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► **To cite this version:**

Noémi Nógrády, Judit Pászti, Henriett Pikó, Béla Nagy. Class 1 integrons and their conjugal transfer with and without virulence associated genes in extra-intestinal and intestinal *Escherichia coli* of poultry. *Avian Pathology*, 2006, 35 (04), pp.349-356. 10.1080/03079450600827007 . hal-00540055

HAL Id: hal-00540055

<https://hal.science/hal-00540055>

Submitted on 26 Nov 2010

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Class 1 integrons and their conjugal transfer with and without virulence associated genes in extra-intestinal and intestinal Escherichia coli of poultry

Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2005-0002.R3
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	20-May-2006
Complete List of Authors:	Nógrády, Noémi; National Centre for Epidemiology, Department of Phage-typing and Molecular Epidemiology Pászti, Judit; National Centre for Epidemiology, Department of Phage Typing and Molecular Epidemiology Pikó, Henriett; Institute of Environmental Health, National Center for Public Health, Department of Molecular Genetics and Diagnostics Nagy, Béla; Veterinary Medical Reserach Institute of the Hungarian Academy of Sciences, Enteric Bacteriology and Foodborn Zoonoses
Keywords:	antibiotic resistance, class 1 integrons, virulence genes, Escherichia coli

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CAVP-2005-0002 Revised-3

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Class 1 integrons and their conjugal transfer with and without virulence

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Short running head: Class 1 integrons in intestinal and extra-intestinal avian *E. coli*

Received: 28 November 2005

Deleted: Keywords: antibiotic resistance, class 1 integrons, virulence genes, *Escherichia coli*¶

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21 CAVP-2005-0002 Revised-3

22 Class 1 integrons and their conjugal transfer with and without virulence
23 associated genes in extra-intestinal and intestinal *Escherichia coli* of poultry

24
25 Noémi Nógrády,¹ Judit Pászti¹, Henriett Pikó² and Béla Nagy^{3*}
26

27 **Abstract**

28
29 Multiple-drug resistance in enteric bacteria is frequently associated with integrons. To
30 determine whether integrons may play a role in avian pathogenic *Escherichia coli*, isolates of
31 extra-intestinal (n=27) and intestinal (n=40) *E. coli* from dead chicks and turkey poult were
32 analysed for the presence of class 1 integrons and of the virulence-associated genes *iss*, *tsh*
33 and *colV*. Eleven extra-intestinal strains possessed a 1.0 kb class 1 integron with a variable
34 region of *aadA1* and were resistant to tetracycline. These traits were indicative of the presence
35 of the Tn21 transposon, which was confirmed by PCR. All extra-intestinal strains had the
36 *colV*, *iss* and *tsh* genes, but none of these genes were cotransferred with the 1.0 kb integron
37 when conjugal transferability was tested. The integron content of the intestinal strains showed
38 considerable variability: one or two of four different class 1 integrons, which varied from 1.0
39 kb to 2.4 kb in size, were detected in the eleven strains. The *aadA7* gene of the 1.0 kb
40 integron, the *dfrA1-aadA1* genes of the 1.6 kb integron and the *folA-catB3-aadA5* genes of the
41 2.4 kb integron were identical to those described by other workers. However, the *orfN682-*
42 *dhfrV-orfD* gene cassette arrangement of the 1.5 kb integron of an intestinal strain of
43 serogroup O5 had no similarity to any previously reported integrons. Conjugal transfer of the

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44 1.6 kb and 2.4 kb integrons was successful, and in a serogroup O33 intestinal *E. coli* strain the
45 *iss* gene was apparently cotransferred with a 1.6 kb integron. The 1.0 kb and the 1.5 kb
46 integrons were not transferable. Our data suggest that intestinal *E. coli* strains of poultry may
47 be a reservoir for emerging multiresistant strains of avian pathogenic *E. coli*.

For Peer Review Only

48 **Introduction**

49

50 Avian pathogenic *Escherichia coli* (APEC) comprise a specific subset of pathogenic *E. coli*
51 that cause extra-intestinal diseases such as air sacculitis, pericarditis or septicaemia in poultry
52 (Barnes *et al.*, 2003). These diseases have traditionally been treated with antimicrobials,
53 which, in turn, has led to an increasing prevalence of antimicrobial resistant *E. coli* strains.
54 Multiple-drug resistance in enteric bacteria is frequently associated with integrons. Integrons
55 were initially described as mobile elements that contain one or more antimicrobial resistance
56 genes at a specific site and also the determinants for the site-specific recombination system
57 responsible for insertion of the resistance genes (Stokes & Hall, 1989). Later integrons
58 without inserted genes were also described. A minimal integron consists of an integrase gene
59 (*intI*), encoding a site-specific recombinase, and an adjacent recombination site, *attI*. However
60 integrons usually also contain one or more gene cassettes at *attI*. The gene cassettes contain a
61 recombination site *attC* (also referred to as the 59 bp element) and an antimicrobial resistance
62 determinant or some other gene. Thus, together integrons and gene cassettes constitute a two-
63 component site-specific recombination system for gene acquisition and dissemination, in
64 which gene cassettes are the mobile elements and integrons are the recombinase-encoding
65 master elements for integration or excision of the cassettes (Recchia & Sherratt, 2002). On the
66 basis of the amino acid sequence of the recombinase five distinct integron classes have been
67 described (Collis *et al.*, 2002), the best characterized and the most frequently detected of
68 which are the class 1 integrons (also referred to as type 1 or *sulI* type integrons) (Hall &
69 Collis, 1998). They consist of three main parts. The 5' conserved segment (5'CS) always
70 contains the integrase (*intI*), its promoter (P_i) and the promoter which is responsible for
71 transcription of the inserted cassettes (P_{ant}). The variable region contains the gene cassette(s):
72 the antimicrobial gene(s) and the *attC* site(s). The 3' conserved segment (3'CS) always

73 contains a *qacEAI* gene (encoding resistance to quaternary ammonium compounds) and a
74 *sulII* type gene (encoding resistance to sulphonamides). The detection of the variable region
75 of a class 1 integron by PCR is based on the use of primers binding to the 5' and 3' conserved
76 segments (Lévesque *et al.*, 1995). Class 2 integrons can also contribute to the spread of
77 antimicrobial resistance genes (Goldstein *et al.*, 2001). The remaining integron classes are not
78 well characterised and have only been detected in single isolates. Bass *et al.* (1999) examined
79 extra-intestinal *E. coli* isolated from chickens, turkeys and quail and found a high prevalence
80 of a 1.0 kb class 1 integron containing the *aadAI* gene cassette, which codes for resistance to
81 streptomycin and spectinomycin. They also confirmed that the majority of these integrons
82 were part of a Tn21-like transposon. Similarly, a high prevalence of class 1 integrons has
83 been found in extra-intestinal *E. coli* in China (Yang *et al.*, 2004). However, no such study
84 has been conducted to examine integron carriage by intestinal *E. coli* of poultry. The aim of
85 this study was to compare the antimicrobial resistance and class 1 integron content of *E. coli*
86 isolates from the caeca of young chicks and turkeys that died of diseases unrelated to *E. coli*
87 infection to isolates of extra-intestinal *E. coli* from chicks and turkeys from the same farms.
88 Some virulence-associated genes of APEC, such as *colV* (colicin V), *iss* (increased serum
89 survival) and *tsh* (temperature sensitive haemagglutinin), have been found in an avian *E. coli*
90 isolate on a conjugative R-plasmid that also codes for the *intI1* gene, the integrase of the class
91 1 integrons (Johnson *et al.*, 2002). Therefore the presence and possible transferability of these
92 genes with the class 1 integrons was also investigated.

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95 **Materials and Methods**

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97 **Bacterial strains, serogroup determination and antimicrobial susceptibility testing.** A

98 total of 67 *Escherichia coli* isolates were used: 27 from the lung, liver or bone-marrow (extra-
99 intestinal organs - EX) of chicks and turkey poults between a few days and a few weeks of age
100 that had died of colibacillosis; and 40 from the caecal contents (intestine - IN) of chicks and
101 turkey poults between a few days and a few weeks of age that had died on the same farms, and
102 were epidemiologically unrelated to the birds from which the extra-intestinal isolates were
103 obtained. Only a single isolate was included from each bird. All *E. coli* isolates were selected
104 from cultures on 5% sheep blood agar and bromothymol blue (BTB) agar (Merck, Darmstadt,
105 Germany) and confirmed as *E. coli* using standard biochemical tests. The isolates were stored
106 at -70°C . Serotyping was carried out using standard tube agglutination techniques (Ørskov &
107 Ørskov, 1984) with 174 O-specific monovalent sera. Antimicrobial susceptibility testing of all
108 isolates was done using the disk diffusion method on Mueller-Hinton agar and with the
109 following antimicrobial disks (Oxoid Ltd., Basingstoke, UK): ampicillin (A, 10 μg),
110 chloramphenicol (C, 30 μg), streptomycin (S, 10 μg), tetracycline (Te, 30 μg), gentamicin
111 (Gm, 10 μg), kanamycin (Km, 30 μg), sulphamethoxazole-trimethoprim (SxT, 25 μg),
112 cefotaxime (Ctx, 30 μg), nalidixic acid (Nx, 30 μg) and ciprofloxacin (Cip, 5 μg). The zones of
113 growth inhibition were evaluated according to the Clinical and Laboratory Standards Institute
114 (CLSI, 2005) recommendations. *E. coli* strain ATCC 25922 was used as a reference strain in
115 antimicrobial susceptibility disk diffusion testing.

116

117 **Bacterial DNA preparation, PCR and DNA sequencing.** Bacterial DNA used for PCR was

118 prepared by boiling a bacterial culture in 200 μl F1 buffer (20 mM Tris-HCl, 2 mM EDTA,

119 pH=8.0, Triton X-100, 0.5%) for 10 minutes. All strains were tested for the presence of class 1
120 integrons by PCR using primers specific for the 5' (forward primer) and the 3' (reverse primer)
121 conserved segments of the class 1 integrons (Lévesque *et al.*, 1995; Table 1.) bordering the
122 variable region. As the number and size of the inserted gene cassettes within the variable
123 region of an integron may vary, the size of the amplicons was expected to be variable. The
124 integron-positive strains were further tested by PCR for the presence of the Tn21 transposon,
125 the mercury resistance gene *merA*, the tetracycline resistance genes *tetA* and *tetB*, and the
126 virulence-associated genes *colV* (colicin V), *iss* (increased serum survival) and *tsh*
127 (temperature sensitive haemagglutinin). The sequences of the primers, the expected size of the
128 PCR products and references for the PCR protocols are summarized in Table 1. Amplifications
129 were performed in an iCycler thermocycler (Bio-Rad Laboratories, CA, USA). The PCR
130 products were detected by horizontal agarose gel electrophoresis at 10 V/cm in 2% agarose
131 gels (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and Tris-acetate/EDTA buffer. The
132 pGEM marker (Promega Corp., MA, USA) was included as a molecular ruler. The gels were
133 stained in ethidium bromide solution and evaluated using the Bio-Rad Gel Documentation
134 system and Quantity One Software (Bio-Rad).

135 | Partial sequencing of selected integrons was carried out on purified PCR amplicons
136 | (QIAquick Gel Extraction Kit, Qiagen Inc., Hilden, Germany) obtained from both the donor
137 | strains and from their transconjugants using the dye-terminator method and a DNA sequencer
138 | (ABI Prism 373). The primers 5'CS and 3'CS were used for sequencing from each end of the
139 | amplicons. In addition, for the 1.5 kb, 1.6 kb and 2.4 kb amplicons, internal primers were also
140 | used to identify the resistance genes inserted in the amplicon. The sequences were compared to
141 | those in GenBank using BLAST at <http://www.ncbi.nih.gov> .

142 |

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143 **Nucleotide sequence accession number:** The partial sequence of the 1.5 kb integron in the
144 serogroup O5 intestinal *E. coli* strain (InEc682) was deposited in the EMBL nucleotide
145 sequence database under the accession number AM231806.

146
147 **Plasmid preparation.** Plasmids were prepared using the alkaline lysis method of Kado & Liu
148 (1981). Agarose gel electrophoresis was performed in 0.75% agarose in a vertical system
149 (Meyers *et al.*, 1976). The approximate sizes of plasmids were estimated by comparing them
150 with the plasmids of *E. coli* V517 (2.0-53.7 kb) and *E. coli* R27 (168 kb) using Quantity One
151 software. For each plasmid, mean sizes were calculated from three independent purifications
152 and electrophoretic gels.

153
154 **Conjugal transfer of plasmids harbouring class 1 integrons.** The strains that were selected
155 for integron sequencing were also used as donors for testing for the possible conjugal transfer
156 of these integrons. The rifampicin (Rif) resistant, plasmidless *E. coli* K12 J-53 strain was used
157 as a recipient in these conjugation experiments. Overnight cultures of the donor and the
158 recipient cells in Luria-Bertani (LB) broth were mixed at a ratio of 1:1 and plated onto the
159 surface of LB plates. Transconjugants were selected on LB agar containing streptomycin,
160 tetracycline or trimethoprim (depending on the resistance of the donor strain) and rifampicin,
161 in final concentrations of 20 µg/ml, 30 µg/ml, 50 µg/ml and 150 µg/ml, respectively. From
162 each conjugation experiment 3-5 colonies suspected to be transconjugants were tested for the
163 transferred antimicrobial resistance plasmid(s) and Tn21, *tetA*, *colV*, *iss* and *tsh* genes as
164 described above.

165
166 **Southern hybridisation.** In order to identify which of the transferred plasmids contained the
167 1.0 kb integron, the plasmids of the EX201 isolate and its transconjugant were probed with

168 the ³²P-labelled 1.0 kb integron. Similarly, the plasmids of the IN664 isolate and its
169 transconjugant were probed with the ³²P-labelled 2.4 kb integron. For the labelling reactions,
170 PCR amplicons obtained from the TC201 and TC664 transconjugants and purified from 2%
171 agarose gels (Agarose Gel DNA Extraction Kit, Roche Diagnostics GmbH, Mannheim,
172 Germany) were used as specific DNA probes. Labelling was performed by random priming
173 using a Megaprime kit (Amersham/Pharmacia Biotech., Buckingham, Hampshire, U.K.) to
174 label 25 ng DNA with 0.8 MBq ³²P-dCTP (220 TBq/mmol) (Feinberg & Vogelstein, 1983).
175 The 12.5 µl reaction volume contained 2 µl of the primer solution (random nonamer primers
176 in an aqueous solution), 2 µl of DNA and 8.5 µl H₂O were boiled for 5 min, then 5 µl
177 labelling buffer (dATP, dGTP and dTTP) and 1 µl Klenow polymerase was added, followed
178 by 2 µl ³²P-dCTP. The labelled probe was added to the hybridisation solution (0.5 M
179 NaH₂PO₄/Na₂HPO₄, 7% SDS). Plasmid DNA isolated from EX201, TC201, IN664 and
180 TC664 was transferred from the agarose gel to a Hybond-N+ membrane (Roche Diagnostics)
181 in 10 x SSC buffer using a Vacuum Blotter (Model 785, Bio-Rad) for 1.5 hours. Transferred
182 plasmid DNA was fixed to the membrane by exposure to UV light for 10 min. The Hybond-
183 N+ membrane was incubated in the hybridisation solution overnight at 65°C in a
184 hybridisation oven. Following hybridisation, the membrane was washed twice for 15 min in
185 washing solution I (2 x SSC, 0.1% SDS), twice for 10 min in washing solution II (1 x SSC,
186 0.1% SDS) and once for 30 min in washing solution III (0.3 x SSC, 0.1% SDS). The
187 membrane was rinsed in 2 x SSC, wrapped in plastic film and exposed to autoradiographic
188 film overnight at -70°C. The film was photographed using the Bio-Rad Gel-Documentation
189 system.

190

191

192 **Results**

193
194 **Multi-drug resistance, integron-associated and virulence-associated genes of the extra-**
195 **intestinal isolates carrying class 1 integrons.** O types, the size of the class 1 integrons and
196 the results of assays for virulence-associated genes in the extraintestinal *E. coli* isolates are
197 summarized in Table 2. All strains were resistant to nalidixic acid and, with one exception, to
198 tetracycline, with the tetracycline resistance determined by the *tetA* gene. In addition to *tetA*,
199 the EX764 isolate also carried the *tetB* gene. Most of the isolates were resistant to
200 streptomycin, but most of the EX isolates were resistant to less than 4 antimicrobials (Table
201 3). Class 1 integrons were detected in 11/27 EX isolates (41%). All the fragments amplified
202 by the 5'CS-3'CS primers were 1.0 kb in size. As there was little variation between the
203 isolates one (EX201) was chosen for integron sequencing. Sequencing of the amplicon
204 obtained from isolate EX201 revealed that it contained the *aadA1*, which encodes resistance
205 to streptomycin and spectinomycin. The sequence had 99% identity with those of the *aadA1*
206 genes in GenBank (Accession numbers X12870.1 and AB188267.1). All integron positive EX
207 isolates yielded products of the expected size in PCRs specific for the Tn21 transposon. All
208 but one of the EX isolates that harboured integrons belonged to serogroup O78, and carried
209 the virulence-associated genes *colV*, *iss* and *tsh*.

210
211 **Multi-drug resistance, integron-associated and virulence-associated genes of the**
212 **intestinal isolates carrying class 1 integrons.** As suggested by their more variable
213 resistance patterns and generally greater spectrum of resistance (Tables 2 and 3), the intestinal
214 isolates had more variable integron patterns than the EX isolates (Figure 1 and Table 2).
215 Eleven IN isolates (28%) harboured one or two of the 1.0, 1.5, 1.6 or 2.4 kb class 1 integrons.
216 The integron positive isolates belonged to four serogroups, O5 (1), O25 (1), O33 (1) or O86
217 (1), or were O-nontypable (ONT) (7). One integron of each size was sequenced. Sequencing

218 of the 1.0 kb integron from isolate IN305 revealed the presence of the *aadA7* gene, the
219 sequence of which had 100% identity with that of the gene in GenBank (Accession number
220 AF224733), which encodes streptomycin and spectinomycin resistance. The 1.6 kb integron
221 obtained from isolates IN306 and IN674 contained the *dfrA1* and *aadA1* genes, which encode
222 resistance to trimethoprim and streptomycin/spectinomycin, respectively. The sequences had
223 99 % identity with that of the integron in GenBank (Accession number AJ884723.1). Isolate
224 IN682 contained a single 1.5 kb integron. It contained three genes. The open reading frame
225 (*orf*) at the 5' end (*orfIN682*) was 309 bp and had no similarity to any previously reported
226 sequences. The *orf* at the 3' end was 291 bp and had 100% sequence identity with *orfD* of
227 InS21 (Accession number AM040709). Between these two orfs lay a *dhfrV* gene, encoding
228 trimethoprim resistance, with 100% identity with GenBank Accession number AJ419169.1).
229 The structure of the 1.5 kb integron (InEc682) is shown in Figure 2. A single 2.4 kb integron
230 was detected in isolate IN664. It also contained three genes: *folA* at the 5' end (encoding
231 trimethoprim resistance), *catB3* in the middle (encoding chloramphenicol resistance) and
232 *aadA5* at the 3' end (encoding streptomycin and spectinomycin resistance). The sequence of
233 the 2.4 kb integron had 98% identity with the corresponding region of the class 1 integron in
234 the pAPEC-O2-R plasmid (Accession number AY214164, bases 89524-91815). Of eleven
235 integron-positive intestinal isolates, eight possessed the *tsh* gene and six the *iss* gene. Five
236 isolates contained *colV*, six *tetA* and three the Tn21 sequences (Table 2).

237

238 **Conjugal transfer of plasmids harbouring class 1 integrons.** The 1.0 kb integron of isolate
239 IN305 and the 1.5 kb integron of isolate IN682 were not transferable by conjugation. Plasmid
240 profiles of isolates EX201, IN306, IN664 and IN674, from which the integron was
241 successfully transferred, and those of their transconjugants are shown in Figure 3.
242 Characteristics of the transconjugants are summarised in Table 4. Three plasmids, of 117, 8.7,

243 and 2.9 kb, were transferred from isolate EX201, resulting in the transfer of the 1.0 kb
244 integron and also the *Tn21* and *tetA* genes to the recipient. However, none of the virulence-
245 associated genes were transferred. Southern blot hybridisation with the ³²P-labelled 1.0 kb
246 probe revealed that in isolate EX201 both the 117 kb and the 28 kb plasmids possessed this
247 integron, and that the 117 kb plasmid was responsible for its conjugal transfer (Figure 3).
248 Isolate IN306 harboured only a single plasmid of 128 kb, which was successfully transferred
249 to the recipient. This conjugative R-plasmid conferred resistance to streptomycin,
250 chloramphenicol, tetracycline, ampicillin and sulphamethoxazole-trimethoprim, and also
251 contained the 1.6 kb integron and the *iss* gene. Three plasmids, of 116, 7.5 and 3.8 kb, were
252 transferable from isolate IN664 and these conferred resistance to tetracycline and
253 sulphamethoxazole-trimethoprim, and also contained the 2.4 kb integron. Southern
254 hybridisation with the ³²P-labelled 2.4 kb probe revealed that the 116 kb plasmid contained
255 the 2.4 kb integron (Figure 3). No other resistance or virulence-associated gene was
256 transferred from this isolate. Of the three plasmids in isolate IN674, only one (158 kb) was
257 cotransferred with the 1.6 kb integron, and this plasmid conferred only sulphamethoxazole-
258 trimethoprim resistance.

259

260

261 Discussion

262

263 The prevalence of multi-drug resistance in the avian *E. coli* isolates described here is similar
264 (Lanz *et al.*, 2003) or lower (Blanco *et al.*, 1997, Yang *et al.*, 2004) than that reported in *E.*
265 *coli* from poultry in other countries. Class 1 integrons were identified with higher frequency
266 among extra-intestinal isolates (41% for the EX and 28% for the IN). All the integron-
267 carrying EX isolates contained a single 1.0 kb integron with an *aadA1* gene cassette. All these

268 isolates contained Tn21 and all but one contained the *merA* gene as well. This uniform pattern
269 of integron, Tn21 and *merA* content in the EX isolates is in good agreement with previous
270 observations for APEC isolates (Bass *et al.*, 1999). The integron-carrying EX isolates
271 belonged to serogroups O78 and O2, which have commonly been associated with
272 colibacillosis (Dho-Moulin- & Fairbrother, 1999). The association of these isolates with
273 disease in poultry may be attributable in part to the fact that all of them possessed the *iss* and
274 *tsh* virulence-associated genes which have been found to increase serum resistance and organ
275 invasion, respectively (La Ragione & Woodward, 2002, Mellata *et al.*, 2003). All of these EX
276 isolates possessed *colV*, which is usually located on large, conjugative plasmids, and often on
277 R-plasmids (Wooley *et al.*, 1992). Conjugation with isolate EX201 resulted in a
278 transconjugant that received the *aadA1* (on a 117 kb plasmid) and *tetA* antimicrobial
279 resistance genes, but not the *iss*, *tsh* or *colV* genes, indicating that the antimicrobial resistance
280 and virulence-associated genes of this isolate were not physically linked. In contrast Johnson
281 *et al.* (2002) reported the transfer of the *iss*, *tsh* and *cvaC* (the ColV structural gene) genes, as
282 well as tetracycline resistance and the *intI1* gene (integrase gene of the class 1 integrons) on a
283 100 kb conjugative plasmid from an APEC isolate.

284 | The IN isolates showed much variability in their content of class 1 integrons and
285 | virulence-associated genes. Among the IN isolates four different class 1 integrons were
286 | identified. Although the resistance genes of the 1.0 kb (*aadA7*), the 1.6 kb (*dhfrI-aadA1*) and
287 | the 2.4 kb (*folA-catB3-aadA5*) class 1 integrons have already been reported in previous
288 | studies (Mazel *et al.*, 2000, Waturangi *et al.*, 2003 and Johnson *et al.*, 2005), the gene cassette
289 | arrangement of the 1.5 kb integron (InEc682, *orfIN682-dhfrV-orfD*) was novel. Although it
290 | would be interesting to investigate the function of these orfs as well as their location and the
291 | genetic environment of the InEc682 integron, it was beyond the scope of this study. It is
292 | worth noting that, although none of the gene cassettes identified within the integrons

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293 conferred resistance to tetracycline, all of the transconjugants that originated from tetracycline
294 resistant donors acquired tetracycline resistance. Clarification of whether this was due to the
295 presence of the *tetA* gene on the same plasmid, or on a different plasmid that was also
296 transferred requires further investigation. It is also not clear why the TC201 and TC674
297 transconjugants were sensitive to streptomycin, even though both of them received the *aadA1*
298 gene from streptomycin resistant donor-strains. It is likely that either the transcription (which
299 is most probably initiated from P_{ant} promoter in the 5' conserved segment), or the translation
300 (which may be dependent on the translation of a short open reading frame (ORF-11) also
301 present in the 5'-CS of many class 1 integrons) of *aadA1* (Hanau-Bercot *et al.*, 2002) might
302 have been reduced in the genetic background of the *E. coli* K12 J-53 recipient. Detailed
303 analysis of the 5' conserved segment of these integrons could clarify this question.
304 Interestingly, many integron-carrying isolates possessed at least one of the three virulence-
305 associated genes (*iss*, *tsh* or *colV*). Conjugal transfer of resistance plasmid(s) possessing the
306 1.6 or the 2.4 kb integron occurred from three different donor strains, but only resulted in the
307 transfer of a virulence-associated gene (*iss*) with a 1.6 kb integron in one case (isolate IN306).
308 Unlike the EX isolates, the presence of Tn21 was not characteristic of the integron-carrying
309 IN isolates, suggesting that the class 1 integrons of the IN isolates may be located on, and
310 mobilized by, genetic elements other than Tn21.

311 | In summary, our results indicate that the intestinal *E. coli* of poultry contain different
312 combinations of antimicrobial resistance elements within their class 1 integrons and different
313 virulence-associated genes. Conjugation experiments suggest that most of these intestinal
314 isolates may serve as potential reservoirs for the spread of antimicrobial resistance genes, and,
315 in some instances, of virulence genes and thus could be a source for emergence of new types
316 of multiresistant avian pathogenic *E. coli*.

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319 **Acknowledgements**

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321 The authors acknowledge the financial support obtained from the National Research and
322 Development Project NKFP 4/040/2001. The excellent technical assistance of Mrs. Margit
323 Király and Mrs. Éva Gönczi is gratefully acknowledged. We thank the Bacteriology
324 Department for serotyping the intestinal isolates. We thank Dr. Anna Kósa for providing the
325 isolates and Dr. Péter Zsolt Fekete for his help during the alignment and submission of the
326 InEc682 integron sequence. Dr. István Tóth provided helpful suggestions and assisted by
327 critically reading the manuscript, Noémi Nógrády is the holder of a Bolyai János stipend from
328 the Hungarian Academy of Sciences.

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330

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428

429 **Figure Legends**

430 **Figure 1.** *PCR products obtained from intestinal E. coli isolates of poultry that produce*
 431 *different patterns of amplicons using the 5'CS and 3'CS primers.*

432 *Lanes 1 and 9, pGEM molecular weight marker (Promega); lane 2, 1.0 kb integron of isolate*
 433 *IN305; lane 3, 1.5 kb integron of isolate IN682; lane 4, 1.0 and 1.6 kb integrons of isolate*
 434 *IN306; lane 5, 1.6 kb integron of isolate IN674; lane 6, 2.4 kb integron of isolate IN664; lane*
 435 *7, 1.0 and 1.2 kb integrons of DT 104 S, Typhimurium isolate M117, used as a positive*
 436 *control; lane 8, distilled water (negative control).*

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438 **Figure 2.** *Structure of the 1.5 kb class 1 integron (InEc682) of isolate IN682.*

439 *5'CS, part of the 5' conserved segment; orfIN682, a hypothetical gene without similarity to*
 440 *any known gene; dhfrV, resistance to trimethoprim; orfD, hypothetical gene, identical to orfD*
 441 *in InS21 (Accession number AJ311891); 3'CS, part of the 3' conserved segment. Only part of*
 442 *the sequences of the 5' and 3' conserved segments were determined. The black boxes*
 443 *represent the open reading frames. Regions located between the open reading frames are*
 444 *indicated by light grey boxes. Dark grey boxes indicate parts of the conserved segments. The*
 445 *numbers above indicate the nucleotide positions within Accession number AM231806.*

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447 **Figure 3.** *Conjugal transfer of plasmids harbouring class 1 integrons.*

448 *A. Plasmid profile of the integron containing isolates EX201, IN306, IN664 and IN674, from*
 449 *which the integron was successfully transferred to the E. coli K12-J53 recipient, and the*
 450 *profile of their transconjugants.*

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451 *Lane 1, plasmids of the control strains R27 (168 kb) and V517 (2.0-53.7 kb); lane 2, TC201;*
452 *lane 3, EX201; lane 4, TC306; lane 5, IN306; lane 6, TC664; lane 7, IN664; lane 8, TC674;*
453 *lane 9, IN674; lane 10, E. coli K12-J53 (recipient, plasmidless).*

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454 **B. a) Plasmid profile and b) Southern blot of the plasmids of isolate EX201 (2) and its**
455 **transconjugant TC201 (1) hybridised with the 1.0 kb integron probe.**

456 *Lane 1; TC201; lane 2, EX201; lane M, plasmids of the control strains R27 (168 kb) and*
457 *V517 (2.0-53.7).*

458 *Arrows indicate the 117 kb plasmid that transferred the 1.0 kb integron.*

459 *There is also a strong signal indicating the presence of the 1.0 kb integron on the 28 kb*
460 *plasmid of isolate EX201.*

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461 **C. a) Plasmid profile and b) Southern blot of the plasmids of isolate IN664 (2) and that of its**
462 **transconjugant TC664 (1) hybridised with the 2.4 kb integron probe.**

463 *Lane 1, TC664; lane 2, IN664; lane M, plasmids of the control strains R27 (168 kb) and V517*
464 *(2.0-53.7).*

465 *Arrows indicate the 116 kb plasmid that transferred the 2.4 kb integron.*

466

Table 1. Primers used for PCR amplifications of integrons and virulence-associated genes of avian *E. coli* isolates

Target gene	PCR primers	DNA sequence (5' to 3')	Expected size of PCR product (bp)	Reference
Integron (variable region)	5'CS 3'CS	GGCATCCAAGCAGCTTTGAC AAGCAGACTTGACCTGA	Variable	Lévesque <i>et al.</i> , 1995.
<i>merA</i>	merAF merA R	ACCATCGGCGGCACCTGCGT ACCATCGTCAGGTAGGGGAACA	1.232	Liebert <i>et al.</i> , 1997.
<i>tnpM</i> of Tn21	TnpMF TnpMR	TCAACCTGACGGCGGCGA GGAGGTGGTAGCCGAGG	348	Villa <i>et al.</i> , 2002.
<i>colV</i>	cvi cvaB	TCTCTGCATTAATGTCTGC GATATGGGGCCAATATCCC	1.203	Gilson <i>et al.</i> , 1990.
<i>iss</i>	iss F iss R	GTGGCGAAAACACTAGTAAAACAGC CGCCTCGGGGTGGATAA	760	Horne <i>et al.</i> , 2000.
<i>tsh</i>	tsh F tsh R	GGGAAATGACCTGAATGCTGG CCGCTCATCAGTCAGTACCAC	420	Maurer <i>et al.</i> , 1998.
<i>tetA</i>	tetA F tetA B	GCTACATCCTGCTTGCCT CATAGATCGCCGTGAAGA	210	Lai-King <i>et al.</i> , 1999.
<i>tetB</i>	tetB F tetB B	GAGACGCAATCGAATTCGG TTTAGTGGCTATTCTTCCTGCC	228	Guillaume <i>et al.</i> ,

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Table 2. Characteristics of avian *E. coli* isolates containing class 1 integrons

Isolate ^a	O type	Size of class 1 integron(s)	PCR for:					
			<i>merA</i>	<i>Tn21</i>	<i>tetA</i>	<i>colV</i>	<i>iss</i>	<i>tsh</i>
EX201*	O78	1.0 kb	+	+	+	+	+	+
EX205	O78	1.0 kb	+	+	+	+	+	+
EX209	O78	1.0 kb	-	+	-	+	+	+
EX211	O78	1.0 kb	+	+	+	+	+	+
EX748	O2	1.0 kb	+	+	+	+	+	+
EX749	O78	1.0 kb	+	+	+	+	+	+
EX761	O78	1.0 kb	+	+	+	+	+	+
EX762	O78	1.0 kb	+	+	+	+	+	+
EX763	O78	1.0 kb	+	+	+	+	+	+
EX764	O78	1.0 kb	+	+	+	+	+	+
EX766	O78	1.0 kb	+	+	+	+	+	+
IN305*	O25	1.0 kb	-	(+) ^e	+	-	-	-
IN306*	O33	(1.0 kb) ^c ; 1.6 kb	+	-	+	-	+	+
IN664*	ONT ^d	2.4 kb	-	-	+	-	+	+
IN668	ONT	1.0 kb	+	-	-	-	-	+
IN669	O86	1.0 kb	-	-	-	+	-	-
IN672	ONT	1.6 kb	-	-	-	-	+	+
IN674*	ONT	1.6 kb	+	-	-	+	-	+
IN675	ONT	(1.0 kb) ^c ; 1.6 kb	-	-	-	+	+	+
IN682*	O5	1.5 kb	+	+	+	-	-	-
IN684	ONT	1.0 kb	-	-	+	+	+	+
IN685	ONT	1.0 kb	+	+	+	+	+	+

*used as donors for conjugation experiments and sequencing of the class 1 integrons.

^a: Isolates designated as EX originated from the liver of diseased poultry except for isolate EX211, which originated from the lung.

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Strains designated as IN originated from caecal contents.

^b: Intermediate resistances are not included.

^c: These integrons appeared as faint bands.

^d: ONT: O-nontypeable.

^e: The specific amplicon appeared as a faint band and non-specific bands were also detected.

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Table 3. *Multi-drug resistance* of avian E. coli isolates*

Isolate	Resistance profile	Class 1 integron (size is given in kbp)
EX747	STeKmNx	-
EX762	STeANx	1.0
IN306	SCTeASxTKmNxCip	1.6
IN650	TeKmNxCip	-
IN656	STeKmNxCip	-
IN657	STeKmNxCip	-
IN662	STeAKmNx	-
IN664	TeASxTNx	2.4
IN668	SAKmSxTNxCip	1.0
IN674	SSxTGm	1.6
IN686	SCTeKmNxCip	-
IN687	SCATeKmNxCip	-
IN688	SATeKmNx	-

*: An isolate was regarded as multi-resistant if showed resistance against 4 or more of the antimicrobials listed.

A: ampicillin (10 µg), C: chloramphenicol (30 µg), S: streptomycin (10 µg), Te: tetracycline (30 µg), Km: kanamycin (30 µg), SxT: sulphamethoxazole-trimethoprim (25 µg), Nx: nalidixic acid (30 µg) and Cip: ciprofloxacin (5 µg).

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Table 4. Characteristics of transconjugants (TC) derived from mating with extra-intestinal and intestinal *E. coli* from poultry

Donor strains (sero-group)	Resistance	Integron(s): gene(s) included	Plasmid(s) (kb) ^a	TC ^b	Assay result for transferred traits							
					Resistance	Plasmid(s) (kb)	Integron: gene(s) included	Tn21	tetA	colV	iss	tsh
EX201 (O78)	STeNx	1.0 kb: <i>aadA1</i>	167, 117, 88, (69), 50, 28, 8.7, 2.9	TC201 Te	Te	117, 8.7, 2.9	1.0 kb: <i>aadA1</i>	+	+	-	-	-
IN306* (O33)	SCTeASxTKmNxCip	1.6 kb: <i>dfrA1-aadA1</i>	128	TC306 SCTeASxT	SCTeASxT	128	1.6 kb: <i>dfrA1-aadA1</i>	NA ^c	+	NA	+	-
IN664 (ONT)	TeASxTNx	2.4 kb: <i>dfrA1-catB3-aadA5</i>	172, 116, 48, (42), 7.5, 3.8	TC664 TeSxT	TeSxT	116, 7.5, 3.8	2.4 kb: <i>dfrA1-catB3-aadA5</i>	NA	+	NA	-	-
IN674 (ONT)	SSxTGm	1.6 kb: <i>dfrI-aadA1</i>	158, 114, 47	TC674 SxT	SxT	158	1.6 kb: <i>dfrI-aadA1</i>	NA	NA	-	NA	-

*: Donor strain IN306 also carried a 1.0 kb integron which appeared as a faint band. It was not transferred to the recipient and therefore was not sequenced.

^a: Plasmids in brackets appeared as faint bands.

^b:TC: transconjugant.

^c: NA: not applicable.

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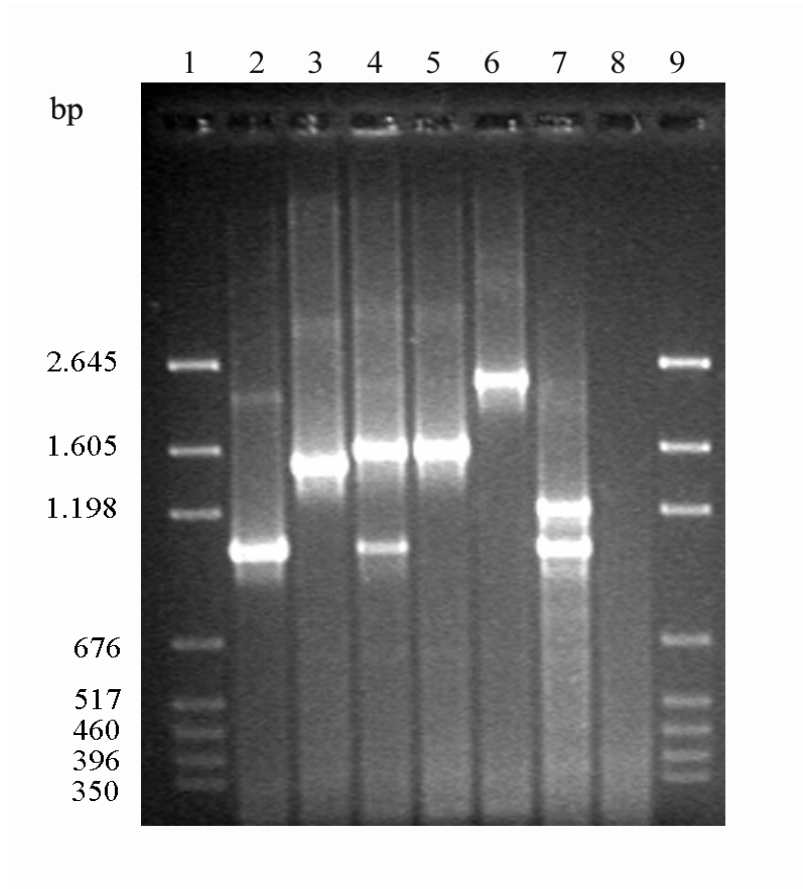
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Figure 1.



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Figure 2.

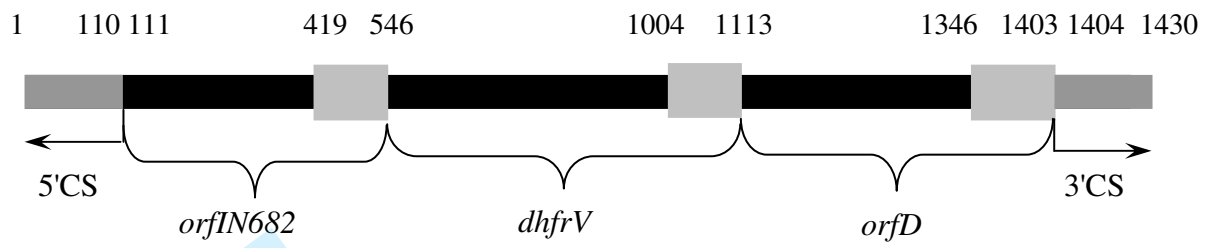
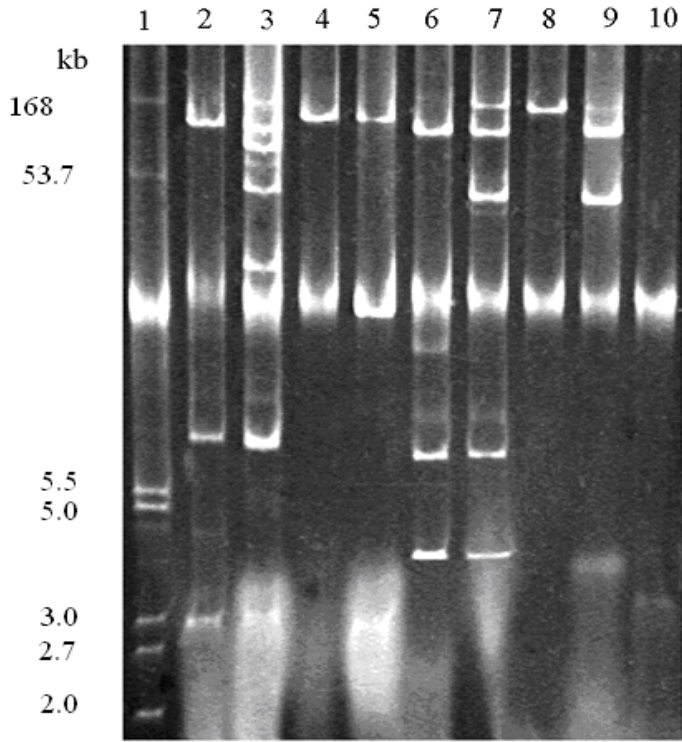


Figure 3. A)



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Figure 3. B)

a)

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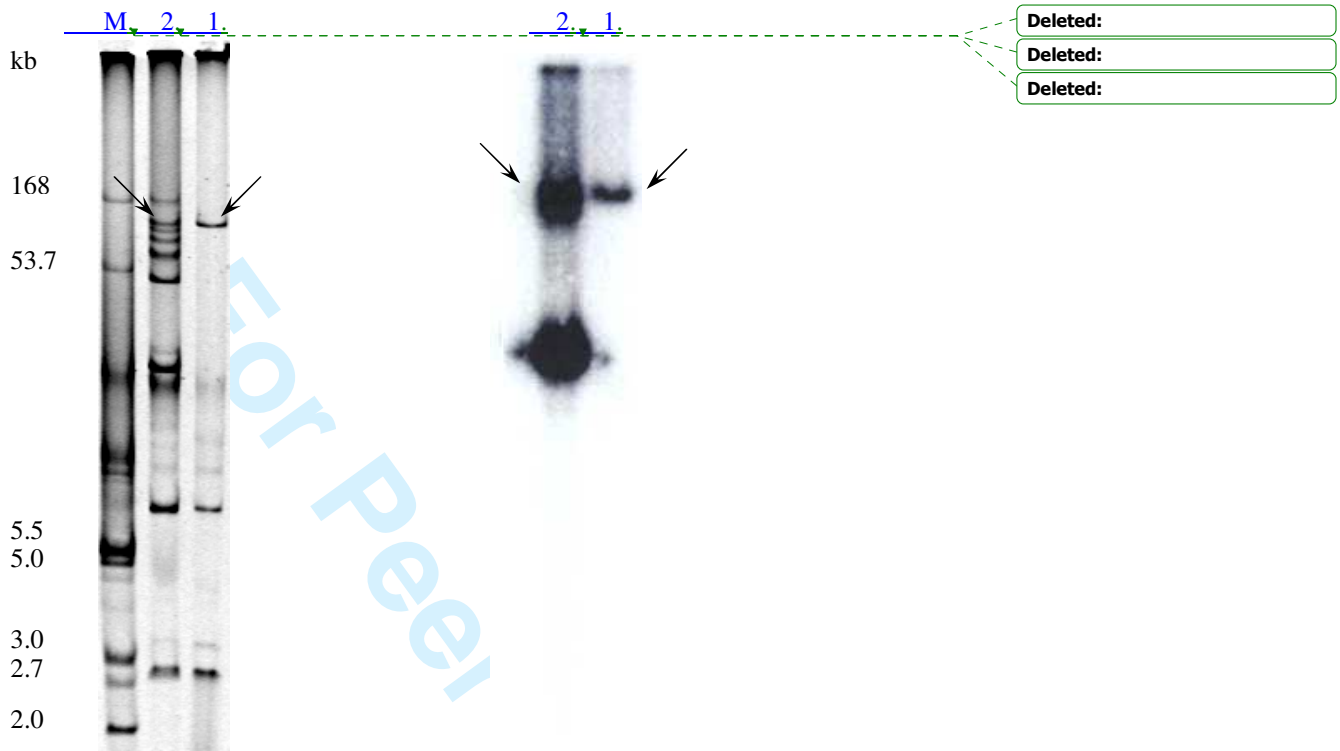
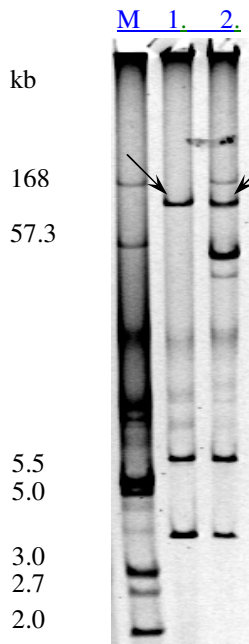
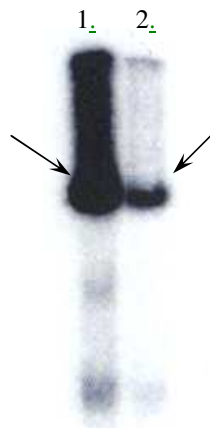


Figure 3. C)

a)



b)



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