ANTIMICROBIAL RESISTANCE GENES AND VIRULENCE GENE ENCODING INTIMIN IN *ESCHERICHIA COLI* AND *ENTEROCOCCUS* ISOLATED FROM WILD RABBITS (*ORYCTOLAGUS CUNICULUS*) IN TUNISIA

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The spread of antimicrobial-resistant bacteria in wildlife must be viewed as a major concern with serious implications for human and animal health. *Escherichia coli* and enterococcal isolates were recovered from faecal samples of 49 wild rabbits (*Oryctolagus cuniculus*) on specific media and were characterised using biochemical and molecular tests. For all isolates, antimicrobial susceptibility testing was performed, and resistance genes were detected by PCR. Molecular typing of isolates was carried out by pulsed-field gel-electrophoresis, and *E. coli* strains were also tested for the presence of intimin (*eae*) gene characteristic of rabbit enteropathogenic *E. coli*. A total of 34 *E. coli* and 36 enterococci [*E. hirae* (52.8%) and *E. faecalis* (47.2%)] were obtained. For *E. coli*, resistance to tetracycline (94%), streptomycin (62%), ciprofloxacin (47%), trimethoprim-sulphamethoxazole (35%) and chloramphenicol (6%) was observed. Resistance to third-generation cephalosporins was detected in one *E. coli* strain that carried the bla_{CMY-2} and bla_{TEM-1} genes. Class 1 integrons were detected in eight isolates. For enterococci, resistance to tetracycline (63.9%), erythromycin (30.5%), streptomycin (18.2%), and chloramphenicol (5.5%) was detected. The *tet*(M)+*tet*(L), *erm*(B) and *ant*(6)-Ia genes were identified in thirteen, seven and three resistant *Enterococcus* strains, respectively. Molecular typing showed a high diversity among our strains. Wild rabbits could represent a reservoir of *E. coli*, and enterococci carrying antimicrobial resistance genes and *E. coli* additionally carrying the *eae* gene of enteropathogenic pathotypes could both contaminate the environment. Our finding seems to represent the first report of *eae*-positive *E. coli* in wild rabbits.

Key words: Wild rabbit, antimicrobial resistance, enterococci, *E. coli*, intimin, third-generation cephalosporin resistance

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Commensal bacteria constitute a source of antimicrobial resistance genes. When they are released into the environment through the faecal material of animals and humans, they can disseminate in different ecosystems, contaminate food products, be transmitted by the food chain, and so reach the intestinal tract of humans (Franz et al., 1999). These microorganisms are considered important as 'indicator bacteria' in order to track the evolution of antibiotic resistance in different ecosystems (Van den Bogaard et al., 2000). *Escherichia coli* and *Enterococcus* are also regarded as opportunistic human pathogens, because they are implicated in a wide diversity of infections, and they might act as reservoirs of antimicrobial resistance genes that could be transmitted to other pathogenic bacteria (Guardabassi et al., 2004). Thus, they might represent a worldwide problem with large repercussions in public health (Van den Bogaard et al., 2000). Wild animals are of importance in relation to antibiotic resistance in several different ways. In fact, many human activities such as human and veterinary clinical settings, farms, landfills and wastewater may give rise to interactions with wildlife, which may have a direct association with the antibiotic resistance profiles of the intestinal bacteria of wild animals in a certain geographic location (Guardabassi et al., 2004; Sayah et al., 2005; Santos et al., 2013). Added to that, wild animal populations could be a possible reservoir of antibiotic-resistant bacteria, and the contact with species of game hunted for their meat may transfer multidrug-resistant bacteria to humans or livestock, which provides a biological mechanism for the increase in antibiotic resistance genes in human populations (Sayah et al., 2005; Allen et al., 2010). In Tunisia, the wild rabbit (*Oryctolagus cuniculus*) represents an important species since it is part of the human food chain. Interaction between wild rabbits and the surrounding ecosystem may represent a source of bacterial dissemination through the environment. To our knowledge, scarce information is available regarding the characteristics of *E. coli* and *Enterococcus* isolates of wild rabbit origin (Figueiredo et al., 2009; Silva et al., 2010; Marinho et al., 2014), and no data exist in Tunisia. Therefore, the aim of this study was to analyse the prevalence of *E. coli* and *Enterococcus* spp. strains in wild rabbits, as well as the phenotypes and genotypes of antibiotic resistance in recovered isolates in order to evaluate the role of wild rabbits as reservoirs of antibiotic resistance genes.

Materials and methods

Sample collection, isolation and identification of bacterial strains

Forty-nine wild rabbits were collected in Southern Tunisia during the rabbit hunting season (in October and November 2016) for human consumption. Swabs of the rectal stumps were collected just when the rabbits were captured. They were transported to the laboratory at 4 °C and were kept refrigerated and processed within 6 h. Faecal samples were added to 5 ml of peptone water, being

incubated at 37 °C for 24 h. After that, several dilutions of the enrichment broth were seeded in a specific medium.

For *E. coli* isolation, the dilutions were seeded in MacConkey agar plates and incubated at 37 °C for 24 h. One colony per sample with typical *E. coli* morphology was selected and identified by classical biochemical methods (indole, citrate and urease), and confirmed by PCR amplification of the *uid* gene (*uid*-F: 5'-ATCACCGTGGTGACGCATGTCGC-3'; *uid*-R: 5'-CACCACGATGCCAT GTTCATCTGC-3').

Additionally, for enterococcal isolation, the dilutions were seeded on Slanetz-Bartley agar plates, that were incubated at 37 °C for 48 h. One colony per plate, with typical enterococcal morphology, was picked from each plate and streaked onto BHI agar plates. The isolates were initially characterised as enterococci based on biochemical tests, including catalase reaction, hydrolysis of esculin in the presence of bile, and capacity to grow in hypersaline medium. The species identification was confirmed by PCR, using primers and conditions for the different enterococcal species (Torres et al., 2003).

Antibiotic susceptibility testing

The antimicrobial susceptibility patterns of the *E. coli* isolates were determined by the disk diffusion test for 16 antimicrobials (in μg/disk) [ampicillin (10), amoxicillin + clavulanic acid (20/10), cefoxitin (30), cefotaxime (30), ceftazidime (30), aztreonam (30), imipenem (10), gentamicin (10), amikacin (30), tobramycin (10), streptomycin (10), ciprofloxacin (5), sulphamethoxazole + trimethoprim (SXT) (1.25/23.75), tetracycline (30), and chloramphenicol (30)] according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2017). *Escherichia coli* ATCC 25922 was used as a quality control strain. The screening for extended-spectrum beta-lactamase (ESBL) producing *E. coli* was performed by the double disk synergy test using disks containing amoxicillin/clavulanic acid on Mueller-Hinton agar plate at a 30-mm distance from the indicator drugs: ceftazidime (30 μg) and cefotaxime (30 μg) (CLSI, 2017). Additionally, susceptibility testing of the enterococcal isolates was performed for the following antimicrobials (in μg/disk): ampicillin (10), vancomycin (30), teicoplanin (30), chloramphenicol (30), tetracycline (30), pristinamycin (15) erythromycin (15), ciprofloxacin (5), gentamicin (120) and streptomycin (300) (CLSI, 2017). *Enterococcus faecalis* strain ATCC 29212 was used for quality control.

Detection of antibiotic resistance genes and E. coli *intimin (eae) gene by PCR*

The resistant *E. coli* isolates were tested by PCR to detect the following genes: *tet*(A) and *tet*(B) genes in all tetracycline-resistant isolates, *bla*$_{TEM}$ gene in ampicillin-resistant isolates, and *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$ genes in cephalosporin-resistant isolates. The presence of the *intI1* gene encoding the integrase of class 1

integrons was analysed by PCR in SXT-resistant isolates. The identification of these genes was performed using specific primers and conditions (Sáenz et al., 2004; Ben Said et al., 2016).

On the other hand, the following resistance genes were tested by PCR in resistant enterococci isolates, using specific primers and conditions (Klibi et al., 2013*a*): *erm*(B) (in erythromycin-resistant isolates), *tet*(M) and *tet*(L) (in tetracycline-resistant isolates), *ant*(6)-Ia (in streptomycin-resistant isolates), and *cat*(A) (in chloramphenicol-resistant isolates).

Molecular typing by pulsed field gel electrophoresis (PFGE) and detection of *eae* virulence gene

Molecular typing by PFGE of *E. coli* and *Enterococcus* isolates was performed as described previously (Sáenz et al., 2004; López et al., 2009). The resulting restriction patterns were analysed by a visual method and by the Gel-Compar II software using the UPGMA algorithm (Turabelidze et al., 2000). Moreover, the presence of the *eae* virulence gene [encoding intimin of enteropathogenic *E. coli* (EPEC)] was tested in all *E. coli* isolates by PCR as described by Alonso et al. (2017).

Results and Discussion

Isolation of bacteria

Escherichia coli isolates were recovered on MacConkey agar plates in 34 of the 49 rabbit faecal samples (69%), while *Enterococcus* strains were obtained from 36 of the samples analysed (73.5%). *Enterococcus hirae* was the most prevalent enterococcal species detected (52.8%, 19/36) followed by *E. faecalis* (47.2%, 17/36). The high incidence of *E. hirae*, in comparison with the incidence of *E. faecalis*, was in contrast with data previously reported about the predominance of *E. faecalis* and *E. faecium* among faecal enterococci of both farmed and wild rabbits (Linaje et al., 2004; Silva et al., 2010) and among those from other wild animals, pets and poultry (Butaye et al., 2001; Poeta et al., 2005, 2006; Jackson et al., 2010; Santos et al., 2013). The presence of *E. hirae* in high proportion was also found among faecal enterococci isolated from camels in arid regions of Tunisia (Klibi et al., 2013*a*).

Antimicrobial resistance among *E. coli*

Analysis of the antimicrobial susceptibility of *E. coli* isolates showed a high frequency of resistance to tetracycline (94%) and streptomycin (62%), which concurred with other studies carried out on wild animals (Costa et al., 2008), food-producing animals (Sáenz et al., 2001), pigs (Teshager et al., 2000) and foods of animal origin (Jouini et al., 2009). Dotto et al. (2014) previously re-

ported very high resistance rates (> 90%) for tetracycline and streptomycin in enterococci recovered from wild and farmed rabbits, although other authors reported lower rates in *E. coli* isolates from wild rabbits (Silva et al., 2010; Marinho et al., 2014). Lower resistance frequencies were recorded to ciprofloxacin (47%), trimethoprim-sulphamethoxazole (35%) and chloramphenicol (6%) (Table 1). The rates of resistance for these antibiotics were higher than those reported in wild animals (Costa et al., 2008; Marinho et al., 2014). Differences in resistance percentages could be explained by the difference of the geographic localisation of the wildlife populations studied (Costa et al., 2008).

Table 1

Distribution of antibiotic resistance in *E. coli* and *Enterococcus* spp. strains isolated from wild rabbits

Antibiotic used	Number of resistant *E. coli* strains (n = 34)	Number of resistant *Enterococcus* strains	
		E. hirae (n = 19)	*E. faecalis* (n = 17)
Tetracycline	32 (94%)	13 (68%)	10 (59%)
Streptomycin	21 (62%)	–	–
Ciprofloxacin	16 (47%)	0	0
Sulphamethoxazole + trimethoprim	12 (35%)	–	–
Chloramphenicol	2 (6%)	0	2 (12%)
Ampicillin	1 (3%)	0	0
Cefoxitin	1 (3%)	–	–
Cefotaxime	1 (3%)	–	–
Ceftazidime	1 (3%)	–	–
Aztreonam	1 (3%)	–	–
Imipenem	0	–	–
Amoxicillin + clavulanic acid	0	–	–
Gentamicin (10 μg)	0	–	–
Amikacin	0	–	–
Tobramycin	0	–	–
Erythromycin	–	1 (5%)	10 (59%)
Vancomycin	–	0	0
Teicoplanin	–	0	0
Streptomycin	–	0	6 (35%)
Gentamicin (120 μg)	–	0	0
Pristinamycin	–	0	0

Dashes mean that the antibiotic was not tested

Multiresistance to three or more different classes of antibiotics was observed in fifteen *E. coli* isolates (44%) (Table 2). Even though wild rabbits were not subjected to the selective pressure associated with the extensive use of antimicrobial drugs for food-producing animals, these high rates of multiresistance found in Tunisian wildlife are worrying. The acquisition of antibiotic-resistant

E. coli strains by wild rabbits could be related to the interactions with farm wastes (Cole et al., 2005; Guerrero-Ramos et al., 2016). All *E. coli* strains resistant to tetracycline harboured the *tet*(A) gene, in association with *tet*(B) in six strains, which indicates that the main mechanism of tetracycline resistance in wild rabbit *E. coli* isolates is by active efflux (Silva et al., 2010). Plasmid-mediated quinolone resistance gene *aac*(6')-Ib-cr was identified in one *E. coli* strain resistant to fluoroquinolones. The gene *int*1 encoding class 1 integrase was detected in eight strains resistant to trimethoprim/sulphamethoxazole; only one type of arrangement was detected in integron-positive isolates, which harboured the gene *aad*A1 encoding resistance to streptomycin. Resistance to broad-spectrum cephalosporins and ampicillin was detected in only one *E. coli* strain, which harboured the genes *bla*$_{TEM-1}$ and *bla*$_{CMY-2}$ encoding for resistance to ampicillin and cephalosporins, respectively. CMY-2 was detected in Tunisia in strains isolated from wastewater (Ben Said et al., 2016), foods of animal origin (Ben Slama et al., 2010) and from food-producing animals (Ben Sallem et al., 2012). Table 3 shows the antimicrobial resistance genes detected in *E. coli* strains. However, none of the *E. coli* isolates were found to be ESBL producers, when the double disc diffusion test was performed for screening.

Table 2

Multiresistant phenotypes detected among *E. coli* and *Enterococcus* isolates obtained from wild rabbits

Species	Multiresistant phenotypes[a]	Number of isolates
E. coli	AMP FOX CTX CAZ AZM STR CIP TET	1[b]
	STR CIP SXT TET	9
	STR SXT TET	2
	CIP SXT TET	1
	CIP STR TET	2
E. faecalis	STR ERY TET CHL	1
	STR ERY TET	4
	ERY TET CHL	1

[a]CTX: cefotaxime; CAZ: ceftazidime; AMP: ampicillin; AZM: aztreonam; CIP: ciprofloxacin; FOX: cefoxitin; STR: streptomycin; TET: tetracycline; SXT: sulphamethoxazole + trimethoprim; STR: streptomycin; TET: tetracycline; ERY: erythromycin; CHL: chloramphenicol; [b]This isolate carried the gene encoding CMY-2 beta-lactamase

Resistance among Enterococcus *isolates*

Antibiotic resistance was detected in 75% (27/36) of enterococcal strains recovered from wild rabbits. High rates of resistance were observed to tetracycline (TET-R; 63.9%) and erythromycin (ERY-R; 30.5%). These frequencies are higher than those reported by Silva et al. (2010) from wild rabbits. Nevertheless,

Guerrero-Ramos et al. (2016) reported a similar rate of TET-R and a higher rate of ERY-R from wild game meat in Spain. Only two *E. faecalis* isolates were resistant to chloramphenicol (5.5%) (Table 1).

Table 3

Antibiotic resistance genes detected in *E. coli* and *Enterococcus* spp. strains recovered from wild rabbits

Species	Antibiotics	Number of resistant strains	Resistance gene and integron detected (number)
E. coli	Ampicillin, cefoxitin, cefotaxime, ceftazidime	1	bla_{TEM-1}, bla_{CMY-2}
	Tetracycline	32	*tet*(A) (26), *tet*(A)+*tet*(B) (6)
	Ciprofloxacin	16	*aac*(6')-Ib-*cr* (1)
	Sulphamethoxazole + trimethoprim	12	*int1* (8)
E. faecalis	Tetracycline	10	*tet*(M) (2), *tet*(M)+*tet*(L) (8)
	Erythromycin	10	*erm*(B) (6)
	Streptomycin	6	*ant*(6)-Ia (3)
E. hirae	Tetracycline	13	*tet*(M) (1), *tet*(M)+*tet*(L) (5)
	Erythromycin	1	*erm*(B) (1)

All enterococcal isolates were sensitive to ampicillin, vancomycin and teicoplanin. This is in agreement with the results of another study that noted the absence of VRE in wild rabbits (Silva et al., 2010). Nevertheless, other authors have found VRE in food-producing animals, including rabbits (López et al., 2009), wild rabbits (Figueiredo et al., 2009), in wild animals in Portugal (Poeta et al., 2005) and in wild meat game in Spain (Guerrero-Ramos et al., 2014). In Tunisia, acquired resistance to vancomycin has been recently described in the hospital setting, and in wild birds (Elhani et al., 2014; Klibi et al., 2015*b*).

High-level resistance to streptomycin (HLR-S) was detected in 18.2% and 12.1% of enterococcal strains, respectively. None of the strains presented a high-level resistance to gentamicin (HLR-G). High-level resistance to aminoglycosides was also found in clinical settings (Klibi et al., 2006), meat products (Klibi et al., 2013*b*; Guerrero-Ramos et al., 2016), as well as in healthy humans, pets and poultry (Poeta et al., 2006). Multiresistance to three or more different families of antibiotics was observed in six *E. faecalis* strains (16.7%) (Table 2).

The acquisition of antibiotic resistance could be explained by a possible exposure in the woodlands to faecal material from wild animals such as wild birds or even from humans (Allen et al., 2010). Moreover, wild rabbits as herbivorous animals could consume contaminated vegetation or water, which supports the hypothesis that antibiotic pressures exist even in natural environments (Da Costa et al., 2013).

The presence of antibiotic resistance genes was studied by PCR in all re-sistant enterococci (Table 3). The combination of *tet*(M)+*tet*(L) genes was found in thirteen strains resistant to tetracycline, while three strains harboured only *tet*(M). These findings are in accordance with the results of other studies that have shown that the *tet*(M) gene is frequently found in enterococcal strains of different origins (Huys et al., 2004). The *erm*(B) gene, conferring resistance to erythromy-cin, was detected in seven strains. The predominance of the *erm*(B) gene is con-sistent with the findings of Santos et al. (2013) related to enterococci isolated from wild animals. Nevertheless, other mechanisms of resistance could be pre-sent in those isolates in which macrolide resistance genes were not found. The gene *ant*(6)-Ia, responsible for resistance to streptomycin, was detected in three strains. These genes were also found in previous reports among HLR-S and HLR-G enterococcal isolates from food-producing animals, vegetables, and farm environments in Tunisia (Klibi et al., 2015*a*; Ben Said et al., 2015). The gene *cat*(A) was not detected among our two chloramphenicol-resistant isolates, which suggests that other mechanisms of resistance may be present in these isolates.
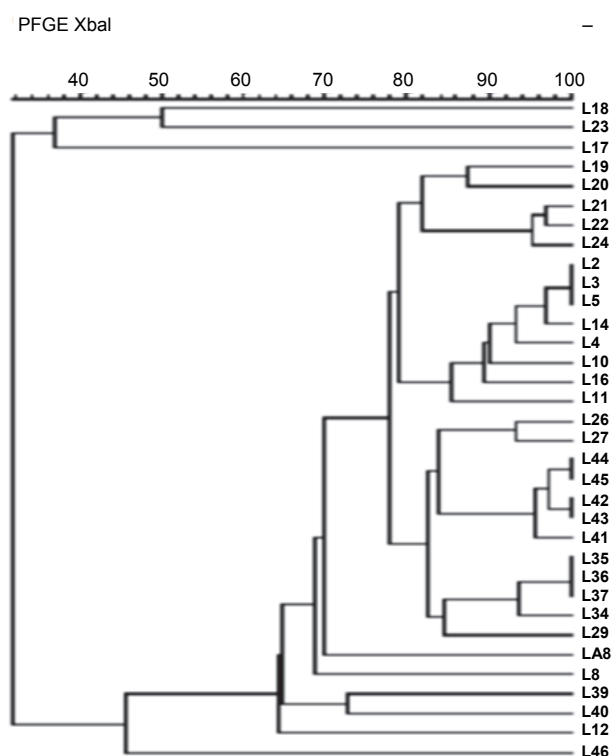

Fig. 1. Dendrogram based on XbaI-PFGE patterns. The GelCompar software (Applied Maths, Kortrijk, Belgium) was used to register macrorestriction patterns and clustering analysis was performed using Dice similarity coefficient and the unweighted-pair group method with arithmetic mean (UPGMA) among *E. coli* isolates

Virulence and molecular typing

The virulence gene *eae* was detected in six isolates (LA8, L4, L5, L20, L21 and L22) among the 34 *E. coli* strains tested (17.6%). This gene encodes intimin, a protein characteristic of enteropathogenic *E. coli* (EPEC) strains, involved in the induction of attaching and effacing *E. coli* (AEEC) lesions in the intestine and causing diarrhoea in rabbits (Ritchie et al., 2003). The gene *eae* was detected in *E. coli* of wild animals (wild boar, deer and owl) in a previous study (Alonso et al., 2017); nevertheless, *eae* was not detected in wild rabbits and hares in another study (Dotto et al., 2014), even though some of the strains came from rabbits with a diarrhoeic syndrome. The origin of the *eae*-positive *E. coli* isolates in our study is not known, although dissemination in the environment could occur. Our finding seems to represent the first report of *eae*-positive *E. coli* in wild rabbits.

Molecular typing by PFGE showed a high level of diversity, 28 different pulsotypes were observed among 34 *E. coli* isolates (Fig. 1). For *Enterococcus*, macrorestriction by SmaI enzyme revealed a genomic diversity for *E. faecalis*, with 12 different pulsotypes detected among seventeen isolates. However, we have noticed a clonality in *E hirae* strains; in fact, only five pulsotypes were detected among nineteen isolates.

In conclusion, this is the first study in Tunisia showing that wild rabbits could represent a reservoir of *E. coli* and *Enterococcus* isolates carrying antimicrobial resistance genes*, which contributes to the spread of antimicrobial resistance into the environment. The use of wild rabbits as a food source for several animals and in the human diet facilitates the transfer of these resistant bacteria to other animals or even to humans. The high incidence of antibiotic resistance found in wild rabbits in the present study raises the question of where these antibiotic-resistant strains originated from. Their origin could be related to the interaction with farm waste, the exposure to faecal material of farm or wild animals and the consumption of contaminated water or vegetation. The same could be postulated for the origin of *eae*-positive *E. coli*. Further studies are needed to determine the prevalence of resistance and virulence genes of *E. coli* and enterococci in different ecosystems, especially in wildlife.

Acknowledgement

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