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Short Communication

Multidrug resistance in *Escherichia coli* carrying integrons isolated from a pig farm with moderate antibiotic use

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Eulalia de la Torre,[1,*] Rocío Colello,[2] Daniel Fernández,[2] Analía Etcheverría,[2] José Di Conza,[3,4] Gabriel O. Gutkind,[5,6] María Ofelia Tapia,[1] Susana N. Dieguez,[1] Alejandro Luis Soraci,[1] and Nora Lía Padola[2]

[1] *Área de Toxicología, Departamento de Fisiopatología, Centro de Investigación Veterinaria de Tandil-Consejo Nacional de Investigaciones Científicas y Técnica - Comisión de Investigaciones Científicas Provincia de Buenos Aires (CIVETAN-CONICET-CICPBA), Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Campus Universitario, Paraje Arroyo Seco s/n, Tandil, Argentina*
[2] *Área de Inmunoquímica y Biotecnología, Departamento de Sanidad Animal y Medicina Preventiva (SAMP), CIVETAN-CONICET-CICPBA, Facultad de Ciencias Veterinarias, UNCPBA, Campus Universitario, Paraje Arroyo Seco s/n, Tandil, Argentina*
[3] *Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (UBA), Ciudad Autónoma de Buenos Aires, Argentina*
[4] *Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina*
[5] *Facultad de Farmacia y Bioquímica, UBA, Ciudad Autónoma de Buenos Aires, Argentina*
[6] *Hospital de Clínicas de la UBA "José de San Martín", Ciudad Autónoma de Buenos Aires, Argentina*

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The emergence and dissemination of antimicrobial resistance in bacteria derived from food-producing animals is a consequence of intensive agricultural and veterinary use of antimicrobial compounds. The inappropriate use and prescribing of antibiotics, along with their use as growth promoters, is the main cause of the development of resistance. The potential risk of the transfer of this resistance through the food chain by bacteria such as *Escherichia coli* (*E. coli*), including food borne pathogens such as Shiga toxin-producing *E. coli*, implies a problem for public health (van den Bogaard and Stobberingh, 2000). Horizontal gene transfer is an important route of the dissemination. Integrons are genetic elements able to capture gene cassettes encoding antibiotic resistance from the environment and incorporate them by site-specific recombination. Integrons are gene-capture and expression systems characterized by the presence of an *intI* gene encoding an integrase, a recombination site (*attI*), and a promoter. The most frequently reported mobile integrons are class 1 and class 2 integrons, which have been shown to contribute to the spread of antimicrobial resistance genes. It is important to remark that the presence of integrase is potentially indicative of strains capable of recruiting antibiotic resistance genes (Cambray et al., 2010). Moreover, resistance genes and resistant bacteria in the environment are considered an ecological problem. Therefore, the investigation of commensal bacteria is important in order to assess the extent of the drug resistance problem. The aim of the present study was to evaluate the susceptibility to antibiotics in commensal integron-positive *E. coli* isolated from pigs from a pig farm in Argentina.

Samples were taken from a commercial farm in Buenos Aires Province, Argentina, between March and June 2012. The use of antibiotics on the farm was considered moderate because subtherapeutic feed-based antibiotics were not used, and only therapeutic concentrations of tetracyclines were used for short periods during disease outbreaks. In addition, injectable antibiotics (fosfomycin, tiamulin, amoxicillin) were not used except for brief periods to control atypical disease outbreaks (less than 15 days per year). Briefly, one sow was randomly selected and fecal material was collected via a rectal swab at 3 h postpartum. Swabs were also obtained from five randomly chosen piglets from the test sow on the same day. The piglets selected were sampled again at day 21 and at day 70. These ages correspond to phases in which the pigs were housed in the farrowing crate (F), weaning (W), and growing/finishing units (G/F), respectively. Fecal samples were grown in 20 ml of LB for 24 h at 37°C, with shaking. Then 10 μl were plated on MacConkey agar and incubated for 18 h at 37°C. From each plate, suspected *E. coli* isolates (5–7 per plate) were randomly selected and were cultured in 0.8

*Corresponding author: Eulalia de la Torre, Campus Universitario, Paraje Arroyo Seco s/n, Tandil, Argentina.
Tel/Fax: +54-0249-4439850 E-mail: delatorreeulalia@gmail.com

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Table 1. Number of antibiotic-resistant *E. coli* isolates (%) containing the class 1 and/or class 2 integrons.

No. of antibiotic	Positive integron	Positive for class 1 integron	Positive for class 2 integron	Positive for class 2 and 1 integrons
Sow				
≤2	8 (72.7)	3 (37.5)	4 (50)	1 (12.5)
>2	3 (27.3)	1 (33.3)	0	2 (66.6)
any antibiotic	11 (100)	4 (36.4)	4 (36.4)	3 (27.2)
Piglets				
F				
≤2	7 (28)	1 (14.3)	6 (85,7)	0
>2	18 (72)	1 (5.6)	16 (88.8)	1 (5.6)
W				
≤2	0	0	0	0
>2	14 (100)	14 (100)	0	0
G/F				
≤2	2 (5.3)	2 (100)	0	0
>2	36 (94.7)	20 (55.6)	11 (30.6)	5 (13.8)
Total				
≤2	9 (11.7)	3 (33.3)	6 (66.7)	0
>2	68 (88.3)	35 (51.5)	27 (39.7)	6 (8.8)
any antibiotic	77 (100)	38 (49.4)	33 (42.9)	6 (7.7)
		42 (47.7%)	37 (42.1%)	9 (10.2%)

≤2: Number of *E. coli* isolates resistant to 2 groups of antibiotics or less.
>2: Number of *E. coli* isolates resistant to more than 2 groups of antibiotics.
F: Farrowing pen stage, W: weaning stage, G/F: Growing/finishing stage.

Table 2. Distribution of resistance (%) of the total isolates relative to the number of antibiotics and the class of integrons.

No. of antibiotic	No. of porcine strains (%)	Positive for class 1 integron	Positive for class 2 integron	Positive for class 2 and 1 integrons	Main profiles of resistance	No. of porcine strains (%)
1	8 (9.1)	1 (12.5)	7 (87.5)	0	TET	8 (9.1)
2	9 (10.2)	5 (55.6)	3 (33.3)	1 (11.1)	ENR, TET	4 (4.5)
3	22 (25)	16 (72.7)	4 (18.2)	2 (9.1)	ENR, TET, TIA	12 (13.6)
4	24 (27.3)	12 (50)	10 (41.7)	2 (8.3)	AMX, ENR, FLOR, TET	11 (12.5)
5	25 (28.4)	8 (32)	13 (52)	4 (16)	AMX, ENR, FLOR, FOSF, TET	20 (22.7)

AMX: amoxicillin, TET: tetracycline, FOSF: fosfomycin, TIA: tiamulin, ENR: enrofloxacin, FLOR: florfenicol.

ml of LB for 24 h at 37°C, with shaking for DNA extraction. *E. coli* gene universal stress protein (*uspA*) was used to confirm *E. coli* (Chen and Griffiths, 1998). Virulence genes *stx1*, *stx2*, *stx2e*, *eae*, *lt*, *sta*, *stb* characteristic of verotoxigenic *E. coli* (VTEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC), respectively, were detected by PCR (Parma et al., 2000). Genes encoding integrase 1 (*Int1*) and integrase 2 (*Int2*) were detected to identify the presence of integrons class 1 or class 2, respectively (de la Torre et al., 2014). Only negative strains for these genes have been considered as commensal and integron positive strains have been included in this study.

The susceptibility of isolates to different antibiotics was tested by the Kirby Bauer disk diffusion method using Muller Hinton Agar against selected antibiotics, namely amoxicillin (AMX; 10 μg), tetracycline (TET; 30 μg), fosfomycin (FOSF; 200 μg), tiamulin (TIA; 30 μg), colistin (COL; 10 μg), enrofloxacin (ENR; 5 μg), florfenicol (FLOR; 30 μg). The inhibition zone size was interpreted by using the standard recommendation of the Clinical Laboratory Standards Institute (2010). An isolate was considered to be multidrug resistant if it was resistant to more than 2 groups of antibiotics.

For confirmation of the organization of resistance gene cassettes in class 1 integrons, PCRs for variable regions were carried out using the following primers: 5′CS: 5′-GCCATCCAAGCAGCAAG, 3′CS: 5′-AAGCAGACTTGACCTGA. Some amplicons were sequenced and/or subjected to restriction endonuclease digestion with *Bts*I or *Pvu*I when the sizes of PCR products were identical. The organization of resistance genes in class 2 integrons was mapped by two specific PCRs. The primer sequences were: *intI*2F: 5′-TTATTGCTGGGATTAGGC, Sat1R: 5′-GTTTCGTTCGAGACTTGAGG and Sat1F: 5′-CTATCTACCAGAAGTGTGAGC, aad1R: 5′-AAGAATGTCATTGCGCTGCC. In addition, some amplicons were also sequenced. The resulting DNA sequences were compared with the sequences in the current GenBank database using an updated version of the BLAST program available at the National Center for Biotechnology Information website (www.ncbi.nih.gov) and in the INTEGRALL database (http://integrall.bio.ua.pt/).

All commensals *E. coli* carrying integrons isolated from the selected sow (n = 11) showed some antibiotic resistance (Table 1). Furthermore, most of the isolates (8/11, 72.7%) were resistant to one or two antibiotics of which

the majority carried class 2 integrons (Table 1). These results are in agreement with Literak et al. (2009) who demonstrated that sows that contained *E. coli* resistant to two antibiotics carried mainly class 2 integrons. From isolates recovered from piglets (n = 77), 49.4% of resistant commensal strains carried class 1 integrons while 42.9% carried class 2 integrons and 7.7% carried both classes of integrons (Table 1). Unlike isolates obtained from sows, those obtained from piglets were mostly multiresistant (88.3%): 51.5% harbored class 1 integrons, 39.7% class 2 integrons and 8.8% carried both classes of integrons (Table 1). However, several studies have demonstrated a broad distribution of multiresistant isolates recovered from production animals and humans (Lanz et al., 2003; Phongpaichit et al., 2007) and a high prevalence of *E. coli* carried class 1 integrons (Phongpaichit et al., 2007).

Isolates from sows were resistant to more than 2 antibiotics (27.3%), while isolates from farrowing piglets showed 72% (18/25) multiresistant strains. This proportion increased at the last stages of production: weaning 100% (14/14) and growth/fattening 94.7% (36/38). In addition, we note that these multiresistant strains are mainly class 1 integron-positive (Table 1). Considering these results, it is important to note that multiresistant *E. coli* isolates are found in piglets less than 12 hours of age, highlighting the importance of transmission of these strains to the litter at birth when microbiota implantation takes place. Mathew et al. (1999) detected higher levels of multiresistant *E. coli* obtained from piglets more than 35 days old compared with those 7 days old and with sows. Similarly, Mazurek et al. (2013) found higher multidrug resistance in piglets compared with sows as a consequence of antibiotic supply in the post-weaning period. Andraud et al. (2011) observed that the transmission of resistant bacterial strains between individuals is a factor that enables their persistence in pig farms. While most studies do not consider the age of the animals, our data indicate that age is an important factor affecting resistance in swine. It must be kept in mind that, at weaning, the liquid or/and semiliquid diet becomes dry food. Thus, the piglets are exposed to antibiotics used in the food as growth promotion in some countries (Barton, 2014).

The highest resistance rates were found for tetracycline (96.6%) and tiamulin (94.1%), followed by enrofloxacin (68.2%), florfenicol (60.2%), amoxicillin (52.3%) and fosfomycin (40.9%). There was no strain resistant for colistin, but disk diffusion test is not the best test to evaluate colistin resistance (Morales et al., 2012). *E. coli* strains resistant to four and five antibiotics simultaneously were the most prevalent (27.3% and 28.4%, respectively) (Table 2). Values obtained in this work agree with other authors whose studies were performed with isolates from countries where the surveillance programs have not been settled and antibiotic use is not properly regulated (Literak et al., 2009; Marchant and Moreno, 2013). The most frequent multiresistance profiles observed were TET-ENR-AMX-FLOR-FOSF (22.7%), TET-TIA-ENR (13.6%) and TET-ENR-AMX-FLOR (12.5%) (Table 2). Overall, in veterinary science, *E. coli* isolates were resistant to five or more antibiotics (Goldstein et al., 2001). Nevertheless, comparison among them should be carefully analyzed because of the different bacterial selection criteria used in these studies.

Eight *E. coli* strains harboring class 1 integrons were randomly selected to characterize the variable region by the digestion of PCR products and sequencing. The variable regions of 5 strains contained one gene cassette, *aadA1*, encoding an aminoglycoside-adenyltransferase that confers resistance to streptomycin and spectinomycin. The *aadA1* genes present more than a 99% identity with the reference *aadA1* (GenBank accession No. X12870). In these genes, two substitutions could be observed: an adenine to guanine transition at base 602 (leading to a lysine to arginine amino acid change at position 201) and a cytosine to thymine transition at base 750 (silent mutation).

It has been shown that gene cassettes belonging to the *aadA* family were mainly found in class 1 integrons in commensal bacteria isolated from farm and nonfarm environments (Yang et al., 2010).

Furthermore, 3 strains did not carry gene cassettes—the so-called empty integrons. This situation has also been described by other authors (Lee et al., 2006), pointing out that these bacteria could have the potential in the future to convert themselves rapidly into multiresistant strains. In this regard, Rosser and Young (1999) proposed that "empty" integrons represent ancestral elements that have not yet acquired gene cassettes inserted between the conserved segments of the integrons.

Likewise, sixteen commensal isolates carrying integrons class 2 were studied. The arrangement of gene cassettes *dhfrI-sat-aadA1* was found within these integrons, which is typically associated in Tn7-like class 2 integrons (accession number NC_002525).

Herein, in the class 2 integrons, the *dhfrI* gene, which confers resistance to trimethoprim, has always been associated with the genes *aadA1* and *sat*. Both of these genes confer resistance to aminoglucosides, and the *sat* gene in particular confers resistance to streptothricin, a much used antibiotic in the food industry and veterinary treatment (Lee et al., 2006).

In one isolate, which contained both integrons, the presence of the same two gene cassettes arrangements were observed: *aadA1* (in class 1 integron) and *dhfrI-sat-aadA1* (in class 2 integron). Kang et al. (2005) indicated that the predominance of the *aadA1* cassette suggests that this could be the first to be acquired by an integron and/or could be more stable than the other genetic cassettes. On the other hand, the high predominance of this gene could be related to the extensive use of these aminoglucosides in the control of infectious diseases in these food-producing animals.

The selected antibiotics are used on farms to achieve a high birth rate and to maximize productive performance in terms of average daily gains and feed efficiency at less cost. Moreover, antibiotics are used with therapeutic purposes, with medicated feeds being the most common mode of administration of the drug (Barton, 2014). Antibacterial growth promoters belonging to different groups of antibiotics structurally unrelated exert their antibacterial activity by several mechanisms. We include the most important groups of antibiotics such as beta-lactams (AMX), tetracyclines (TET), quinolones (ENR), epoxides (FOSF),

phenicoles (FLOR), pleuromutilin (TIA) and glycopeptides (COL) which have been used for this purpose (Cromwell, 2002).

There is growing evidence that the environment plays a role in the spread of antibiotic resistance among pathogenic species. Many questions have been raised concerning the impact of the release of antibiotics and antibiotic-resistant bacteria on the environment or over human and animal health. The use of antimicrobial agents could select resistant bacteria nonpathogenic, e.g. those that recruit resistant determinants mediated by integrons, which later may transfer the acquired resistance to different pathogenic bacteria species (van den Bogaard and Stobberingh, 2000) by horizontal gene transfer. Keeping this in mind, we managed to obtain resistant transconjugants, indicating the ability of the mobile genetic element bearing integron to be transferred from commensal microflora to potential zoonotic pathogens (data not shown).

The abundance of commensal *E. coli* harboring integrons and resistance levels reported in this study is alarming and clearly indicates that unmonitored use of antibiotics on swine farms could expand the abundance of the antibiotic resistance reservoir in the farm environment. Antibiotic resistance in bacteria associated with pigs not only affects pig production but also has an impact on the environment, by contamination soil and water, and on human health through the transfer of resistant organisms and associated genes via the food chain and/or direct contact of farm personnel and workers in the food industries.

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