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MULTIDRUG RESISTANCE AND INTEGRONS IN *ESCHERICHIA COLI* ISOLATED FROM CHICKEN IN GREECE

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Enteric faecal flora of food-producing animals such as poultry is a potential reservoir for antimicrobial resistance genes which can be transferred to human pathogens via the food chain. The present study investigated 47 strains of Enterobacteriaceae recovered from a variety of chicken specimens for their resistance to 18 antimicrobial agents and the presence of integrons, and analyzed the association between integrons and antimicrobial susceptibility. Multidrug resistance was found in 82.9% of the isolates. The presence of integrons was shown in 68.1% of the strains tested: 42.5% carried a class 1 integron, 10.6% carried a class 2 integron, and 14.9% had both class 1 and 2 integrons. An unusual cassette aacA4-catB3-dfrA1 was revealed in two class 1 integron-positive isolates. The association between the presence of an integron and multidrug resistance was significant ($p<0.05$). The mercury resistance gene, merA, was found in 44.4% of strains with class 1 integron, indicating the role of Tn21 transposon in dissemination of integrons within the samples studied. The study gives baseline information on the resistance problem and its genetic background in contemporary poultry Enterobacteriaceae in Greece, and suggest the need for the introduction of surveillance programs to monitor antimicrobial resistance that can be potentially transmitted to humans.

Key words: Enterobacteriaceae, integrons, multiresistance, poultry

INTRODUCTION

Antibiotic usage is considered the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant bacteria in both veterinary and human medicine (Miles *et al*., 2006). The acquired resistance occurs not only in pathogenic bacteria, but also in the endogenous microflora of exposed animals and humans (Miles *et al*., 2006). Resistant animal pathogens

may also be a threat to human health if these resistant bacteria enter the food supply or otherwise serve as reservoirs of resistance genes for human pathogens (Miles, *et al*., 2006; Bywater, *et al*., 2004). Because gram-negative bacilli of the family *Enterobacteriaceae* are common agents of food-borne diseases, great attention is focused on antibiotic resistance genes in this group of intestinal bacteria. *Escherichia coli* is a common inhabitant of the gastrointestinal tract of most animals, and in poultry it is known as a cause of extra-intestinal infections such as respiratory tract infections, septicemia and soft tissue infections (Maurer *et al*., 1998). Diseases of poultry caused by *Escherichia coli* result in significant economic losses every year (Maurer *et al*., 1998).

The major agents of gene transfer in the *Enterobacteriaceae* include the conjugative plasmids in which antibiotic resistance genes typically occur within genetic elements such as transposons and integrons. Integrons are genetic units containing elements for site-specific recombination, capture and mobilization of genes, including genes encoding resistance (Ploy *et al*., 2000). The defining gene of an integron is the *intI* gene encoding an integrase, a site-specific recombinase that inserts and removes small DNA cassettes, each encoding an antibiotic resistance gene (Ploy *et al*., 2000). More than 60 distinct resistance gene cassettes have been described (Kang *et al*., 2005). At least six classes of integrons, determined according to their *intI* gene, are known to have a role in the dissemination of antibiotic-resistance genes (Ploy MC *et al*., 2000). Class 1 and class 2 are the most frequently found in members of the family *Enterobacteriaceae* from both clinical isolates and normal flora of food animals, as well as in human clinical specimens (Goldstein *et al*., 2001). These classes are often located within transposable elements, Tn*21* and Tn*7* respectively (Goldstein *et al*., 2001; de la Cruz and Grinsted, 1982; Hall and Collis, 1995).

The purpose of this study was to investigate antimicrobial susceptibility among *Enterobacteriaceae* isolated from healthy and sick broiler chickens, and to investigate the association of reduced susceptibility to antimicrobial agents with the presence of integrons. The study was conducted in the region of Attiki, Greece, and included poultry husbandries belonging to few poultry enterprises that provide the broad market of Athens with fresh and frozen chickens.

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility testing

Forty-seven *Enterobacteriaceae* isolates were recovered from healthy (29) and sick (18) broiler chickens grown on 13 commercial broiler poultry farms in Greece over a three-month period (December 2005 through February 2006). The farms are situated in neighbouring regions belonging to three Prefectures, namely Evia, Viotia and Attiki. Feeding with addition of anticoccidioides is in use on all farms, and together with water supply, animal health and husbandry conditions are under control of the Central Veterinary Service of Chalkis (CVSC). Isolates were obtained from a variety of specimens including stool, liver, lower intestine, and gastrointestinal fluid. Specimens were taken either in cage or slaughterhouse from healthy chicken or after diseased chickens were sacrificed. All the isolates,

belonging to the family *Enterobacteriaceae*, were identified to the species level by a commercial biochemical identification kit API 20E (bioMérieux, France). Antimicrobial sensitivity profiles of the isolates were established by the disk diffusion method in accordance with the recommendations given by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2006). The isolates were tested for susceptibility to 18 antibiotics listed in Table 1. *Escherichia coli* ATCC 25992 was used as the control strain. All strains displaying intermediate susceptibility were considered to be resistant.

Typing with random amplification of polymorphic DNA (RAPD)

RAPD by polymerase chain reaction (PCR) was used as a rapid screening method to identify genetic differences among *Escherichia coli* isolates and to exclude potential repeated isolates as previously described (Maurer *et al*., 1998). Samples were distinguished by considering data from one PCR with the primer AP1290, 5'-CTGGATGCGA-3'. For each PCR, 5 μL DNA was added to a reaction mix containing 10 pmol of primer, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U Taq polymerase, made up to a total volume of 50 μL. Target sequences were amplified by PCR consisting of a 4 min denaturation step at 94°C, followed by an initial amplification of 4 cycles of 1 min at 94°C, 1 min at 26°C and 1 min at 72°C. A second round of amplification, consisting of 40 cycles of 45 s at 94°C, 45 s at 40°C, and 2 min at 72°C, was used.

Bacterial DNA extraction and PCR amplification of integrase genes

Bacterial DNA was extracted by the Qiagen mini prep kit (Qiagen, Germany). Integrons were detected using PCR with degenerate primers designed to hybridize to conserved regions of integron-encoded integrase genes *intI1, intI2* and *intI3* as previously described (White *et al*. 2001). The class of the integron was determined by analysing integrase PCR products by restriction fragment length polymorphism (RFLP) following digestion using *Rsa*I restriction enzyme as previously described (White *et al*., 2001). Previously described *Escherichia coli* strains with and without *intI1* and *intI2* were used as the positive and negative control strains.

Amplification and sequencing of gene cassette regions

Primers used to amplify class 1 integron cassette regions were: IL1 (5'-GGCATCCAAGCAGCAAG-3') and IL2 (5'-AAGCAGACTTGACCTGA-3') as described previously (*Levesque C, Roy PH*, 1993). Class 2 integron cassette regions were amplified using hep74 (5'-CGGGATCCCGGACGGCATGC ACGATTTGTA-3') and hep51 (5'-GATGCCATCGCAAGTACGAG-3') as described previously (White *et al*., 2001). PCR amplifications were carried out in 50 μL reaction mixtures containing 3 μL of purified DNA, 10 pmol of each oligonucleotide primer, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U Taq polymerase. All PCR reactions were performed for 35 cycles, each cycle consisted of 94°C for 1 min, 55°C for 1 min and extension at 72°C for 1 min for amplification of the integrase

genes, or 4 min for amplification of the cassette regions. Sequencing of the cassettes was performed at Lark Technologies, Essex, United Kingdom, after extracting, purifying and cloning of the amplicons using the Qiagen Cloning kit (Qiagen, Germany).

*Amplification and sequencing of mer*A *gene*

Detection of mercuric reductase gene *mer*A, as a marker for the presence of transposon Tn*21*, was performed by PCR amplification using the protocol previously described by Bass *et al*. (1999). Sequencing of the amplicon was performed after extracting, purifying and cloning of the amplicon using the Qiagen Cloning kit (Qiagen, Germany). Previously described *Escherichia coli* strains with and without *mer*A gene were used as the positive and negative control strains.

Statistical methods

The significance of association of the presence of integrons and susceptibility to different antimicrobials was analyzed by using χ^2 test.

RESULTS

Characterisation of the isolates

Out of 47 isolates of enteric bacteria recovered from the examined chicken samples, 43 were identified as *Escherichia coli*, one as *Salmonella* Typhimurium, one as *Enterobacter sakazakii*, and two as *Proteus mirabilis.* Based on RAPD patterns, 26 *E. coli* analyzed strains appear to be genetically diverse, and fell into three major RAPD types and several unique types.

Incidence of integrons, Tn*21 and resistance patterns*

All of the isolates were screened for the presence of integrase genes *intI1, intI2*, and *intI3*, resulting in 491 bp amplicons. Thirty-two strains were *intI*-PCR positive, indicating the prevalence of integron-containing isolates in the collection examined of 68.1%. *IntI1* amplicon does not contain an *Rsa*I restriction site and remains intact after digestion. *IntI2* amplicon contains one restriction site producing two restriction fragments of 334 and 157 bp, whereas *intI3* contains two restriction sites producing three fragments of 290, 104 and 97 bp (White *et al*., 2001). Amplification and *Rsa*I digestion analyses revealed that 20 (42.5%) strains carried a class 1 integron, five strains (10.6%) carried a class 2 integron, and seven strains (14.9%) had both class 1 and 2 integrons. No class 3 integron-positive isolates were detected. In total, 27 class 1 integrons and 12 class 2 integrons were identified in 32 of the 47 isolates.

The amplification of the *mer*A gene, which is considered a marker for the presence of transposon Tn*21,* resulted in a 1230 bp fragment. Since non-specific PCR amplification products have been detected by agarose gel electrophoresis, sequencing was performed to confirm the presence of the mercury reductase A gene. Out of 20 strains carrying class 1 integron and seven carrying both classes 1 and 2, nine and three strains were positive for *mer*A respectively. In total, 44.4% were positive for *mer*A.

Out of 47 isolates, 39 (82.9%) exhibited multidrug resistance, i.e. resistance to three or more antimicrobial agents with different mechanism of action. Most *E. coli* isolates recovered from healthy as well as from sick chicken were resistant to multiple classes of antimicrobials. Regardless of integron carriage high rates of resistance were found for the following antibiotics: ampicillin, cotrimoxazole and trimethoprim (74.5%, 35 strains), sulfamethoxazole (53.2%, 25 strains), tetracycline (46.8%; 22 strains), nalidixic acid (38.3%; 18 strains), and streptomycin (29.8%, 14 strains).

Table 1. Association between antibiotic resistance and integrons in 47 isolates of *Enterobacteriaceae*

Antibiotic	% Resistance of 32 *int*-positive isolates (no. of resistant isolates)	Association with integron*	% Resistance of 15 *int*-negative isolates (no. of resistant isolates)	% Resistance of total (no. of resistant isolates)
ampicillin	75% (24)	0.832	73.3% (11)	74.5% (35)
amoxicillin-clavulanic acid	12.5% (4)	0.950	6.7% (1)	10.6% (5)
piperacillin	6.2% (2)	0.825	(0)	4.2% (2)
aztreonam	3.1% (1)	0.499	13.3% (2)	6.4% (3)
cefoxitin	12.5% (4)	0.950	6.7% (1)	10.6% (5)
cefotaxime	6.2% (2)	0.609	6.7% (1)	6.4% (3)
streptomycin	37.5% (12)	0.17	13.3% (2)	29.8% (14)
gentamicin	3.1% (1)	0.668	(0)	2.1% (1)
tobramycin	3.1% (1)	0.668	(0)	2.1% (1)
kanamycin	28.1% (9)	0.059**	(0)	19.1% (9)
cotrimoxazole	87.5% (28)	0.009	46.7% (7)	74.5% (35)
sulfamethoxazole	62.5% (20)	0.120	33.3% (5)	53.2% (25)
trimethoprim	87.5% (28)	0.009	46.7% (7)	74.5% (35)
chloramphenicol	28.1% (9)	0.059**	(0)	19.1% (9)
ciprofloxacin	12.5% (4)	0.415	26.7% (4)	17% (8)
enrofloxacin	3.1% (1)	0.887	6.7% (1)	4.2% (2)
nalidixic acid	43.7% (14)	0.427	26.7% (4)	38.3% (18)
tetracycline	59.3% (19)	0.027	20% (3)	46.8% (22)

*Significant values are in bold

**Kanamycin and chloramphenicol (0.059) are only slightly above 0.05 significance limit

The susceptibilities of integron-positive and integron-negative isolates, and the association between the presence of integrons and reduced susceptibility to antimicrobials are presented in Table 1. Out of 39 multiresistant isolates, 30 (76.9%) were integron-positive, while nine (23%) were integron-negative. The association between the presence of integrons and multidrug resistance was

significant $p<0.05$ ($p=0.013$). Namely, among 15 integron-negative isolates, nine (60%) were multidrug resistant while 30 out of 32 integron-positive strains (93.7%) displayed multidrug resistance. In addition, resistance to tetracycline (59.3%; 19 strains) and trimethoprim (87.5%; 28 strains) was also significantly associated with the presence of integrons, $p=0.027$ and $p=0.009$ respectively. Resistance rates to kanamycin and chloramphenicol were slightly above the significance limit ($p=0.059$) for the association with integrons.

Characterization of cassette arrays

Out of 27 class 1 integron-positive isolates, cassettes were identified in 21 integrons. The cassette regions of six remaining class 1 integrons could not be amplified by PCR, possibly due to the lack of a hybridization site. Class 1 integrons harboured different cassette arrays from 1000 bp to 2700 bp comprising of *aadA1, aadA2, aadA5, dfrA1, dfrA12, dfrA17*. The most common types of cassettes carried by class 1 integrons were those conferring resistance to streptomycin and spectinomycin. These cassettes represented 53.7% of all cassettes found and included *aadA1* (39% of cassettes), *aadA2* (4.9%), and *aadA5* (4.9%). *dfr* cassettes (*dfrA1*, *-A12*, *-A17*) that confer resistance to trimethoprim represented 41.5% of cassettes detected. The most common combination was *aadA1, dfrA1* (52.4 %; 11 strains) conferring resistance to both streptomycin and trimethoprim. Five strains (23.8%) contained a single antibiotic resistance gene, *aadA1* (streptomycin-spectinomycin resistance). An unusual cassette *aacA4-catB3-dfrA1* was revealed in two isolates, one carrying class 1 integron and one carrying both class 1 and 2 integrons, within a cassette of 2700 bp. Analyses of 12 class 2 integrons identified cassettes of ~1700 bp in five strains and 2000 bp in four strains, but sequencing data are not available. The cassette regions of three class 2 integrons generated no amplicons probably due to rearrangement or absence of a primer hybridization site.

DISCUSSION

The present study investigated 47 isolates of *Enterobacteriaceae* from broiler chickens with respect to their antimicrobial resistance and the presence of integrons as a potential basis for this resistance.

The overall data, including the result obtained in our study, show that antibiotic resistance among avian *Enterobacteriaceae* isolates is common and is of great concern to the poultry industry (Kang *et al.* 2005; Bass *et al*., 1999; Yang *et al*., 2004; Singh *et al*., 2005). This has also been shown in our study since over 80% of strains tested exhibited multidrug resistance.

A number of studies investigated the occurrence of integrons in selected populations of avian isolates of gram-negative bacilli (Bywater *et al*., 2004; Bass L *et al*., 1999; Johnson *et al*. 2005; Saenz *et al*., 2004), and prevalence ranging from 16% to 63% were reported (Kang HY *et al*., 2005; Bass *et al*., 1999; Yang *et al*., 2004; Singh *et al*., 2005). As far as rates of different classes of integrons are concerned, class 1 integrons appear to be prevalent in nature (Goldstein *et al*., 2001). The clear predominance of class 1 integrons we found is consistent with

the results of previous studies (Kang *et al*., 2005; Goldstein *et al*., 2001; Bass *et al*., 1999; Yang *et al*., 2004; Kang *et al*., 2005) which also showed the highest frequency of occurrence of this class in poultry isolates. The dissemination of class 1 integrons has been attributed to the spread of a large (19.7 kb) integron-containing transposon Tn*21* (Goldstein *et al*., 2001; Bass *et al*. 1999). In addition to drug resistance, Tn*21* confers mercury resistance through its mercuric reductase gene, *mer*A (Bass *et al.*, 1999). We searched for the presence of this gene in all strains containing class 1 integron and looked into the possibility that integrons in our isolates are actually part of Tn*21*. The gene was detected in 44.4% of the strains carrying class 1 integron, which indicates that at least a part of class 1 integrons spread within our isolates is related to Tn*21*. One possible explanation for this finding is the presence of a truncated derivative or a Tn*4*, a Tn*21* derivative which does not confer mercury resistance because Tn*3* has inserted into and disrupted the *mer* locus of Tn*21* (Bass *et al*., 1999). The low incidence of class 2 integrons in the present study was similar to the distribution of this class in previously examined veterinary (Goldstein *et al*., 2001) and human urinary isolates (Chang CY *et al*., 2000). The results do not provide the basis for conclusions regarding the distribution of integrons among different bacterial species, since there were only four isolates of species other than *Escherichia coli*.

Many studies have shown a correlation between the presence of integrons and multiple-antibiotic resistance phenotype in enteric bacteria, with the class 1 integrons being the most prevalent (Singh *et al*., 2005; Fluit and Schmitz, 2004; Martinez-Freijo *et al*., 1998; Leverstein van-Hall *et al*., 2003; Grape *et al*., 2005; Mathai *et al*., 2004). This has also been shown in our study. Nevertheless, although the association between multidrug resistance and the presence of integrons was significant, 60% of integron-negative strains were multidrug resistant. This result indicates that the presence of integrons represents only one among many factors influencing the development of multidrug resistance. Several other factors promoting the emergence, selection and dissemination of resistant enteric strains have also been identified such as antibiotic usage, incomplete therapy, use of nontherapeutic antimicrobial growth promotants as feed additives for poultry (Miles *et al*., 2006), inappropriate use of disinfectants in farm environments (Randall *et al*., 2005), and mutations in the *mar* (multiple-antibiotic-resistant) locus regulation (Saenz *et al*., 2004). On the farms included in the present study, antibiotics are administered not only to individual diseased chicken but at the same time to the flock prophylactically which is considered potentially infected. This acquired resistance occurs not only in pathogenic bacteria but also in the intestinal microbial flora of exposed animals that can be passed onto people via food or through direct contact with animals (Miles *et al*., 2006).

It has been proposed that integrons are significantly associated with resistance to the older antibiotics (White *et al*., 2001; Fluit, 2004; Mathai *et al*., 2004). This was in part confirmed by results of the present study as indicated by elevated levels of resistance in integron-positive strains towards antibiotics such as chloramphenicol, tetracycline, ampicillin or kanamycin (Table 1). A high percentage of isolates were also resistant to antifolates (cotrimoxazole, sulfamethoxazole and trimethoprim). In contrast, only two isolates have exhibited resistance to enrofloxacin, a fluoroquinolone antibiotic approved for poultry use.

The most prevalent types of cassettes carried by class 1 integrons were those conferring resistance to streptomycin and spectinomycin, and/or trimethoprim. This finding is in agreement with the high prevalence of these genes found in *Enterobacteriaceae*, including those isolated from human clinical specimens (Kang HY *et al*. 2005; White *et al*., 2001; Bass L *et al.* 1999; Yang *et al*., 2004). These aminglycosides are seldom used therapeutically, yet *aadA* gene cassettes remain prevalent within integrons and could demonstrate a form of genetic memory if reexposed to these antibiotics (White *et al*., 2001). Persistence of a specific resistance gene also occurs in human populations after decades without exposure to that antibiotic (Nandi *et al*., 2004), and it has been proposed that the likely basis for this persistence is physical linkage of genes for resistance to an older antibiotic with genes for resistance to a currently used antibiotic (Nandi *et al*., 2004). On the other hand, the high prevalence of *dfr* cassettes might be attributed to the usage of trimethoprim in human medicine or the related veterinary analogue ormetoprim (White *et al*., 2001). Resistance gene cassettes were similarly abundant in strains isolated from all the farms included in the study. This result is consistent with results of previous studies investigating the incidence of genes associated with integrons among avian enteric bacteria, showing that a high prevalence of integron-related genes is not limited to farms using antibiotic growth promotants (Nandi *et al*., 2004). It is noteworthy that sequencing of two 2700 bp class 1 integrons revealed an arrangement of gene cassettes *aacA4-catB3-dfrA1* that has recently been described as a novel rearrangement in the class 1 integron in a *Pseudomonas aeruginosa* respiratory isolate (Li X *et al*., 2006). The explanation for the acquisition of this cassette type by the two species of unrelated genera and ecological niche does not seem evident.

In conclusion, the present study showed relatively high prevalence of integrons in isolates of enteric bacteria obtained from broiler chickens on Greek farms, and their significant association with reduced susceptibility to a range of antibiotics. These results further confirm the potential of integrons to contribute to development of resistance in *Enterobacteriaceae*. Their potential for transfer of antimicrobial resistance from enteric zoonotic bacteria of food animals to the human population is a cause of concern. It is, therefore, essential to continuously monitor bacterial susceptibility to antimicrobials and to study temporal trends among isolates from healthy animals. It is desirable that such studies should be repeated in the future years.

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MULTIREZISTENCIJA I INTEGRONI KOD SOJEVA BAKTERIJE *ESHERICHIA COLI* IZOLOVANIH KOD BROJLERA U GRČKOJ

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SADRŽAJ

Crevna mikroflora životinja koje se koriste u ishrani ljudi, uključujući i crevnu mikrofloru živine, predstavlja potencijalni rezervoar gena rezistencije na antibiotike za bakterije koje su humani patogeni. U toku ove studije je iz različitih uzoraka uzetih od 29 zdravih i 18 bolesnih brojlera izolovano ukupno 47 sojeva *Enterobacteriaceae* od kojih je čak 43 identifikovano kao *Escherichia coli*. Kod svih izolata ispitana je osetljivost na 18 antibiotika, prisustvo integrona, kao i moguća povezanost integrona i rezistencije na antibiotike. Preko 80% izolata (82,9%) je bilo multirezistentno. Integroni su bili prisutni kod 68,1% izolata i to: integroni klase 1 kod 42,5%, integroni klase 2 kod 10,6%, a obe klase kod 14,9% sojeva. Treba istaći da je kod dva izolata sa klasom 1 integrona ustanovljeno prisustvo kasete *aacA4-catB3-dfrA1,* koja je do sada opisivana samo kod vrste *Pseudomonas aeruginosa*. Statistička analiza pokazala je značajnu povezanost prisustva integrona i multirezistencije ($p<0,05$). Gen *mer*A, odgovoran za rezistenciju na živu, detektovan je kod 44,4% izolata sa klasom 1 integrona, što ukazuje na ulogu Tn*21* transpozona u diseminaciji integrona unutar ispitivane grupe izolata. Ova studija pružila je prvi uvid u problem rezistencije i genetičku osnovu rezistencije izolata enetrobakterija kod živine u Grčkoj. Dobijeni rezultati ukazuju na potrebu uvođenja programa kontinuiranog praćenja rezistencije ovih bakterija, s obzirom na postojanje mogućnosti za transfer njihovih gena rezistencije na humane patogene.