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Antimicrobial Resistance of *Escherichia coli* isolated in one day old chickens and effect of amoxicillin treatment during its growth

Ana Jiménez-Belenguer (Corresponding author)

Affiliation: Biotechnology Department. Centro Avanzado de Microbiología de Alimentos. Universitat Politècnica de València. Camino de Vera 14, P.O. Box 46022, Valencia, Spain. Tel.: +34 963877423; fax: +34 963877429

E-mail address: anjibe@upvnet.upv.es

Eva Doménech

Affiliation: Institute of Food Engineering for Development (IUIAD), Food Technology Department (DTA), Universitat Politécnica de Valencia. Camino de Vera 14, P.O. Box 46022 Valencia, Spain

E-mail address: evdoan@tal.upv.es

Arantxa Villagrá

Affiliation: Centro de Tecnología Animal CITA-IVIA. Polígono La Esperanza nº 100, P.O. Box 12400 Segorbe, Castellon, Spain

Telf: +34964712566

E-mail address: Villagra_ara@gva.es

Alejandro Fenollar

Affiliation: Biotechnology Department. Universitat Politècnica de València. Camino de Vera 14, P.O. Box 46022, Valencia, Spain

E-mail address: alfepe@etsiamn.upv.es

Maria Antonia Ferrús

Affiliation: Biotechnology Department. Centro Avanzado de Microbiología de Alimentos Universitat Politècnica de València. Camino de Vera 14, P.O. Box 46022, Valencia, Spain

E-mail address: mferrus@btc.upv.es

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Abstract

The use of antimicrobials in food animals is the major determinant for the propagation of resistant bacteria in the animal reservoir. However, other factors also play a part. In particular, vertical spread between the generations has been suggested to be an important transmission pathway. The objective of this paper was to determine the resistance patterns of *E. coli* isolated from farmed newborn chickens as well as to study the antibiotic pressure effect when amoxicillin was administered during their growing period. With this aim, meconium from 22 one day old Ross chickens was analyzed. In addition, during their growth period, amoxicillin treatments on days 7, 21 and 35 were carried out. Results showed a high number of *E.coli* resistant strains isolated from one day chickens, being the highest rates for beta-lactams group, followed by quinolone and tetracyclines. Moreover, as expected, analysis during the productive cycle of chickens after treatment with amoxicillin showed that the highest percentage of resistances were detected for this antibiotic. Moreover, significant differences in resistance percentages between control and treated broilers were detected in relation to ampicillin, cephalothin streptomycin, kanamycin, gentamicin, chloramphenicol and tetracycline. Differences in resistances to ciprofloxacin and nalidixic acid between control and treated animals were not observed. Finally, no resistance was detected for amikacin and ceftriaxone. These results suggest the possibility of vertical transmission of resistant strains to newborn chickens from parenteral flocks, and seem to indicate that the treatment with amoxicillin had a cross effect, increasing the resistances of *E. coli* to other antibiotics.

Key words: Newborn chickens, antimicrobial resistance, vertical transmission, *E coli*.

1. Introduction

Antimicrobial resistance is a main concern, since resistant bacteria can pose a greater risk to human health as a result of potential treatment failure, loss of treatment options and increased likelihood and severity of disease. In fact, treatment failures by multiresistant strains are responsible for half of the approximately 27,000 annual deaths from infections in the European Union (Watson, 2008).

The use of antimicrobials drugs to treat and prevent diseases in animals or to promote their growth has been accompanied by the development of antimicrobial resistances. Transmission of resistance from animals to humans can take place through a variety of routes, where the food-borne route probably is the most important. Reduction in the use of antimicrobial agents can have a positive effect on the occurrence of antimicrobial resistance in humans and nowadays, the use of sub-therapeutic doses in order to increase the body weight is completely banned in the European Union since January 2006 (Regulation 1831/2003/EC).

Resistant strains selected by animal antimicrobial treatments can reach humans via other animals, sewage, or other humans, such as farmers or slaughterers (Phillips et al., 2003, Miranda, et al., 2007). Thus, transmission of resistant isolates between animals and environment makes necessary go ahead and invest in preventive measures. In this sense, addressing zoonotic transmission of pathogens that are resistant to antimicrobials is crucial to stablish effectice risk management policies.

Amoxicillin (AMX) is a broad spectrum β-lactam penicillin, introduced in human medicine in the early 1970s and used for the treatment of infections caused by Gram negative and positive bacteria. In fact, AMX is recommended by the European guidelines as the first-choice antibiotic for treating mild respiratory and other common

81 infections in non-hospitalized patients. For all these reasons, when investigating
82 resistance spreading among farm animals, amoxicillin is one of the most interesting
83 antibiotics to study.

84 On the other hand, poultry was the most dynamic meat sector during the last decade.
85 In fact, the total poultry meat production increased from 69 million tons in 2000 to 94
86 million tons in 2008, which corresponds to an increase of 35% (FAO, 2010). Nowadays,
87 this kind of meat represents 30% of the global meat consumption and chickens,
88 followed by far for turkeys, are the most common sources of poultry meat (87% and
89 6.7%, respectively) (FAO, 2010).

90 Surveillance of resistance in commensal bacteria is important because they can be
91 reservoirs of resistance determinants and because they are more ubiquitous than
92 pathogens. Exchange of resistance genes occurs between pathogens and nonpathogens,
93 even between gram-positive and gram-negative organisms. Of particular interest are
94 enterococci and *E. coli*, that can play a role in transmission of mobile resistance genes
95 (McEwen and Fedorka-Cray, 2002). *E. coli* isolated in healthy animals, carcasses or
96 meat thereof, provides valuable data for investigating relationship with the selective
97 pressure exerted by the use of antimicrobials on the intestinal population of bacteria in
98 food-producing animals and determining the occurrence of resistance to antimicrobials.
99 *E. coli* is also useful as representative of *Enterobacteriaceae* to monitor the emergence
100 and changes in the proportion of bacteria possessing ESBL (EFSA 2013).

101 Therefore, the objective of this paper was to determine the antibiotic resistances
102 pattern of *E. coli* in newborn chicken, in order to assess the possibility of vertical
103 transmission from parenteral flocks, and to study the antibiotic pressure effect that the
104 administration of amoxicillin through their growth has in the development of
105 antimicrobial resistance rates in their comensal gut microbiota.

2. Materials and methods

2.1. Breeding chickens

A total of one day old 22 healthy Ross male chickens were obtained from the same commercial hatchery. Reception of chickens was carried out in the "Centro de Investigación Tecnológica Animal" in Segorbe, Castellón (Spain). Chickens were labeled and randomly assigned to six groups of three chickens and one with four chicks. Each group was conducted separately and between-group transmission was avoided by having a solid separation between pens. All the pens had a trough and three water nipple dispensers and the floor was covered with 10 cm of wood shavings. The chickens were fed with the same free-antimicrobial feed. All parts of this study were carried out according to EC council directives (2010/63/EU) concerning the laws, regulations, and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes (2015/VSC/PEA/00178).

2.2. Antibiotic administration

One of the groups (the one with four chicks) was considered the control and was kept untreated. The six remaining groups were treated with amoxicillin (Neudiaval oral powder (50x118 g Laboratory Mevet, Lérida, Spain) according to manufacturer's instructions. Chickens were kept in the experimental facilities from day of arrival (day 0) until day of slaughter (day 49) and they were weighed and treated with different doses of amoxicillin: D1, 24 mg amoxicillin/kg of animal; D2, 12 mg amoxicillin/kg of animal and D3, 8 mg amoxicillin/kg of animal. Each dose was administered to two different groups. Dose administration was carried out with an oral syringe. The

131 treatments (T) were administered over 3 days. The treatment one (T1) started in day 7,
132 treatment two (T2) day 21 and treatment three (T3) day 35 of chickens life cycle. No
133 other antimicrobial treatment was administered during the course of the experiment.
134
135 *2.3. Sample collection*
136 Four sample collections were carried out during the experimental period,
137 immediately before administration of antibiotic doses. First sampling was on day 0 on
138 meconia. Second and third samplings took place on days 21 and 35. Samples were taken
139 directly from the cloaca using a sampling swab (Deltalab Collection and transport
140 system. Amies swab ps+viscose). The final sampling was on day 49 and samples were
141 taken directly from the cecum after slaughtering all the animals with over-doses with
142 embutramide. In all cases, the samples were refrigerated until they were processed in
143 the laboratory.
144
145 *2.4. Escherichia coli isolation*
146 TBX agar plates (T.B.X. Medium, OXOID Ltd., England, UK) were inoculated and
147 incubated at 44ºC for 24h. Two randomized colonies of each plate were isolated,
148 transferred to PC agar (Plate Count Agar, Scharlau, Barcelona, Spain) and incubated to
149 37ºC for 24h. The isolates from the PC agar plates were then checked as *E. coli* with the
150 API-20 E system (Biomérieux, France). Colonies identified as *E. coli* were selected for
151 the antibiogram test.
152
153 *2.5. Antimicrobial susceptibility*
154 Antimicrobial susceptibility determination of isolated *E. coli* was completed by a
155 standard disc diffusion assay (Antimicrobial Susceptibility Test Disc, OXOID Ltd.,

England, UK) on Mueller Hinton agar (BBL™ Mueller-Hinton II Agar, BD). The MIC breakpoints and levels of resistance were determined according to the recommendations of the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS, 2002). The *E. coli* strains were tested against 12 antibiotics of veterinary and sanitary significance: gentamycin (CN) 10µg, amoxicillin/clavulamic acid (AMC) 3µg, ampicillin (AMP) 10µg, amikacin (AK) 30µg, kanamycin (K) 30µg, cloranphenicol (C) 30µg, cephalothin (KF) 30µg, ciprofloxacin (CIP) 5µg, ceftriaxone (CRO) 30µg, tetracycline (TE) 30µg, nalidixic acid (NA) 30µg and streptomycin (S) 10µg.

2.6. Statistical analysis

Statistical analyses of the data were undertaken using Statgraphics Centurion XVI.II (Statpoint Technologies, Inc. Warrenton, Virginia). The possible relationship between treatments with amoxicillin and the increase of *E. coli* resistance to different groups of antibiotics were carried out with a Multiple Correspondence Analysis (MCA) (Greenacre, 2007). In MCA, subjects (rows) and variables (columns) can be depicted simultaneously on a graphical display; where a close position between different points indicates a relatively high level of association. On the contrary, when the points are in different parts of the axis, the association is low. Relative proportions were compared using the Chisquared test (X^2) and Fisher's exact test. Comparisons of means were also performed. A probability value of less than 5% was deemed to be significant.

3. Results and discusion

3.1. Resistances found in in one-day-old chickens

E. coli strains isolated from one-day-old chicks' meconium showed resistance in 63.3% (14 out of 22) and multiresistance, defined as resistance to at least three different antimicrobial classes, was observed in 95% (13 out of 14 meconia). The highest percentage of resistance was found against nalidixic acid (80%), ampicillin, and amoxicillin/clavulanic, both of them in 70% of samples, followed by tetracycline (30%) cephalothin (23.3%) and ciprofloxacin (16.7%). Lower than 10% resistances were found against streptomycin, gentamicin and kanamycin. Finally, none of the *E. coli* isolates was resistant to amikacin, chloramphenicol and ceftriaxone (Figure 1). Similar results were found by Martins da Costa et al. (2011) who found no *E. coli* resistant to chloramphenicol but observed resistant strains from one day-old-chicks against ampicillin, cephalothin, tetracycline, streptomycin, gentamicin and enrofloxacin. As this study was performed from chickens that had not been exposed to antimicrobial agents previously, a vertical transmission of resistant strains from parental flocks (Giovanardi et al., 2005a; 2005b) or by contamination in the hatchery environment (Dierikx et al., 2013) could be the main causes. In our work, environmental exposition to antibiotics was limited by the conditions of expertimental design: "Centro de Investigación Tecnológica Animal" is not a conventional, but an experimental farm, in which chickens were maintained into high hygienic pens, thus reducing as much as possible the spreading of antibiotic exposure through environment. Thus, contamination of chickens via vertical transmission seems to be the most probable explanation to the high resistance rates found.

According to Petersen et al. (2006) parents represent an extensive bacterial reservoir from which transmission may occur through contamination of the eggs shell during lay. The study performed by Bortolaia et al. (2010) was also consistent with vertical transmission of ampicillin and nalidixic acid resistant *E. coli* through the broiler

production system. These authors concluded that clones among parent and broiler flocks were indistinguishable, which indicated that transmission of ampicillin and nalidixic resistant *E. coli* occurred from parents to broilers. Bortolaia et al (2010) suggested, according to their findings, that resistance to beta-lactams and fluoro-quinolone in *E. coli* was due to vertical transmission through parent chickens. In the same framework, Baron et al. (2014) suggested that *E. coli* resistance may be introduced in the hatchery facilities, either form true vertical transmission when parental poultry stocks are contaminated or form very early contamination in the hatchery itself, or during transport, which is a period when the immature digestive flora is probably very receptive to early colonization, although other possible contamination events occurring thereafter on the production farm cannot be excluded.

3.2. Resistances patterns changes during experimental treatments with amoxicillin

During the growth period, three amoxicillin treatments were administrated. Results of resistant *E. coli* isolated strains showed no significant differences between them (p-value 0,1760), however, significant differences were found between isolates from control and treated broilers (p-value 0,0013). Finally, no significant differences existed in the resistances found between the three doses administrated (p-value 0.9025), consequently, values from the three doses were managed together.

Figure 2 shows the Multiple Correspondence Analysis (MCA) carried out to evaluate the global effect of each amoxicillin treatments on the profile of *E. coli* response (sensitive, intermediate and resistant) to different groups of antibiotics used. Two dimensional MCA solution accounts for 23.6% of the inertia (the first dimension accounts for 15.1% and the second 8.5%). Considering the x-axis, resistance and intermediate resistance of *E. coli* to antibiotics were located on the left; however,

antibiotic sensitivity was located on the right. Moreover, considering that the closer position the closer relationship, results showed that control samples had a higher percentage of *E. coli* strains sensitive to antibiotics and those obtained from chicken treated had a higher percentage of resistances. In addition, the antibiotics more related with resistance response were ampicillin, amoxicillin, cephalothin, which means that although only one antibiotic was used, it could be influencing other β lactam antibiotics. On the other hand of the axis, corresponding to antimicrobial sensitive response, ceftriaxone, kanamicin, gentamicin, ciprofloxacin and chloranphenicol presented a near position and consequently a high antimicrobial sensitivity. Considering the y-axis, fewer differences can be observed. However, the high distance of intermediate response indicates a low percentage of intermediate resistance.

Figure 3 shows the relationship between broiler chickens treatment with amoxicillin and antimicrobial resistance of *E. coli* to beta-lactams (amoxicillin, ampicillin, ceftriaxone and cephalothin); aminoglycosides (amikacin, gentamicyn, kanamycin and streptomycin); phenicol (chloranphenicol); quinolones (ciprofloxacin and nalidixic acid) and tetracycline (tetracycline).

Results showed that no animal (control and treated) presented *E. coli* strains resistant to amikacin and ceftriaxone. Similar results were found by Saenz et al. (2001) and Bywater et al. (2004). Taking into account the critical importance for human medicine of ceftriaxone (WHO, 2012; Domenech et al., 2015) the obtained results are encouraging.

Slight differences in resistances between control and treated flocks were found for ciprofloxacin and nalidixic acid. In addition, antimicrobial resistance in control birds (not exposed to antimicrobials) exhibited interesting changes over time in ciprofloxacin. In this cases, no resistant strains are present in control 1 (C1), corresponding to day 7 of

life cycle, however they appear in controls 2 and 3. In a previous study, Apajalahti et al., (2004), reported that microbial composition of the chicken gut can change according to the diet and the environment, directly by providing a continuous source of bacteria, or indirectly by influencing bird defense mechanisms. In general, a large numbers of chicks bearing resistant *E. coli* strains shed huge numbers of resistant isolates, resulting in rapid contamination of the other individuals in the same flock and production barn environment. This high level of contamination is probably difficult to eliminate even with strict disinfection procedures, particularly on farms with outdoor runs (Baron et al., 2014).

In this work, obtained values for resistance to nalidixic acid in treated chickens were 64±8.2% . The resistance rates of *E. coli* reported by EFSA, (2013) vary considerably between countries, from 0.6%, (n= 316) in Finland to 85.1% (n= 101 samples) in Spain. In relation to ciprofloxacin, obtained values were 16.1±4.8%. These values are higher than those reported in Denmark 9% (n=134 samples), and lower that the European mean 53.1 % (n=1703) (EFSA, 2013).

On the other hand, resistant *E. coli* to ampicillin, cephalothin, all aminoglycosides studied, chloramphenicol and tetracycline showed high differences between treated and no treated flocks. Moreover, only treated animals presented resistance to gentamicin, kanamycin streptomycin and chloramphenicol. When an antibiotic pressure is acting, those genes that are capable to permit the survival of the strains are selected. These selected genes can be horizontally transferred between different bacterial species (Van den Bogaard et al., 2001). Horizontal gene transfer is the most characteristic acquisition of resistance genes (Binnewies et al. 2006). Plasmids carrying genetic determinants which confer resistance to different classes of antibiotics confer a selective advantage for the carrier strains. This kind of plasmids are large, self-conjugative and control their

copy number by regulation of the replication rate in the cell (Nordstrom, 2005), what could lead to their persistence and spread in the microbial community of the intestine. Moreover, a study carried by Miller et al. (2004) showed that the exposure to beta-lactams induce the *dpiBA* operon, which inhibits the replication of the bacterial chromosome, inducing the SOS response and induce an enhancement of the genetic variability. These facts could explain the general higher rates of resistance for the treated group versus the control group.

The higher resistance rates for tetracyclines determined in this work was 67.6%, although the mean value was 37.4%. These values were relatively low as compared with a 75% of resistance reported by Sáenz et al., (2001) and was around the European mean value reported by EFSA, 2013 45.2% (n=2019). Multiresistant strains were more prevalent in each successive trial. This might be explained by the persistence of farm indigenous *E. coli* strains that were repeatedly exposed to antimicrobials (Furtula et al., 2010; Smith et al., 2007).

With regard to public health, amoxicillin, ampicillin, and nalidixic acid are on the list of Critically Important Antimicrobials published by the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO, 2012), by acomplishing the criterion 1 (the antimicrobial agent is the sole, or one of limited available therapy, to treat serious human disease) and also the criterion 2 (Antimicrobial agent is used to treat diseases caused by either: (a) organisms that may be transmitted to humans from non-human sources or, (b) human diseases causes by organisms that may acquire resistance genes from non-human sources). Hence, the increasing of antimicrobial resistance in general and resistance to these antibiotics in particular, may represent a major threat to human health (EU Commission, 2010).

To conclude, in this work we have demonstrated the existence of a high percentage of resistant *E. coli* strains in one old day chickens, not exposed previously to any antibiotic, what strongly suggest the possibility of vertical transmission from parenteral flocks. On the other hand, influence of amoxicillin treatments in increasing resistances to beta-lactams, aminoglycosides and cloramphenicol has been shown. Critical importance to human health of the antimicrobial resistances found highlights the importance to take immediately control measures focusing on reducing vertical and horizontal transmission in farm environment.

Conflict of interest

None to declare

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406 Figure captions

407

408 Figure 1. Percentage of *E. coli* resistant resistant (■), intermediate (▒) and sensitive
409 (□) in meconia of day-old chicks. Where AK= Amikacin; AMC= Amoxicilin/
410 clavulamic acid; AMP=Ampicillin; C= Chloramphenicol; CIP= Ciprofloxacin; CN=
411 Gentamicin; CRO= Ceftriaxone; K= Kanamycin; KF= Cephalothin; NA= Nalidixic
412 acid; TE= Tetracycline; S= Streptomycin

413

414 Figure 2. MCA bi-plot. Antibiotics tested: A. AMP =Ampicillin; A. C =
415 Chloramphenicol; A. CN = Gentamicin; A. CRO = Ceftriaxone; A. K= kanamycin; A.
416 NA = Nalidixic acid; A. SXT = Trimethoprim/sulphametoxazol; A. TE = Tetracycline.
417 Before any dose administrations (C0), after the three dose administrations (T1, T2 and
418 T3), and the same sampling for the control group (C1, C2, C3). VR = (resistant), VI =
419 (intermediate), VS = (sensitive).

420

421 Figure 3: Percentage of *E. coli* resistant (■), intermediate (▒) and sensitive (□) for
422 the group of control (C1, C2, and C3) and after the three treatments (T1, T2 and T3).
423 Where AK= Amikacin; AMC= Amoxicilin/ clavulamic acid; AMP=Ampicillin; C=
424 Chloramphenicol; CIP= Ciprofloxacin; CN= Gentamicin; CRO= Ceftriaxone; K=
425 Kanamycin; KF= Cephalothin; NA= Nalidixic acid; TE= Tetracycline; S= Streptomycin

426