ANTIMICROBIAL RESISTANCE IN COMMENSAL *Escherichia coli* ISOLATES FROM RABBITS

Resistencia antimicrobiana en aislados comensales de *Escherichia coli* de conejos

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ABSTRACT

Food animals may serve as a reservoir of bacteria that carry antimicrobial resistance genes that may be transferred to microorganisms found in humans and thereby limit the medical value of antimicrobials. In contrast to *Escherichia coli* from humans and several other animal species, there is little information on the frequency and mechanisms of antimicrobial resistance of that bacteria isolated from rabbits. The objective of the present study was to determine the antimicrobial resistance profile of 478 *E. coli* isolates from healthy rabbits. Another objective was to assess the diversity and distribution of the major resistance genes [*tetA, tetB, tetC*, and *tetM* for doxycycline, bla_{TEM}, bla_{OXA-1}, bla_{SHV}, and $bla_{CTX-M-9}$ for amoxicillin, *aac(3)II*, *aac(3)IV*, and *ant(2")I* for gentamicin, and *qnrA*, *qnrB*, *qnrS*, *aaC(6)Ib*, and *qepA* for enrofloxacin], as well as the mutations in the quinolone resistance-determining region of the *gyrA* and *parC* genes, in these isolates. The percentage of isolates resistant to doxycycline was very high (89.3%). However, relatively few isolates were resistant to amoxicillin (16.1%), gentamicin (2.9%), and enrofloxacin (4.2%). Predominant resistance genes were *tetA* and *tetB* in the isolates resistant to doxycycline, and bla_{TEM} in the isolates resistant to amoxicillin. Most *E. coli* isolates with intermediate resistance to enrofloxacin presented a single mutation in *gyrA,* while most isolates resistant to it presented double mutations in *gyrA* and single mutations in *parC*, or double mutations in both *gyrA* and *parC*. The present study provides baseline data on frequency and molecular basis of antimicrobial resistance in *E.coli* isolates from rabbits. In addition, the results of this study suggest that commensal *E. coli* isolates from rabbits may be a reservoir of antimicrobial resistance genes.

Key words: Antimicrobial resistance, resistance genes, *Escherichia coli*, rabbits.

RESUMEN

Los animales de producción pueden ser un reservorio de bacterias que portan genes de resistencia que pueden ser transferidos a microorganismos presentes en humanos y, por tanto, limitar la utilidad médica de los antimicrobianos. A diferencia de *Escherichia coli* de humanos y diversas especies animales, hay escasa información sobre la frecuencia y mecanismos de resistencia antimicrobiana de esta bacteria aislada de conejos. El objetivo del presente estudio fue determinar el perfil de resistencia a los antimicrobianos de 478 aislados de *E. coli* de conejos sanos. Otro objetivo fue analizar la diversidad y distribución de los principales genes de resistencia [*tetA, tetB, tetC* y *tetM* para doxiciclina, bla_{TEM}, bla_{OXA-1}, bla_{SHV} y $bla_{CTX-M-9}$ para amoxicilina, *aac(3)II*, *aac(3)IV* y *ant(2")I* para gentamicina y *qnrA*, *qnrB*, *qnrS*, *aaC(6)Ib* y *qepA* para enrofloxacina], así como las mutaciones en la región determinante de resistencia a quinolonas de los genes *gyrA* y *parC*, en esos aislados. El porcentaje de aislados resistentes a doxiciclina fue muy elevado (89,3%). Sin embargo, relativamente pocos aislados fueron resistentes a amoxicilina (16,1%), gentamicina (2,9%) y enrofloxacina (4,2%). Los genes de resistencia más frecuentes fueron *tetA* y *tetB* en los aislados resistentes a doxiciclina y bla_{TEM} en los aislados resistentes a amoxicilina. La mayoría de los aislados de *E. coli* con resistencia intermedia a enrofloxacina presentaron una mutación simple en *gyrA*, mientras que la mayoría de los aislados resistentes a la misma presentaron dobles mutaciones en *gyrA* y una mutación simple en *parC* o dobles mutaciones, tanto en *gyrA* como en *parC*. Este estudio proporciona datos de referencia sobre la frecuencia y bases moleculares de la resistencia antimicrobiana en aislados de *E. coli* de conejos. Además, los resultados de este estudio sugieren que los aislados comensales de *E. coli* de conejos pueden ser un reservorio de genes de resistencia antimicrobianos.

Palabras clave: Resistencia antimicrobiana, genes de resistencia, *Escherichia coli*, conejos.

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INTRODUCTION

Food animals may serve as a reservoir of resistant bacteria that carry antimicrobial resistance genes that may be transferred to microorganisms found in humans and thereby limit the medical value of antimicrobials. Thus, interventions reducing this reservoir of resistance genes among food animals may prolong the lifetime of these drugs for human use [1]. These human food safety concerns have been influential in pushing the European Union to ban the use of antimicrobials as growth promoters in food production and to increase surveillance of bacterial resistance in food-borne pathogens and indicator organisms such as *Escherichia coli* [2, 22].

In contrast to *E. coli* from humans and several other animal species, only a limited number of studies published to date have examined the frequency of antimicrobial resistance and/or the mechanisms of antimicrobial resistance in commensal and pathogenic *E. coli* strains isolated from rabbits [3, 9, 17, 21]. The objective of the present study was to determine the antimicrobial resistance profile of commensal *E. coli* isolates from rabbits (*Oryctolagus cuniculus*). Another objective was to assess the diversity and distribution of the major resistance genes, as well as the mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes, in these isolates.

MATERIALS AND METHODS

A total of 478 *E. coli* isolates were examined in this study. These isolates were obtained in an ongoing research project carried out in Spain designed to evaluate the effect of the oral administration of different doses of doxycycline on the frequency of resistance to different antimicrobials among *E. coli* and enterococci isolates from healthy rabbits. In this project, 20 45-day-old crossed New Zealand x Californian rabbits 45 days old that had never received antimicrobials before the administration of doxycycline were examined. Fecal samples from individual rabbits were taken before initiating the treatment, at the end of the treatment, and at four weeks post-treatment. This study was carried out in a controlled environment in which no medication had been administered for approximately four years before the trial, and the areas located on either side of the study area were empty. Rabbits had free access to a commercial feed (CUNIUNIC, NANTA, S.A., Madrid, Spain).

Antimicrobial testing was performed using the disc diffusion method, according to the recommendations of the Clinical and Laboratory Standards Institute [7]. The following antimicrobials belonging to four different classes frequently used in veterinary medicine were tested: doxycycline (tetracyclines), amoxicillin (β-lactams), gentamicin (aminoglycosides), and enrofloxacin (fluoroquinolones). All antimicrobial susceptibility discs were obtained from a commercial source (Rosco Diagnostica A/S, Taastrup, Denmark). Measurement of growth inhibition areas allowed the classification of each isolate as susceptible, intermediate or resistant, according to data provided by the manufacturer of the discs. As reference strain, *E. coli* ATCC 25922 was used.

The presence of the major resistance genes for doxycycline (*tetA, tetB, tetC*, and *tetM*), amoxicillin (bla_{TEM}, bla_{OXA-1}, bla_{SHV}, and $bla_{CTX-M-9}$), and gentamicin [*aac(3)II*, *aac(3)IV*, and *ant(2")I*] was determined in resistant and intermediate resistant isolates by polymerase chain reaction (PCR) as previously described [4, 5, 8, 11, 19, 20]. *E. coli* isolates resistant and intermediate resistant to enrofloxacin were screened for mutations in the QRDR of the *gyrA* and *parC* genes and for the presence of plasmid-mediated quinolone resistance (PMQR) genes [*qnrA*, *qnrB*, *qnrS*, *aaC(6)Ib*, and *qepA*] by PCR, as previously described [6, 10, 16, 18, 24].

RESULTS AND DISCUSSION

The antimicrobial susceptibility of the 478 *E. coli* isolates was summarized in TABLE I. These isolates were obtained in three different stages, but since no significant differences in the frequencies of antimicrobial resistance among stages were observed (data not shown), the isolates were considered as a whole. The percentage of isolates resistant to doxycycline was very high (89.3%). However, relatively few isolates were resistant to amoxicillin (16.1%), gentamicin (2.9%), and enrofloxacin (4.2%). The frequency of doxycycline resistance in the present study was similar to the frequency of tetracycline resistance (86.4%) found in a recent study carried out on enteropathogenic *E. coli* strains from diarrheic rabbits in Portugal [17], but higher than those previously reported for resistance to tetracycline in *E. coli* strains from diarrheic and healthy rabbits in Spain (50%) [3] and in wild rabbits in Portugal (11.4%) [21]. In agreement with the results of this study, the percentages of *E. coli* strains resistant to ampicillin and gentamicin found previously in diarrheic and healthy rabbits in Spain (6.4 and 0.9%, respectively) [3] and in wild rabbits in Portugal (11.4 and 2.3%, respectively) [21] were very low. However, the percentages of enteropathogenic *E. coli* strains from diarrheic rabbits resistant to ampicillin, gentamicin, and ciprofloxacin found in Portugal (86.4, 18.2, and 9.1%, respectively) [17] were higher than those reported in the present study for amoxicillin, gentamicin, and enrofloxacin. The antimicrobials licensed for use in rabbits in Spain are enrofloxacin, colistin, apramycin, tiamulin, tilmicosin, and several sulfonamides [23]. As the use of tetracyclines in rabbits is not permitted in Spain, the high resistance to doxycycline found in this study is probably due to the previous use of these antimicrobials in rabbits. On the other hand, and in contrast to *E. coli* from poultry [15], the use of enrofloxacin in rabbits in Spain seems not to be associated with a high frequency of *E. coli* isolates resistant to that antimicrobial.

Because of the high number of doxycycline-resistant isolates (427) identified in this study, a sample of 70 was ran-

TABLE I

NUMBER (PERCENTAGE) OF COMMENSAL *Escherichia coli* ISOLATES FROM RABBITS FECES THAT WERE RESISTANT (R), INTERMEDIATE RESISTANT (I), OR SUSCEPTIBLE (S) TO FOUR ANTIMICROBIAL AGENTS

Doxycycline			Amoxicillin			Gentamicin			Enrofloxacin		
R	I	S	R	I	S	R	I	S	R	I	S
427 (89.3)	39 (8.2)	12 (2.5)	77 (16.1)	0 (0)	401 (83.9)	14 (2.9)	5 (1)	459 (96)	20 (4.2)	11 (2.3)	447 (93.5)

TABLE II

MECHANISMS OF ANTIMICROBIAL RESISTANCE IN COMMENSAL *Escherichia coli* ISOLATES FROM RABBITS FECES

Antimicrobial	Mechanism of antimicrobial resistance	Number (%) of resistant isolates	Number (%) of intermediate resistant isolates
	Resistance genes		
Doxycycline	*tetA*	33 (47.1)	
	tetB	34 (48.6)	
	tetA + *tetB*	1 (1.4)	
	Not identified	2 (2.9)	
Amoxicillin	bla_{TEM}	60 (77.9)	
	bla_{TEM} + $bla_{CTX\text{-}M\text{-}9}$	3 (3.9)	
	Not identified	14 (18.2)	
Gentamicin	*aac(3)II*	1 (7.1)	0 (0)
	aac(3)IV	1 (7.1)	0 (0)
	Not identified	12 (85.7)	5 (100)
	Amino acid changes in the QRDR* of GyrA / ParC		
Enrofloxacin	Ser83 → Leu / No mutation		9 (81.8)
	Ser83 → Leu + Asp87 → Asn / No mutation	2 (10)	
	Ser83 → Leu + Asp87 → Asn / Ser80 → Ile	9 (45)	
	Ser83 → Leu + Asp87 → Asn / Ser80 → Ile + Ser50 → Phe	1 (5)	
	Ser83 → Leu + Asp87 → Asn / Ser80 → Ile + Glu84 → Gly	6 (30)	
	No mutation / No mutation	2 (10)	2 (18.2)

* Quinolone resistance-determining region.

domly selected for genotypic analysis to pinpoint the antimicrobial resistance genes responsible. At the same time, mechanisms of antimicrobial resistance were characterized in all isolates resistant to amoxicillin (77), gentamicin (14), and enrofloxacin (20), as well as in all isolates intermediate resistant to gentamicin (5) and enrofloxacin (11).

tetA and *tetB* genes predominated among *E. coli* isolates from rabbits resistant to doxycycline (TABLE II, FIG. 1). This is consistent with studies showing that most human and animal *E. coli* strains resistant to tetracyclines carry one of these two genes [5, 14, 17, 21]. The results in the present study further suggest a negative association between *tetA* and *tetB* genes, which has been observed previously and probably results from plasmid incompatibilities [4, 14].

Most *E. coli* isolates resistant to amoxicillin in the present study possessed the bla_{TEM} gene, consistent with previous studies on human and animal *E. coli* isolates resistant to β-lactams [8, 12, 14, 17, 21]. Resistance genes could not be

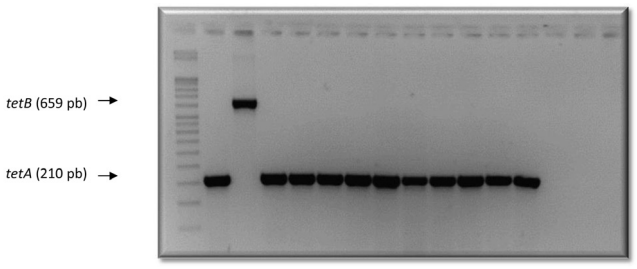

FIGURE 1. AGAROSE GEL IMAGE OF AMPLICONS OBTAINED FROM PCR WITH PRIMERS DESIGNED FOR *tetA* AND *tetB*. Lane 1, molecular size marker (50 bp); lane 2 and 3, positive controls for *tetA* and *tetB*, respectively; lanes 4-13, *Escherichia coli* isolates obtained in this study positive to *tetA*; lanes 14 and 15, *E. coli* isolates obtained in this study negative to *tetA* and *tetB*; lane 16, negative control.

identified in 14 of 77 (18.2%) isolates resistant to amoxicillin. In a similar study in which ampicillin-resistant enteropathogenic *E. coli* strains from diarrheic rabbits were analyzed for the presence of the same β-lactam resistance genes as in the present work, investigators found that 47.4% were negative for all these genes [17]. These findings suggest that other genes in addition to the ones thoroughly reviewed in the literature may make significant contributions to the circulation of antimicrobial resistance in rabbits. Future studies should seek to identify the full range of resistance genes in these populations.

Similar to the case of β-lactam resistance genes, resistance genes could not be identified in 17 of 19 (89.5%) isolates resistant and intermediate resistant to gentamicin.

Nine of the 11 isolates with intermediate resistance to enrofloxacin presented a single amino acid substitution in the GyrA protein (Ser83 → Leu), while 18 of the 20 *E. coli* isolates resistant to enrofloxacin presented a double amino acid substitution in GyrA (Ser83 → Leu and Asp87 → Asn). Mutations in *gyrA* at codons Ser83 and Asp87 confer greater resistance to quinolones than do mutations at other codons within the quinolone resistance-determining region and are the most common *gyrA* mutations found in human and animal isolates of *E. coli* [13]. Nine of the 20 isolates resistant to enrofloxacin presented a single amino acid substitution in the ParC protein (Ser80 → Ile), and 7 isolates resistant to enrofloxacin showed a double amino acid substitution in ParC (6 isolates: Ser80 → Ile + Glu84 → Gly; 1 isolate: Ser80 → Ile + Ser50 → Phe). To the authors' knowledge, this is the first report of a Ser50 → Phe substitution in the ParC protein of *E. coli* isolates. Mutations in *parC* were always found together with mutations in *gyrA* in the present study, as reported for other *E. coli* isolates [13]. Consistent with these results, mutations in *parC* at codon 80 are the most frequently mutations identified in *E. coli* isolates resistant to quinolones [13]. In two of 11 isolates (18.2%) with intermediate resistance and two of 20 isolates (10%) resistant to enrofloxacin, no mutations in *gyrA* and *parC* were found. None of the isolates intermediate resistant or resistant to enrofloxacin carried PMQR genes. However, the frequency in another study [9] of *oqxAB*, a PMQR gene not investigate in this work, in *E. coli* isolates from domestic and wild lagomorphs in Italy was relatively high (15%).

CONCLUSIONS

The present study provides baseline data on frequency and molecular basis of antimicrobial resistance in *E.coli* isolates from rabbits. In addition, the results of this study suggest that commensal *E. coli* isolates from rabbits can serve as reservoirs of antimicrobial resistance genes.

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