Incidence and Characterization of Integrons, Genetic Elements Mediating Multiple-Drug Resistance, in Avian *Escherichia coli*

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Antibiotic resistance among avian bacterial isolates is common and is of great concern to the poultry industry. Approximately 36% ($n = 100$) of avian, pathogenic *Escherichia coli* isolates obtained from diseased poultry exhibited multiple-antibiotic resistance to tetracycline, oxytetracycline, streptomycin, sulfonamides, and gentamicin. Clinical avian *E. coli* isolates were further screened for the presence of markers for class 1 integrons, the integron recombinase *intI1* and the quaternary ammonium resistance gene *qacEΔ1*, in order to determine the contribution of integrons to the observed multiple-antibiotic resistance phenotypes. Sixty-three percent of the clinical isolates were positive for the class 1 integron markers *intI1* and *qacEΔ1*. PCR analysis with the conserved class 1 integron primers yielded amplicons of approximately 1 kb from *E. coli* isolates positive for *intI1* and *qacEΔ1*. These PCR amplicons contained the spectinomycin-streptomycin resistance gene *aadA1*. Further characterization of the identified integrons revealed that many were part of the transposon Tn21, a genetic element that encodes both antibiotic resistance and heavy-metal resistance to mercuric compounds. Fifty percent of the clinical isolates positive for the integron marker gene *intI1* as well as for the *qacEΔ1* and *aadA1* cassettes also contained the mercury reductase gene *merA*. The correlation between the presence of the *merA* gene with that of the integrase and antibiotic resistance genes suggests that these integrons are located in Tn21. The presence of these elements among avian *E. coli* isolates of diverse genetic makeup as well as in *Salmonella* suggests the mobility of Tn21 among pathogens in humans as well as poultry.

Escherichia coli adversely affects avian species through infections of the blood, respiratory tract, and soft tissues. Diseases resulting from *E. coli* infections, such as colibacillosis, air sacculitis, and cellulitis, cause high morbidity and mortality in poultry (21), which have a significant economic impact on the poultry industry (15). These infections have traditionally been treated with antibiotics. Antibiotics once effective at controlling *E. coli* infections are now ineffective due to the bacterium's acquired resistance to these compounds. Resistance in microbial pathogens like *E. coli* to two or more classes of antibiotics is now commonplace in both veterinary (19, 23, 26) and human (13) medicine. Although antibiotic susceptibility may return with the discontinuance of antibiotic use, antibiotic-resistant bacteria can still persist long after the removal of the selection pressure (9). Even with the rotation of antibiotics, antibiotic resistance may persist due to the genetic linkage of several antibiotic and heavy-metal resistance genes provided for their perpetuation as selection pressures change.

Multiple-drug resistance in enteric organisms like *E. coli* is known to be associated with integrons. Integrons generally contain an integrase gene (*intI*) (31, 32) and a cassette integration site (*attI*) (48), into which antibiotic resistance gene cassettes have integrated. A gene cassette contains an antibiotic resistance gene and a 59-bp element, a short inverted repeat element with a core recombination site (48). At least four classes of chromosomal and plasmid-borne integrons in gram-negative bacteria have been described (1, 36, 49, 51). Class 1 integrons commonly contain the quaternary ammonium compound resistance gene *qacEΔ1* and the sulfonamide

resistance gene *sulI* in the 3' conserved region (39, 49). The conserved 5' and 3' regions flank gene cassettes, which contain single or multiple antibiotic resistance gene(s). The integron acquires or exchanges antibiotic resistance genes with its specialized recombinase, *intI* (11, 38, 59). These genetic elements are responsible for linking antibiotic resistance genes together to form large multiple loci of antimicrobial resistance within microbial genomes (4, 45). Class 1 integrons have primarily been found on complete or truncated derivatives of the Mu-like transposon Tn402, which reside in broad-host-range plasmids (16, 41, 47, 52, 53) or within Tn21 or Tn21-like transposons (20). Tn21, a large (19.7-kb) class II replicative transposon, carries a mercury resistance (*mer*) operon, an integron (*Int2*), and a transposition module (*tnpA*, *tnpR*, and *res* site). Variants of Tn21 included those transposons similar to Tn21 but with different or additional cassettes and/or the insertion and/or deletion of insertion sequences (IS) in regions 3' of the inserted cassettes (29). The prototypical Tn21 carries the *aadA1* gene cassette in its integron. In addition to drug resistance, Tn21 confers mercury resistance through its mercuric reductase gene, *merA* (30). In the case of Tn4, which does not confer mercury resistance, it appears that transposon Tn3 inserts into and disrupts the *mer* locus of Tn21 (29).

Resistance to specific antibiotics, like streptomycin, continues to be prevalent among avian *E. coli* isolates, despite the discontinuance of a given antibiotic as a therapeutic agent. The presence of integrons among clinical avian *E. coli* isolates may account for multiple-antibiotic resistance and continued resistance to antibiotics that have been withdrawn from use in poultry medicine. In this study, we report the high incidence of this genetic element among avian *E. coli* isolates and determine that the majority of these integrons are part of a Tn21-like transposon.

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TABLE 1. PCR primers^a

Gene	PCR primers	Expected size of PCR product (bp)	Reference
5' CS	GGCATCCAAGCAGCAAG		28
3' CS	AAGCAGACTTGACCTGA		28
<i>intI1</i>	F: CCTCCCGCACGATGATC R: TCCACGCATCGTCAGGC	280	28
<i>qacEΔ1</i>	F: AAGTAATCGCAACATCCG R: AAAGGCAGCAATTATGAG	250	This study
<i>merA</i>	F: ACCATCGGCGGCACCTGCGT R: ACCATCGTCAGGTAGGGGAACA	1,232	30

^a F, forward; R, reverse.

MATERIALS AND METHODS

Bacterial strains and plasmids. One hundred *E. coli* and eight *Salmonella* isolates were obtained from the tracheas, lungs, air sacs, livers, and spleens of chickens, quail, ostriches, and turkeys. Clinical avian *E. coli* and *Salmonella* isolates were stored as 20% glycerol stocks at -80°C .

Colony blots. DNA probes for Southern analysis were generated by PCR with primers specific for *int*, *aadA1*, *qacEΔ1*, and *merA*. *E. coli* SK1592(pDU202) (57) that contains Tn21 served as the template for generating PCR-based DNA probes for DNA-DNA hybridizations. Oligonucleotides were synthesized by the Molecular Genetics Instrumentation Facility at the University of Georgia, Athens (Table 1). The total DNA template for PCR was prepared as follows. A 1.5-ml overnight culture was centrifuged to pellet the bacteria, resuspended to its original volume in distilled H_2O , and boiled for 10 min. The cell debris was pelleted by centrifugation at $12,000 \times g$ for 30 min, and the supernatant was diluted 10-fold in distilled H_2O to serve as a template for PCR. The DNA template was stored at -20°C . One hundred nanograms of *E. coli* chromosomal DNA served as a template in a 10-μl PCR mixture. This reaction mixture consisted of 0.2 mM deoxynucleoside triphosphates, 2.0 mM MgCl_2 , 1× PCR buffer (50 mM Tris [pH 7.4]), bovine serum albumin (0.25 mg/ml), 50 pmol (each) of forward and reverse PCR primers, and 0.5 units of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.). The program parameters for the Idaho Technology Rapidcycler (Idaho Falls, Idaho) (58) were (i) 94°C for 0 s, (ii) 55°C for 0 s, and (iii) 72°C for 15 s for 30 cycles. DNA products from PCR were analyzed by gel electrophoresis. DNA was separated on a 1.5% agarose-1× Tris-acetate-EDTA gel at 70 V. The 100-bp ladder (Promega, Madison, Wis.) served as the molecular weight (MW) standard for determining the MW of the PCR products. The DNA fragment corresponding in size to the MW expected for specific primer pairs was extracted from the agarose gel slice with a Supelco GelElute agarose spin column (Bellefonte, Pa.). The procedure for DNA-DNA hybridizations was performed as described by Sambrook et al. (44) with hybridizations and washes done at 68°C . Hybridizing DNA fragments were detected with digoxigenin antibody-alkaline phosphatase conjugate provided with the Genius system (Boehringer Mannheim).

Southern hybridization. Plasmid DNA was isolated from avian *E. coli* (44) and digested with *EcoRI*. The DNA was separated on a 1% agarose-1× Tris-acetate-EDTA gel and ethidium bromide (5 μg/ml) and transferred to a nylon membrane (44). Single-stranded DNA was cross-linked to membranes with ultraviolet light (optimal cross-linking setting; Fisherbiotech UV Crosslinker). Membranes were hybridized with DNA probes specific for *aadA* or *merA*. DNA probes were prepared as outlined above. Procedures for DNA-DNA hybridizations and detection were performed as specified in the protocol for the Genius 3 kit (Boehringer Mannheim). The annealing temperature for hybridizations and washes was 68°C (44).

Mercury resistance assay. Bacterial isolates were streaked onto tryptone broth agar plates, containing 1.0×10^{-4} M mercuric chloride, and Luria-Bertani plates (50). Growth of isolated, single colonies on the mercuric chloride plates indicated the presence of the mercury resistance phenotype.

RESULTS AND DISCUSSION

Antibiotic resistance patterns in avian *E. coli*. One hundred clinical avian *E. coli* isolates were examined for their general susceptibility to a battery of antibiotics of human and veterinary significance. The prevalence of resistance to the aminoglycosides ranged from 27% for kanamycin to 97% for streptomycin among these isolates (Table 2). Most *E. coli* isolates (86%) were resistant to the tetracyclines, tetracycline and oxytetracycline. Avian *E. coli* isolates were generally resistant to both streptomycin and the sulfonamides (97 of 100). A high percentage of the *E. coli* strains isolated were also resis-

tant to ampicillin (30%) and chloramphenicol (10%). Similar observations have been reported, in other countries, for coliforms isolated from avian species (3, 7, 54, 55). It is difficult to determine, however, whether the higher incidence of antibiotic resistance corresponds to the continued use of ampicillin and chloramphenicol in these countries. Neither antibiotic is currently recommended as a therapeutic agent in avian medicine, although ampicillin resistance may be a reflection of cross-resistance to ceftiofur, a third-generation cephalosporin currently available as a therapeutic for poultry (17). On the other hand, chloramphenicol has been banned from use in food animals since the 1980s in the United States and Canada (18). The majority (64%) of clinical *E. coli* strains isolated from diseased birds at the Poultry Diagnostic and Research Center, University of Georgia, exhibited multiple resistance to five or more antibiotics (Table 3). The common multiple-antibiotic resistance profile among these isolates includes resistance to oxytetracycline, tetracycline, gentamicin, streptomycin, and sulfonamide. Gentamicin resistance may be due to the inclusion of this antibiotic with the Marek's vaccine that is administered to almost all poultry in ovo (14, 42). Since class 1 integrons typically assemble arrays of antibiotic resistance genes, it was logical to determine the integron incidence among avian clinical isolates.

The incidence of genes associated with integrons among avian *E. coli*. One hundred avian *E. coli* isolates were screened for the presence of *intI1* and *qacEΔ1*, markers for class 1 integrons (32, 39). Sixty-three clinical isolates were positive for

TABLE 2. Antibiotic susceptibility patterns of avian *E. coli*

Class and antibiotic	No. of resistant strains (<i>n</i> = 100)
β-Lactams	
Ampicillin	30 ^a
Chloramphenicols	
Chloramphenicol	11
Tetracyclines	
Tetracycline	1
Oxytetracycline	5
Tetracycline and oxytetracycline	86
Aminoglycosides	
Streptomycin	97
Gentamicin	65
Neomycin	30
Kanamycin	27

^a β-lactamase positive.

TABLE 3. Antibiotic resistance patterns of avian *E. coli* strains

Antibiotic resistance ^a	No. of resistant strains (n = 100)
Ap Cm Tc Au Gm S Su.....	3
Ap Tc Au Gm S Su.....	9
Tc Au S Su.....	6
Tc Au Gm S Su	36
Tc Au Km Gm Ne S Su.....	8
Tc Au Gm Ne S Su.....	8
Drug resistance to ≥ 5 antibiotics.....	64
S-Su-resistant <i>E. coli</i> strains with class 1 integron gene, <i>intI1</i> ... 84 ^b	

^a The most common pattern of multiple-drug resistance among strains of *E. coli* isolated from poultry is shown in line 4 of the table (boldface type). Ap, ampicillin; Au, oxytetracycline; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Ne, netilmicin; S, streptomycin; Su, sulfonamide.

^b No. of strains tested, 97 (87% resistant).

both *intI1* and *qacEΔ1* (Table 4). However, 27 avian *E. coli* isolates did not possess either marker (Table 4).

Integron-mediated, antibiotic resistance genes are common among clinical *Enterobacteriaceae* associated with disease in humans (33, 43). Globally disseminated Tn21-like transposons which carry class 1 integrons, as well as close relatives of In2 which are found in other independent locations (typically conjugative plasmids) (5, 29), account for the high incidence of this element among commensal (57), environmental (12), and clinical (62) bacterial isolates. To further characterize the integrons of avian *E. coli*, PCR was used to amplify and sequence the gene cassettes associated with the identified class 1 integrons (27, 28). With previously published class 1 integron PCR primers (28), several avian *E. coli* isolates that were positive for *intI1* and *qacEΔ1* yielded amplicons of approximately 1.0 kb each (data not shown). The DNA sequences of the PCR amplicons were nearly identical to the nucleotide sequence for the *aadA1* gene, which encodes streptomycin and spectinomycin resistance. There was 99% identity between the PCR amplicon's DNA sequence and *aadA1* genes from *E. coli* (25), *Shigella flexneri* (10), *Klebsiella* spp. (40, 52), and *Pseudomonas aeruginosa* (GenBank accession no. L36547). The class 1 integrons examined did not possess the complete resistance phenotype observed among the clinical avian *E. coli* isolates. This does not rule out the existence of other class 1 integrons in avian *E. coli* strains that we may not have been able to detect.

A large percentage of *E. coli* isolates with streptomycin and sulfonamide resistance were also positive for the class 1 inte-

grase gene *intI1* (84 of 97) (Table 3). Since the antibiotic resistance gene *aadA1* was present in several avian *E. coli* isolates, we assessed its distribution among the clinical *E. coli* isolates by DNA hybridization. All *E. coli* isolates positive for *intI1* and *qacEΔ1* (n = 63) also had the *aadA1* resistance gene (Table 4). These same genetic markers were also evident in 6 of 8 avian *Salmonella* isolates examined (data not shown). In assessing the distribution of class 1 integron markers and *merA* among avian *E. coli* isolates, we also noted other genotypes where *intI1*, *qacEΔ1*, *aadA1*, or *merA* genes were absent. These unusual genotypes (*intI1 aadA1 merA*, *aadA1 qacEΔ1 merA*, *intI1 merA*, and *aadA1*) might represent recombinational events that occur in class 1 integrons and the *mer* operon (29).

The integrons of avian *E. coli* are part of the mercury resistance transposon Tn21. Since the *aadA1* gene was associated with all *E. coli* isolates positive for *intI1* and *qacEΔ1* and since this gene has been associated with the mercury resistance transposon Tn21 (22), we looked into the possibility that the integrons in avian *E. coli* are actually part of a Tn21-like transposon. We initially examined the occurrence of the Tn21 mercury reductase gene *merA* among these isolates. Fifty avian *E. coli* strains were positive for two known Tn21 markers, *aadA1* and *merA*. The mercury resistance gene *merA* was present in 79% of *E. coli* isolates that were also positive for *intI1*, *qacEΔ1*, and *aadA1* genes. The phenotypes associated with Tn21, mercury resistance and streptomycin resistance, were also present in several group B and E *Salmonella* isolates (5 of 8) examined. Among the *E. coli* isolates that were positive for *merA*, 86% were resistant to mercuric chloride. To demonstrate that the genes conferring these phenotypes, mercury resistance and streptomycin resistance, are part of a Tn21-like transposon in these isolates, the linkage between *aadA1* and *merA* was determined by Southern analysis. Plasmid DNA was isolated from 14 avian *E. coli* strains positive for *aadA1* and *merA*, digested with the *EcoRI* restriction enzyme, and probed with either the *aadA1* or *merA* probe. The *merA* probe hybridized to two DNA fragments of 9.5 and 7 kb in 7 of 10 avian *E. coli* isolates positive for *mer* as expected, since *EcoRI* cuts in the center of most *merA* genes (Fig. 1B). The second DNA probe for *aadA1* hybridized to a single DNA fragment of 9.5 kb in 7 of 10 avian *E. coli* isolates positive for *intI1*, *aadA*, and *merA*. *merA* and *aadA1* (Fig. 1A) are most likely linked, since both DNA probes recognized the same 9.5-kb *EcoRI* DNA fragment in several of the isolates examined (Fig. 1, lanes 1, 3 to 5, 8, 11, and 12). This evidence lends support to the idea that the incidence of these genes among avian *E. coli* strains is due to the presence of a Tn21-like element. However, these avian *E. coli* isolates do not contain the canonical Tn21 but a derivative. According to the genetic map of Tn21, *aadA* and *merA* sequences are expected to map to a 13-kb *EcoRI* DNA fragment (GenBank accession no. AF071413) (29), not the 9.5-kb *EcoRI* DNA fragment observed in avian *E. coli* strains positive for *intI1*, *aadA1*, and *merA*. The smaller 9.5-kb *EcoRI* DNA fragment suggests a probable deletion of the IS1326 and IS1353 sequences of canonical Tn21 (5, 29) to generate these Tn21-like elements. An additional *EcoRI* restriction enzyme site, present in *tniBΔ1* of the canonical Tn21 (29), is also absent from these Tn21-like sequences in avian *E. coli*.

This study is the first to document the dissemination of Tn21 among an important group of pathogenic avian *E. coli* isolates. The dissemination of mercury resistance in avian *E. coli* appears to be due to Tn21. This element has also been reported in gram-negative clinical isolates associated with illness in humans (62). Resistance to the heavy metal, mercury, is not unprecedented among *E. coli* strains of veterinary significance. Mercury resistance among avian *E. coli* isolates (7), as well as

TABLE 4. Distribution of class 1 integrons and the mercury resistance gene *merA* among clinical avian *E. coli* isolates

Genotype	No. positive (n = 100)
<i>intI1 aadA1 qacEΔ1 merA</i>	50
<i>intI1 aadA1 qacEΔ1</i>	13
<i>intI1 aadA1 merA</i>	4
<i>aadA1 qacEΔ1 merA</i>	1
<i>intI1 aadA1</i>	0
<i>intI1 merA</i>	1
<i>merA</i>	3
<i>aadA1</i>	1
No class 1 integron or <i>mer</i> genes.....	27

FIG. 1. The physical linkage between the integron-associated drug resistance genes *aadA1* and *merA* of Tn21 in avian *E. coli*. The plasmid DNA, cut with restriction enzyme *EcoRI*, was probed for *aadA1* (A) or *merA* (B). Avian *E. coli* strains examined in this study were positive (+) by colony blots for *intI1*, *aadA1*, *merA* (lanes 3 to 5, 7 to 9, 11 to 12, and 16), *int*, and *aadA* (lanes 6, 10, 13, and 17) or negative (-) for all class I integron genes and *merA* of Tn21 (lanes 14 and 15). *E. coli* K-12 with pDU202 served as the positive control (lane 1), while the same *E. coli* K-12 strain without this plasmid served as the negative control (lane 2). Both DNA probes recognized a 9.5-kb DNA fragment (lanes 1, 3, 4, 5, 8, 11, and 12), demonstrating the physical linkage of *aadA1* and *merA*.

in the pathogenic *E. coli* that is associated with disease in other domestic animals (23), has previously been reported, although the genetic determinant responsible for resistance has never been identified. It is currently uncertain what selective advantage the organism may gain from mercury resistance, since mercuric compounds are not used in hatchery disinfectants. One possible source may be the fish meal present in poultry feed, since fish can accumulate toxic compounds, including mercury (6). The use of fish meal in chicken feed varies according to region, availability, and market price (16a). Since MerA mercury reductase does not confer cross-resistance to other heavy metals (46), the selection pressure is probably not due to the arsenic compounds that are added to feed as coccidiostats (37). There is presumably an as yet-unidentified selection pressure that favors Tn21's widespread dissemination among poultry pathogens, since this transposon was also identified among *Salmonella* strains isolated from poultry (data not shown).

The widespread distribution of the transposon Tn21 among clinical isolates is possibly attributable to the conjugative plasmids on which they reside. Many of these avian *E. coli* isolates contain ColV plasmids (60, 61), which may give the bacterial host a selective advantage in its competition with normal flora (8). This transposable element may also be carried by a virulence plasmid, which might explain the dissemination and persistence of Tn21 in avian *E. coli*. Previous examples of physical linkages between antibiotic resistance and heavy-metal resistance and virulence factors on conjugative plasmids in *E. coli* have been described (24, 35). We are currently looking at other factors linked to the same plasmid(s) associated with Tn21, which may explain the continued propagation of this element without obvious selection pressure.

Currently the only antibiotics approved for poultry use and proven effective at combating infection are two fluoroquinolones, enrofloxacin and sarafloxacin. Effectiveness of these drugs may be short lived, as *E. coli* is becoming resistant to quinolones and fluoroquinolones (2, 3, 55, 56). In addition to continuing development of new antimicrobial agents, another key to solving the problem of multiple-antibiotic resistance may be identifying and exploiting conditions unfavorable to the persistence and dissemination of integrons in the bacterial cell.

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