT	P12	A22
	226	CT, OT	P4	A29
	227	CT, OT	P4	A33
	228	CT, OT	P12	A41
After immersion chilling (n = 8)	231	CT, OT	P2	A40
	232	Susceptible	P25	A35
	233	NM, CT, OT	P2	A27
	235	CT, OT	P20	A39
	236	CT, OT	P7	A28
	238	CT, OT	P11	A23
	239	CT, OT	P2	A15
	240	CT, OT	P11	A25
After packaging (n = 1)	248	CT, OT	P8	A26

[a] Abbreviations: CT, chlortetracycline; OT, oxytetracycline; NM, neomycin.
[b] n, number of strains.

cline, and oxytetracycline. An arbitrary MIC breakpoint for resistance to colistin of ≥16 μg/ml was used, since NCCLS interpretative standards have not been established for the polymyxin class of antibiotics, to which colistin belongs. Using these breakpoints, all but two of the strains (strains 217 and 232 [Table 1] were resistant to at least one and at most three of the antimicrobial agents. The majority (76%) of the strains were resistant to the two tetracyclines only, while 14% were resistant to the tetracyclines as well as neomycin. The remaining three isolates were resistant to neomycin only (Table 1). All the strains were susceptible to danofloxacin and colistin, with MIC_{90}s of ≤0.125 and 1 μg/ml, respectively (Table 2). Similarly, Watts et al. (38) reported the MIC_{90} of danofloxacin for *E. coli* isolates of veterinary origin to be ≤0.015 μg/ml. Danofloxacin belongs to the new fluoroquinolone class of antimicrobials, which are highly effective against gram-negative bacilli (6, 12). Their use in the poultry industry, however, is thought to be inappropriate due to cross-resistance with fluoroquinolones used to treat important human enteric infections (10, 11). Fluoroquinolone resistance has been reported for *Salmonella* serotypes (21, 28), *Campylobacter jejuni* (10), and *E. coli* (11). The susceptibilities of the strains in this study to colistin were in agreement with those reported in a Spanish study where 468 *E. coli* strains of avian origin were susceptible to this antimicrobial agent (6). Resistance to colistin reportedly does not commonly develop in bacteria originally susceptible to this antimicrobial agent (22), which could possibly explain the narrow range and low MICs obtained for the *E. coli* strains in this study. Neomycin is an aminoglycoside and is primarily active against *Escherichia* spp., but it is also effective against other genera of the *Enterobacteriaceae* (22). In our study, 20% of the *E. coli* strains tested were resistant to this antimicrobial agent. Conversely, 90% of the strains were resistant to the two tetracyclines, chlortetracycline, and oxytetracycline (MIC_{90}s of 128 and >512 μg/ml, respectively) (Table 2). This high level of resistance is of concern due to possible cross-resistance with antibiotics used in human medicine. Recent studies have suggested a link between the use of antimicrobial agents in poultry and other food-producing animals, and the emergence of human pathogens with decreased susceptibilities or complete resistance to antibiotics used for treatment of human infections (4, 5, 28).

Antimicrobial resistance typing was a poor tool for differentiating between strains in this study since the majority (76%) shared the same profile, that is, resistance to the two tetracyclines (Table 1).

Plasmid profiles. Plasmid DNA was extracted by the alkaline lysis method from overnight cultures grown in TSB at 37°C (18). Plasmids were separated on 0.8% agarose gels, viewed under UV transillumination, and photographed. *Lactococcus lactis* subsp. *lactis* DSM 4645 plasmids were used as molecular size markers (3).

All but one of the strains contained between one and six plasmids, with sizes ranging from 1.5 to 89 kb. One, two, or four plasmids were harbored by almost equal proportions of the strains (24, 28, and 24%, respectively). Overall, however, plasmid profiles obtained for all the strains were diverse, with

TABLE 2. MICs of five antimicrobial agents for 50 *E. coli* strains associated with poultry carcasses

Antimicrobial agent	MIC (μg/ml)		
	Range	50%[a]	90%[b]
Danofloxacin	≤0.125–1	≤0.125	≤0.125
Colistin	0.5–1	1	1
Neomycin	2–>512	8	16
Chlortetracycline	4–128	64	128
Oxytetracycline	4–>512	>512	>512

[a] 50%, MIC_{50}.
[b] 90%, MIC_{90}.

30 profiles emanating from the 50 isolates (Table 1). Twenty of these profiles were unique, while the remaining 10 were shared by at least two and at most nine strains. These nine strains contained a single 89-kb plasmid (profile P2 [Table 1]) which was also present in 86% of strains containing more than one plasmid. Profile P19 was shared by five strains, while profiles P4, P7, P9, P11 through P14, and P27 were shared by two strains each (Table 1).

Seven of the nine strains isolated from carcasses after the defeathering stage had different plasmid profiles, while the profiles of all the strains originating from carcasses before evisceration were different (Table 1). Conversely, 3 and 4 of the 12 strains from carcasses after evisceration shared profiles P2 and P19, respectively, while two strains each from carcasses after spray washing shared profiles P4, P12, and P13. Furthermore, three and two strains from carcasses after the immersion chilling stage displayed profiles P2 and P11, respectively (Table 1).

No apparent correlation was found between the plasmid profiles of the strains and their resistance patterns to the antimicrobial agents (Table 1).

AFLP analysis. The NucleoSpin C & T kit (Macherey-Nagel, Düren, Germany) was used to extract genomic DNA from 1-ml cultures grown in TSB at 37°C for 18 h. DNA concentrations were estimated by agarose gel electrophoresis with diluted samples of λ DNA (Boehringer Mannheim GmbH, Mannheim, Germany). The AFLP ligation and preselective amplification kit (Perkin-Elmer, Foster City, Calif.) was used for AFLP reactions, which were each performed on 250 to 500 ng of DNA as described previously (15). Amplified fragments were separated on denaturing 4% polyacrylamide sequencing gels, which were run on a model S2 sequencing gel apparatus (Gibco, BRL Life Technologies, Gaithersburg, Md.) at 50 W with 1× Tris-borate-EDTA (TBE) buffer in the upper compartment and 1× TBE supplemented with 0.5 M sodium acetate in the lower compartment (2). AFLP fingerprints were detected by the modified silver staining method described previously (15). Gels were air dried overnight and then scanned with a Hewlett-Packard ScanJet IIcx scanner. AFLP patterns were analyzed with the GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). Gels were normalized by including the 1-kb Plus ladder (Gibco) at four-lane intervals on every gel as a standard. After conversion, normalization, and background subtraction, levels of similarity between the AFLP fingerprints were calculated by using the Pearson product-moment correlation coefficient (*r*). Strains were clustered by using the unweighted pair group method with arithmetic averages (UPGMA) (33).

AFLP reactions generated between 26 and 44 detectable bands per strain. The AFLP fingerprints of one *E. coli* strain from carcasses after evisceration and three strains from carcasses after spray washing are shown in Fig. 1 as typical examples. The dendrogram generated by clustering of the AFLP data by UPGMA is shown in Fig. 2. Clearly, the AFLP fingerprints of the 50 strains analyzed were highly heterogeneous, with the highest level of homology observed between the strains being 96% and the lowest level of homology being 27%. Thus, none of the AFLP fingerprints were shown by the software to be 100% homologous, even though they appeared to be identical by visual inspection. Slight variations in band width and mobility as well as background intensities could explain this discrepancy. Similarity levels obtained here are thus not absolute but were nevertheless useful for determining relationships among the *E. coli* strains. For purposes of this study, therefore, strains whose AFLP patterns were >90% similar were assumed to be closely related genetically. At a delineation level of 90%, therefore, 41 different AFLP fingerprints were

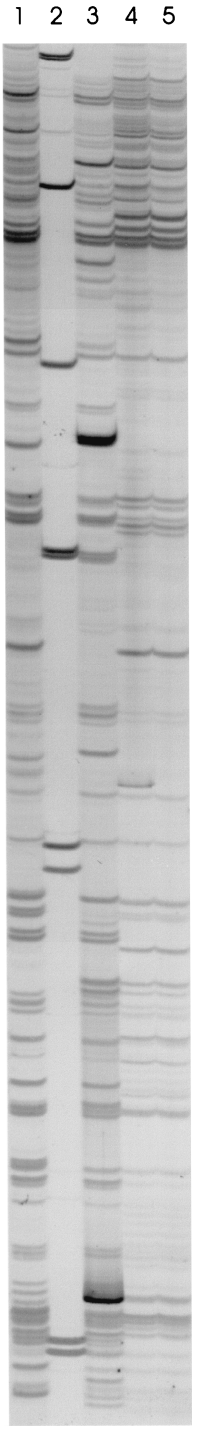

FIG. 1. AFLP patterns of one *E. coli* strain from carcasses after evisceration (strain 216) and three strains from carcasses after spray washing (strains 217, 218, and 219). Lane 1, strain 216; lane 2, 1-kb Plus ladder; lane 3, strain 217; lane 4, strain 218; lane 5, strain 219.

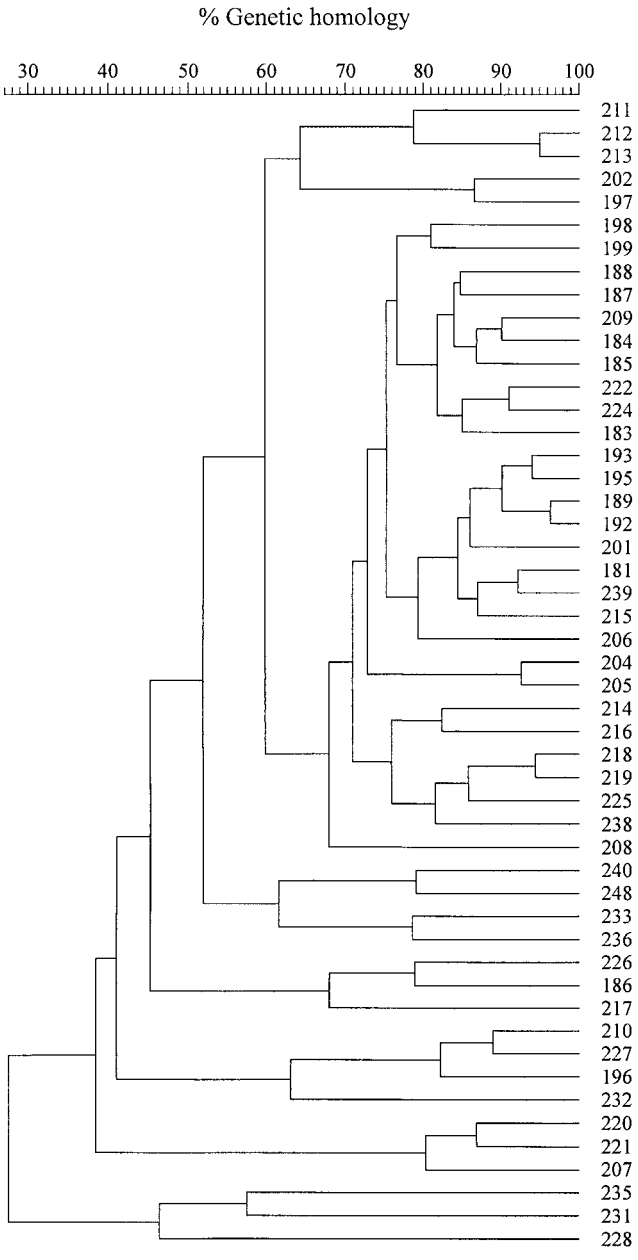

FIG. 2. Dendrogram based on AFLP fingerprints of 50 *E. coli* strains from poultry carcasses. The dendrogram was constructed by using UPGMA. Levels of similarity between AFLP fingerprints were calculated using the Pearson product-moment correlation coefficient.

generated for the 50 *E. coli* strains (Table 1). Strains with homology levels of >90% included strains 212 and 213 from carcasses after evisceration, strains 218 and 219, as well as strains 222 and 224 from carcasses after spray washing (Fig. 2; Table 1). Strains isolated from carcasses at different stages of processing were also found to be genetically related. These included strains 181 and 239 from carcasses after defeathering and immersion chilling, respectively; strains 184 and 209 from carcasses after defeathering and evisceration, respectively; and strains 204 and 205 from carcasses before and after evisceration, respectively (Fig. 2; Table 1). Finally, strains 189, 192, 193, and 195 formed the largest cluster of related strains, with two of the strains originating from carcasses after defeathering and two strains from carcasses before evisceration (Fig. 2; Table 1). The heterogeneous nature of the AFLP fingerprints of the strains possibly indicates a large number of contamination sources of carcasses with *E. coli*. Sources could include the farm and processing environments as well as the processing equipment.

Comparison of the plasmid and AFLP profiles obtained for each of the strains showed that in almost all cases, strains that shared plasmid profiles did not also share the same AFLP profiles (Table 1). Plasmid profiling has previously been shown to be of limited value as a genotyping method compared to other molecular typing techniques, mainly due to the instability of plasmids, poor reproducibility due to the variable presence of extra bands from open and linear forms of the plasmids, the presence of plasmids that appear to be similar, or simply the absence of plasmids in some of the strains (1, 35). In the present study, some of the strains that were regarded as genetically closely related by AFLP analysis also shared plasmid profiles, that is, strains 204 and 205 (level of homology, 93%), strains 212 and 213 (level of homology, 95%), and strains 218 and 219 (level of homology, 95%) (Fig. 2; Table 1). The remainder of the related strains, however, displayed different plasmid profiles. For instance, strains 189 and 192, whose AFLP fingerprints were 96% similar, differed not only in their plasmid profiles but also in their antimicrobial resistance profiles (Fig. 2; Table 1). In order for the AFLP technique to be sensitive enough to discern these polymorphisms in small genomes, such as those in bacteria, it may have to be modified to cover more alleles. This could be achieved by using a restriction endonuclease that cuts more frequently than the ones used in the present study.

In conclusion, the high-resolution genotyping method of AFLP analysis showed that the strains isolated from poultry carcasses during processing were genetically diverse. This suggests multiple sources of contamination of carcasses with *E. coli*. To pinpoint these sources, a study including isolates from the environment and equipment, as well as intestinal contents of carcasses and workers' hands, would have to be conducted.

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