

MOLECULAR CHARACTERISATION OF ANTIMICROBIAL RESISTANCE AND VIRULENCE GENES IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM DIARRHOEIC AND HEALTHY RABBITS IN TUNISIA

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Abstract: The purpose of this study was to identify *Escherichia coli* isolates in diarrhoeic and healthy rabbits in Tunisia and characterise their virulence and antibiotic resistance genes. In the 2014-2015 period, 60 faecal samples from diarrhoeic and healthy rabbits were collected from different breeding farms in Tunisia. Susceptibility to 14 antimicrobial agents was tested by disc diffusion method and the mechanisms of gene resistance were evaluated using polymerase chain reaction and sequencing methods. Forty *E. coli* isolates were recovered in selective media. High frequency of resistance to tetracycline (95%) was detected, followed by different levels of resistance to sulphonamide (72.5%), streptomycin (62.5%), trimethoprim-sulfamethoxazole (60%), nalidixic acid (32.5%), ampicillin (37.5%) and ticarcillin (35%). *E. coli* strains were susceptible to cefotaxime, ceftazidime and imipenem. Different variants of *bla*_{TEM}, *tet*, *sul* genes were detected in most of the strains resistant to ampicillin, tetracycline and sulphonamide, respectively. The presence of class 1 integron was studied in 29 sulphonamide-resistant *E. coli* strains from which 15 harboured class 1 integron with four different arrangements of gene cassettes, *dfrA17+aadA5* (n=9), *dfrA1 + aadA1* (n=4), *dfrA12 + addA2* (n=1), *dfrA12+orf+addA2* (n=1). The *qnrB* gene was detected in six strains out of 13 quinolone-resistant *E. coli* strains. Seventeen *E. coli* isolates from diarrhoeic rabbits harboured the enteropathogenic *eae* genes associated with different virulence genes tested (*fimA*, *cnf1*, *aer*), and affiliated to B2 (n=8) and D (n=9) phylogroups. Isolated *E. coli* strains from healthy rabbit were harbouring *fimA* and/or *cnf1* genes and affiliated to A and B1 phylogroups. This study showed that *E. coli* strains from the intestinal tract of rabbits are resistant to the widely prescribed antibiotics in medicine. Therefore, they constitute a reservoir of antimicrobial-resistant genes, which may play a significant role in the spread of antimicrobial resistance. In addition, the *eae* virulence gene seemed to be implicated in diarrhoea in breeder rabbits in Tunisia.

Key Words: antibiotic resistance genes, breeding rabbits, *E. coli* isolates, integrons, virulence genes, Tunisia.

INTRODUCTION

Breeding rabbits in Tunisia is difficult and expensive; the sector is facing health vulnerability related to various microbial diseases. Indeed, high economic losses due to enteric diseases are often attributable to intestinal colonisation by *Escherichia coli* isolates in commercial rabbit farms (Camarda *et al.*, 2004).

Although *E. coli* belongs to normal microflora colonising the gastrointestinal tracts of most mammals and birds, certain strains have been associated with intestinal or extra-intestinal infections. Amongst these, enteropathogenic *E. coli* (EPEC) are the major cause of infant diarrhoea in developing countries and they could be responsible for recurrent diarrhoeas in wild and domestic birds (Panteado *et al.*, 2002; Zhao *et al.*, 2018).

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Indeed, EPEC is important cause of mortality in weaned and in suckling rabbits (Peantido *et al.*, 2002; Blanco *et al.*, 1996; Pohl *et al.*, 1993). In a recent report, Enteropathogenic *E. coli* infection was associated with up to 10.5% of diarrhoea cases in a large laboratory Dutch rabbit, and this infection was caused by *E. coli* isolates harbouring *eae* virulence factor (Swennes *et al.*, 2012).

Rabbits are used for research and food production. They are exposed to interspecies pathogen transmission and the zoonotic potential of animal EPEC strains emphasises the need for virulence determinant-based screening of *E. coli* isolates from diarrhoeic animals (Swennes *et al.*, 2012).

The virulence of such germs is associated with the presence of a chromosomal pathogenicity island called LEE (Locus of Enterocyte Effacement), which determines the 'attaching and effacing' (A/E) lesions described as the main factor responsible for the diarrhoea (Swennes *et al.*, 2012; Blanco *et al.*, 2006).

Over the last decade, selective pressure caused by intensive use of antimicrobial drugs in human and veterinary medicine, livestock, aquaculture, agriculture and food technology, associated with several mechanisms of bacterial genetic transfer, could have contributed to the emergence and spread of antimicrobial resistance in different bacteria groups (Barbosa and Levy, 2000; Coque *et al.*, 2008; Werner *et al.*, 2008). Antimicrobial agents exert a selection pressure not only on pathogenic, but also on commensal bacteria, especially *E. coli* which is considered to be a reservoir of antimicrobial resistance genes (Alonso *et al.*, 2017). Besides, *E. coli* are very efficacious in horizontal gene transfer to phylogenetically distant bacteria. Resistant bacteria from animals can infect humans by direct contact as well as via food products of animal origin (Da Costa *et al.*, 2013). Thus, they might represent a worldwide problem with severe repercussions on public health (Guardabassi *et al.*, 2004; Van den Bogaard *et al.*, 2000).

E. coli is intrinsically resistant to therapeutic levels of penicillin G, the first β -lactam introduced into clinical practice, related to its outer membrane barrier. *E. coli* is also resistant to several different classes of antibiotics with distinct mechanisms of action (Johnson *et al.*, 2012). In *E. coli*, β -lactamase production is the most important mediator of resistance to broad spectrum β -lactams. The β -lactamase enzymes are often carried in plasmids and are most commonly produced by *Enterobacteriaceae*, in general and by *E. coli*, in particular (Poirel *et al.*, 2012). They confer resistance to penicillin and cephalosporin and they are an emerging cause of multidrug resistance of Gram-negative bacteria (Poirel *et al.*, 2012).

In addition, animal breeding could represent a possible reservoir of antibiotic resistant and virulent bacteria, as observed in rabbits (Laukova *et al.*, 2019; Wang *et al.*, 2019), and contact with species of game hunted for their meat may transfer multidrug-resistant (MDR) and virulent bacteria to humans or to livestock. This would provide a biological mechanism for the increase of antibiotic resistance and virulence genes in human population (Allen *et al.*, 2010; Santos *et al.*, 2013).

The aim of the present study is the comparison of *E. coli* isolates collected from rabbits with a different health status, in Tunisia, and characterisation of the genes implicated in virulence and antimicrobial resistance.

MATERIAL AND METHODS

Sample collection, isolation and identification of *E. coli* isolates

Sixty weaned rabbits (4-12 wk of age) were collected from six different intensive rabbitries in Tunisia, during 2014-2015. Rectal swabs were collected from 20 and 40 diarrhoeic and healthy rabbits, respectively. Samples from diarrhoeic animals were collected from two different farms (F1 and F2) situated in two different regions (Monastir and Tunis); samples from healthy animals were recovered from four farms (F3, F4, F5, F6) located in two other regions (Sfax and Nabeul). Sampling information is shown in Table 1.

Faecal samples were suspended in 5 mL of peptone water and then incubated at 37°C for 24 h. Serial dilutions in enrichment broth were seeded in MacConkey agar plates and incubated 24 h at 37°C for *E. coli* recovery. One colony per sample with typical *E. coli* morphology was selected and identified by classical biochemical methods (Gram staining, indole, citrate and urease), and confirmed by species-specific polymerase chain reaction (PCR) of *uidA* gene

Table 1: Sequence primers used.

Primer name	Sequence (5'-3')	References
<i>tetA</i>	F: GTAATCTGCACTGTCCG	Guardabassi <i>et al.</i> , 2000
	R: CTGCCTGGACAACATTGCAA	
<i>tetB</i>	F: CTCAGTATTCCAAGCCTTTG	Guardabassi <i>et al.</i> , 2000
	R: CTAAGCACTTGTCTCCTGT	
<i>BlaTEM</i>	F: ATTCTTGAAGACGAAAGGGC	Belaouaj <i>et al.</i> , 1994
	R: ACGCTCAGTGAACGAAAAC	
<i>BlaSHV</i>	F: CACTCAAGGATGATATGTG	Pitout <i>et al.</i> , 1998
	R: TTAGCGTTGCCAGTGCTCG	
<i>aad A</i>	F: GCAGCGCAATGACATTCTTG	Sanez <i>et al.</i> , 2004
	R: ATCCTTCGGCGCGATTITG	
<i>Sul 1</i>	F: TGGTGACGGTGTTCGGCATTG	Mazel <i>et al.</i> , 2000
	R: GCGAGGGTTTCCGAGAAGGTG	
<i>Sul 2</i>	F: CGGCATCGTCAACATAACC	Maynard <i>et al.</i> , 2003
	R: GTGTCCGGATGAAGTCAG	
<i>Sul 3</i>	F: GAGCAAGATTTTTGGAATCG	Perreten and Boerlin, 2003
	R: CATCTGCAGCTAACCTAGGGCTTTGGA	
<i>qnrA</i>	F: GGGTATGGATATTATGATAAA	Oktem <i>et al.</i> , 2008
	R: CTATCCGGCAGCACTATTA	
<i>qnrB</i>	F: GGMATHGAAATTCGCCACTG	Oktem <i>et al.</i> , 2008
	R: TTTGCYGYCGCCAGTCGAA	
<i>aac(6)-Ib-cr</i>	F: TTGCATGCTCTATGAGTGGCTA	Park <i>et al.</i> , 2006
	R: CTCGAATGCCTGGCGTGTGTT	
<i>Int I</i>	F: GGGTCAAAGGATCTGGATTTGCG	Mazel <i>et al.</i> , 2000
	R: ACATCGCTAAATCATCGTCCG	
<i>Int II</i>	F: CQCGGATATGCGACAAAAGGT	Mazel <i>et al.</i> , 2000
	R: GTAGCAAACGAGTGACGAAATG	
<i>qacΔ1+sul1</i>	F: GGCTGGCTTTTTCTGTTATCG	Sanez <i>et al.</i> , 2004
	R: GCGAGGGTTTCCGAGAAGGTG	
<i>chuA</i>	F: GACGAACCAACGGTCAGGAT	Clermont <i>et al.</i> , 2000
	R: TGCCGCCAGTACCAAAGACA	
<i>yjA</i>	F: TGAAGTGCAGGAGACGCTG	Clermont <i>et al.</i> , 2000
	R: ATGGAGAATGCGTTCCTCAAC	
<i>tspEAC2</i>	F: GAGTAATGTCGGGGCATTCA	Clermont <i>et al.</i> , 2000
	R: CGCGCCAACAAGTATTACG	
<i>fimA</i>	F: GTTGTCTGTGCGGCTGTGCR	Joaquim.R <i>et al.</i> , 2002
	R: ATGGTGTGGTTCGGTATTTC	
<i>papG</i>	F: CATTATCGTCCTCCTCAACTTAG	Ruiz <i>et al.</i> , 2002
	R: AAGAAGGGATTTGTAGCGTC	
<i>aer</i>	F: TACCGGATTGCATATGCAGACCGT	Yamamoto <i>et al.</i> , 1995
	R: AATATCTTCTCCAGTCCGGAGAAG	
<i>eae</i>	F: CATTATGGAACGGCAGGT	Beaudry M, 1996
	R: ATCTTCTGCGTACTGCGTTCA	
<i>cnf1</i>	F: AAGATGGAGTTTCTATGCAAGGAG	Yamamoto <i>et al.</i> , 1995
	R: CATTAGAGTCTGCCCTCATTATT	
<i>papC</i>	F: GACGGCTGACTGCAGGGTGTGGCG	Ruiz <i>et al.</i> , 2002
	R: ATATCCTTTCTGCAGGGATGCAATA	
<i>bfp</i>	F: ACAAAGATACAACAACAAAAA	Ruiz <i>et al.</i> , 2002
	R: TTCAGCAGGAGTAAAAGCAGTC	
<i>sxt 1</i>	F: GAA CGA AAT AAT TTA TAT GT	Ruiz <i>et al.</i> , 2002
	R: TTT GAT TGT TAC AGT CAT	
<i>hlyA</i>	F: AACAAAGGATAAGCACTGTTCTGGCT	Ruiz <i>et al.</i> , 2002
	R: ACCATATAAGCGGTCAATCCCGTCA	

(Jouini *et al.*, 2009). Extraction of isolate DNA was performed by the boiling method and *E. coli* ATCC 25922 was used as a control strain.

Antimicrobial susceptibility testing

The susceptibility of identified strains to 14 antimicrobial agents was performed using the disc diffusion method in accordance with CLSI recommendations (Clinical and Laboratory Standards Institute, 2015). Briefly, a standardised inoculum of bacterial suspension (standardised at 0.5 McFarland turbidity) was swabbed onto the surface of Mueller-Hinton agar. Antibiotic discs were placed on the surface and the size of the inhibition zone around the disc was measured after overnight incubation at 37°C. Antimicrobial agents tested (µg/disc) were as follows: ampicillin (10), amoxicillin-clavulanic acid (20/10), ticarcillin (75), cefotaxime (30), ceftazidime (30), imipenem (10), gentamicin (10), tobramycin (10), streptomycin (10), nalidixic acid (30), ciprofloxacin (5), trimethoprim-sulfamethoxazole (SXT) (1.25/23.75), tetracycline (30) and sulphonamides (200). *E. coli*-ATCC 25922 was used as a quality control strain.

Detection of antimicrobial resistance genes

The presence of genes associated with resistance to tetracycline (*tetA*, *tetB*), β- lactams (*bla*_{TEM}, *bla*_{SHV}), streptomycin [*aadA1*, *aadA2*], sulfamethoxazole [*sul1*, *sul2*, *sul3*] was investigated by PCR (Sáenz *et al.*, 2004), including positive and negative controls from the Institute Pasteur laboratory collection. The *bla*_{TEM} amplicons were sequenced to determine the type of β-lactamase gene. In addition, the quinolone resistance genes (*qnrA*, *qnrB*, *aac(6')-Ib*) were detected by PCR and DNA sequencing (Ben Slama *et al.*, 2011; Jacoby *et al.*, 2011). Primers and experimental conditions are listed in Table 1.

Detection of integrons

The presence of *int1* and *int2* genes (encoding class 1 and class 2 integrases, respectively) as well as the 3'-conserved segment (3'-CS) (*qacED1-sul1* genes) of class 1 integron was examined by PCR in all sulphonamide-resistant isolates. The variable regions of class 1 integron were characterised by PCR and sequencing and their sequences compared to those from the GenBank to identify gene cassettes (Sáenz *et al.*, 2004).

Phylotyping and virulence genotyping of *E. coli*

The identification of the major phylogenetic groups of *E. coli* isolates was determined by PCR using a combination of three gene sequences (*chuA*, *yjA* and *tspEAC2*) (Clermont *et al.*, 2000).

Nine virulence factors often found in pathogenic *E. coli* (ExPEC), namely *fimA* (encoding type 1 fimbriae), *papG* allele III (adhesin PapG, class III), *hlyA* (haemolysin), *cnf1* (cytotoxic necrotising factor), *papC* (P fimbriae), *aer* (aerobactin iron uptake system), *eae* (intimin), *bfp* (bundle forming pilus) and Shiga toxin (*sxt1* and *sxt2*) were amplified using primers and single or multiplex PCR assays as previously reported (Ruiz *et al.*, 2002).

RESULTS

Bacteria isolation

A total of 40 *E. coli* strains were isolated from 60 samples of which 18 and 22 strains from diarrhoeic and healthy rabbit, respectively (Table 2). The other tested samples (2 and 22 from diarrhoeic and healthy rabbit respectively) showed a negative culture of *E. coli* isolates.

Resistance among *E. coli* isolates

Analysis of antimicrobial resistance detected in our collection showed a high frequency of resistance to tetracycline (95%) followed by resistance to sulphonamide (72.5%), streptomycin (62.5%), trimethoprim-sulfamethoxazole (60%), nalidixic acid (32.5%), ampicillin (37.5%) and ticarcillin (35%). Multi-resistance to three or more different classes of antibiotics was observed in 27 *E. coli* isolates (67.5%) (Table 2). Lower resistance frequency was recorded

Table 2: Resistance phenotype, integrons and resistance genes in *E. coli* from rabbit faecal samples in Tunisia

Strains	Origin	farm	Health status	Antimicrobial resistance phenotype ^a	Resistance genes	Class 1 integrons	
						Int1/qac-sul1	Genes cassette
EC1	Monastir	F1	Diarrhoea	NA,S,SXT,TET,AMP,SUL	<i>tet A, bla_{TEM-1b}</i>	+/+	<i>dfrA17+aadA5</i>
EC2	Monastir	F1	Diarrhoea	NA,S,SXT,TET,SUL	<i>tet B</i>	+/+	<i>dfrA17+aadA5</i>
EC3	Monastir	F1	Diarrhoea	SXT, TET	<i>tet A</i>	-/-	
EC4	Monastir	F1	Diarrhoea	NA,S,SXT,TET,AMP,TIC,SUL	<i>tet A- bla_{TEM-1b'} qnrB</i>	+/+	<i>dfrA1+aadA1</i>
EC5	Monastir	F1	Diarrhoea	NA,S,SXT,TET,SUL	<i>tet B</i>	+/+	<i>dfrA17+aadA5</i>
EC6	Monastir	F1	Diarrhoea	NA,S,SXT,TET,SUL	<i>tet A, qnrB</i>	+/+	<i>dfrA17+aadA5</i>
EC7	Monastir	F1	Diarrhoea	S, TET, AMP, TIC, SUL	<i>tetB- bla_{TEM-1b'} sul1, sul2</i>	-/-	
EC8	Monastir	F1	Diarrhoea	SXT,TET,AMP,TIC,SUL	<i>tetB- bla_{TEM-1b'} sul2</i>	+/+	<i>dfrA17+aadA5</i>
EC9	Monastir	F1	Diarrhoea	S,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b'} sul2</i>	+/+	<i>dfrA1+aadA1</i>
EC30	Tunis	F2	Diarrhoea	S,SXT, TET, SUL	<i>tetA, sul1, sul2</i>	-/-	
EC31	Tunis	F2	Diarrhoea	AMC		-/-	
EC32	Tunis	F2	Diarrhoea	TET	<i>tetB</i>	-/-	
EC33	Tunis	F2	Diarrhoea	S,SXT, TET, SUL	<i>tetB</i>	+/+	<i>dfrA1+aadA1</i>
EC34	Tunis	F2	Diarrhoea	S,SXT, TET, SUL	<i>tetA</i>	+/+	<i>dfrA1+aadA1</i>
EC35	Tunis	F2	Diarrhoea	S,SXT, TET, SUL	<i>tetA</i>	+/+	<i>dfrA17+aadA5</i>
EC36	Tunis	F2	Diarrhoea	NA,S,SXT,TET,SUL	<i>tetB</i>	+/+	<i>dfrA17+aadA5</i>
EC37	Tunis	F2	Diarrhoea	SXT,TET	<i>tetA</i>	-/-	
EC38	Tunis	F2	Diarrhoea	TET	<i>tetA</i>	-/-	
EC39	Nabeul	F3	Healthy	TET, AMP, TIC	<i>tetB- bla_{TEM-1b}</i>	-/-	
EC40	Nabeul	F3	Healthy	TET	<i>tetA</i>	-/-	
EC10	Sfax	F4	Healthy	NA,S,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b}</i>	+/+	<i>dfrA17+aadA5</i>
EC11	Sfax	F4	Healthy	NA,S,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b'} qnrB</i>	+/+	<i>dfrA17+aadA5</i>
EC12	Sfax	F4	Healthy	S,SXT,TET,AMP,TIC,SUL	<i>tetB- bla_{TEM-1b}</i>	-/-	
EC13	Sfax	F4	Healthy	NA,S,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b'} qnrB</i>	+/+	<i>dfrA12+aadA2</i>
EC14	Sfax	F4	Healthy	S,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b'} sul1, sul2</i>	-/-	
EC15	Sfax	F4	Healthy	NA,S,SXT,TET,AMP,TIC,SUL	<i>tetB- bla_{TEM-1b'} qnrB, sul1, sul2</i>	-/-	
EC16	Sfax	F4	Healthy	NA,S,TOB,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b}</i>	+/+	<i>dfrA12+orf+aadA2</i>
EC17	Sfax	F4	Healthy	S, TET, AMP, TIC, SUL	<i>tetB- bla_{TEM-1b'} sul2</i>	-/-	
EC18	Sfax	F5	Healthy	NA,TET,SUL	<i>tetA, sul2</i>	-/-	
EC19	Sfax	F5	Healthy	TET,SUL	<i>tetA, sul3</i>	-/-	
EC20	Sfax	F5	Healthy	S, TET, SUL	<i>tetA, sul3</i>	-/-	
EC21	Sfax	F5	Healthy	TET	<i>tetA</i>	-/-	
EC22	Sfax	F5	Healthy	NA,S,TOB,SXT,TET,AMP,TIC,SUL	<i>tetA- bla_{TEM-1b'} qnrB, sul1, sul2</i>	-/-	
EC23	Sfax	F5	Healthy	CN		-/-	
EC24	Sfax	F6	Healthy	S, TET, SUL	<i>tetB, sul1, sul2</i>	-/-	
EC25	Sfax	F6	Healthy	S,SXT, TET, SUL	<i>tetA, sul3</i>	-/-	
EC26	Sfax	F6	Healthy	TET	<i>tetA</i>	-/-	
EC27	Sfax	F6	Healthy	S, TET, SUL	<i>tetA, sul 3</i>	-/-	
EC28	Sfax	F6	Healthy	TET, SUL	<i>tetB, sul3</i>	-/-	
EC29	Sfax	F6	Healthy	TET	<i>tetA</i>	-/-	

^aTET, tetracycline; SXT, trimethoprim-sulfamethoxazole; SUL, sulphonamide; S, streptomycin; AMP, ampicillin; NA, nalidixic acid; CN, gentamycin; TIC, ticarcillin, AMC, amoxicillin/clavulanic acid; TOB, tobramycin.

for tobramycin (5%), gentamicin (2.5%) and amoxicillin-clavulanic acid (2.5%). None of these strains were resistant to cefotaxime, ceftazidime and imipenem and all the strains were resistant to at least one antibiotic. The three most common antimicrobial resistance profiles of these isolates were NA-S-SXT-TET-AMP-TIC-SUL (n=5), S-SXT-TET-SUL (n=5), NA-S-SXT-TET-SUL (n=4).

Detection of resistance genes

The resistance genes detected among our antimicrobial-resistant *E. coli* isolates are shown in Table 2. Fifteen ampicillin-resistant isolates were detected in this study, and all of them harboured a *bla*_{TEM} gene (encoding a TEM beta-lactamase). The *bla*_{TEM} amplicon was sequenced in these isolates, and the *bla*_{TEM-1b} gene was identified in all of them. All of our 38 tetracycline-resistant isolates contained *tet*(A) (n=25) or *tet*(B) (n=13). For the 29 sulphonamide-resistant isolates, they all harboured the *sul1* gene inside the integron, *sul2* and *sul3* genes were detected respectively in 4 and 5 strains; only one combination of *sul1-sul2* was found in 6 strains. The mechanisms of resistance of 13 isolates to quinolones were confirmed by the presence of only *qnrB* gene in 6 strains; *aac* (6')-Ib-cr and *qnrA* genes were not detected.

Detection of integrons

Class I integrons was detected in 15 *E. coli* isolates out of 29 sulphonamide-resistant ones. *Int1* and *qacEsul1* genes in the 3' conserved region was detected in all studied isolates. The variable regions of class 1 were amplified by PCR and sequenced for all *int1* positive isolates, and their sequences were compared with those in GenBank to identify gene cassettes. Four kinds of gene cassette arrangement were found in Class I integron as follows (number of strains): *dfrA17+aadA5* (n=9), *dfrA1+aadA1* (n=4), *dfrA12+addA2* (n=1), *dfrA12+orf+addA2* (n=1). Class 2 integron was not detected among the studied isolates.

Phylotyping and virulence genotyping of *E. coli*

Eight and nine studied strains were shown to belong to B2 or D phylogenetic groups, respectively, and contained at least three out of eight virulence genes tested. Seventeen *E. coli* isolates recovered from diarrhoeic rabbits harboured *eae* gene and affiliated to B2 or D phylogenetic groups; nevertheless, strains isolated from healthy rabbits were *eae* gene negative and contained only *fimA* (n=15) gene. These strains were affiliated to A (n=14) and B1 (n=8) phylogenetic groups (Table 3). In addition, *E. coli* positive isolates for *eae* gene harboured the majority *fimA* gene with different virulence factor tested. Four types of combination of virulence genes were detected in *E. coli* isolates recovered from diarrhoeic rabbits, as follows: *aer+fimA*, *hly+eae* (n=4), *cnf+aer+fimA+eae* (n=8), *eae+cnf1+fimA* (n=3) and *aer+papGIII+eae* (n=3).

DISCUSSION

The high presence of *E. coli* isolates in faecal samples from weaned rabbit, whether diarrhoeic or healthy ones, suggested that the isolated germs should be considered as part of the resident coli-flora of rabbit (Kaper *et al.*, 2004). The detection of *E. coli* isolates in rabbits was reported in previously studies (Sweenes *et al.*, 2012; Zhao *et al.*, 2018). Indeed, *E. coli* is the most important and prevalent pathogen of the gastrointestinal tracts of humans and warm-blooded animals (Kaper *et al.*, 2004). As commensal bacteria, it lives in a mutually beneficial association with its host, is often responsible for a broad spectrum of diseases such as enteritis or urinary tract infections, and could be considered the most common pathogen for animals (Yoo *et al.*, 2009).

The results of our study showed alarming resistance frequencies expressed by *E. coli* in rabbit, especially to tetracycline (95%), sulphonamide (72.5%), streptomycin (62.5%) and trimethoprim-sulfamethoxazole (60%). Similarly, high percentages of resistant *E. coli* isolated from wild rabbits (Miranho *et al.*, 2014), food-producing animals (Sáenz *et al.*, 2001) and pigs (Teshageret *et al.*, 2000) were detected for these types of antibiotics in other countries. A high tetracycline resistance was also reported in rabbit farms in China, which has been related to the fact that tetracycline is being widely used to control and prevent rabbit diseases (Zhao *et al.*, 2018). In Africa and especially in Tunisia, the information on therapeutic or prophylactic antibiotic use in livestock is often missing and confidential. In the tested

Table 3: Phylogenetic groups and virulence genes in *E. coli* from rabbit faecal samples in Tunisia.

Strains	Origin	Farm	Health Status	Virulence Factors	Phylogenetic Groups
EC1	Monastir	F1	Diarrhoea	<i>aer, fimA, hly, eae</i>	B2
EC2	Monastir	F1	Diarrhoea	<i>aer, fimA, hly, eae</i>	D
EC3	Monastir	F1	Diarrhoea	<i>aer, papGIII, eae</i>	B2
EC4	Monastir	F1	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	D
EC5	Monastir	F1	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	D
EC6	Monastir	F1	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	B2
EC7	Monastir	F1	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	D
EC8	Monastir	F1	Diarrhoea	<i>eae, cnf1, fimA</i>	D
EC9	Monastir	F1	Diarrhoea	<i>eae, cnf1, fimA</i>	D
EC30	Tunis	F2	Diarrhoea	<i>eae, cnf1, fimA</i>	B2
EC31	Tunis	F2	Diarrhoea	-	A
EC32	Tunis	F2	Diarrhoea	<i>aer+ fimA, hly, eae</i>	D
EC33	Tunis	F2	Diarrhoea	<i>aer+ fimA, hly, eae</i>	B2
EC34	Tunis	F2	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	B2
EC35	Tunis	F2	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	B2
EC36	Tunis	F2	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	D
EC37	Tunis	F2	Diarrhoea	<i>cnf1, aer, fimA, eae</i>	B2
EC38	Tunis	F2	Diarrhoea	<i>aer, papGIII, eae</i>	D
EC39	Nabeul	F3	Healthy	<i>aer, papGIII</i>	B1
EC40	Nabeul	F3	Healthy	<i>cnf1, fimA</i>	B1
EC10	Sfax	F4	Healthy	<i>cnf1, fimA</i>	B1
EC11	Sfax	F4	Healthy	<i>cnf1, fimA</i>	B1
EC12	Sfax	F4	Healthy	<i>cnf1, fimA</i>	B1
EC13	Sfax	F4	Healthy	<i>cnf1, fimA</i>	B1
EC14	Sfax	F4	Healthy	<i>fimA</i>	B1
EC15	Sfax	F4	Healthy	<i>fimA</i>	B1
EC16	Sfax	F4	Healthy	<i>cnf1, fimA</i>	B1
EC17	Sfax	F4	Healthy	<i>cnf1, fimA</i>	A
EC18	Sfax	F5	Healthy	<i>fimA</i>	A
EC19	Sfax	F5	Healthy	<i>fimA</i>	A
EC20	Sfax	F5	Healthy	<i>fimA</i>	B1
EC21	Sfax	F5	Healthy	<i>fimA</i>	A
EC22	Sfax	F5	Healthy	<i>cnf1,</i>	A
EC23	Sfax	F5	Healthy		B1
EC24	Sfax	F6	Healthy	<i>cnf1</i>	A
EC25	Sfax	F6	Healthy	<i>cnf1</i>	B1
EC26	Sfax	F6	Healthy	<i>cnf1</i>	B1
EC27	Sfax	F6	Healthy	<i>cnf1,</i>	A
EC28	Sfax	F6	Healthy	<i>fimA</i>	A
EC29	Sfax	F6	Healthy	<i>fimA</i>	A

Tunisian farms, we managed to form an idea about the consumption of antibiotics thanks to information from the breeders, showing that the tetracycline and sulphamide families were the antibiotics most used in rabbit breeding. The overuse of antibiotics in farms could cause the high antibiotic resistance percentages for these families detected in this study.

Nevertheless, all identified strains were susceptible to cefotaxime, ceftazidime and imipenem; this might be due to the less frequent use of these antibiotics in rabbit farms. In addition, the β -lactam antibiotics are highly toxic for rabbits and are not frequently used to treat the infection in breeding rabbits. In recent decades, the prevalence of multidrug-resistant (resistant at least to 3 antibiotic families) *Enterobacteriaceae* has been increasing worldwide and constitutes a potential concern for public health. Our results showed that the frequency of MDR strains among all isolated *E. coli* strains was 67.5%, which is higher than those observed in China (50.9%) (Zhao *et al.*, 2018). This could be due to the uncontrolled overuse of antibiotics in breeder farms in the African continent, where the information on antibiotics administration was often not confirmed by veterinarians (Eager *et al.*, 2012).

The *bla*_{TEM} gene encodes TEM enzymes, which are the predominant plasmid mediated β -lactamases in Gram-negative bacteria, previously found in ampicillin-resistant *E. coli* isolates from different origins (Brinas *et al.*, 2002). The *bla*_{TEM} amplicon was sequenced in 15 isolates, and the variant *bla*_{TEM-1b} gene was identified in all of them. This gene was also the most prevalent one in ampicillin-resistant *E. coli* isolates from food-producing animals in Tunisia (Jouini *et al.*, 2009; Ben Salama *et al.*, 2010) and from wild rabbits in Portugal (Marinho *et al.*, 2014).

The main mechanism of tetracycline resistance in *E. coli* isolates from rabbits is based on active efflux (Silva *et al.*, 2010), which was confirmed in our study, as *tet(A)* or *tet(B)* genes were detected in all the tetracycline-resistant strains. These genes have frequently been reported in human and animal isolates (Bryan *et al.*, 2004).

Interestingly, a *qnrB* gene was identified in six nalidixic acid-resistant isolates, and neither *qnrA* nor *aac(6')-Ib-cr* genes variant were detected. In fact, in Tunisia, Qnr plasmid-carrying *E. coli* strains were not described in animal isolates except in companion pets (Ben Sallem *et al.*, 2013). However, in China, *qnrS* and *aac(6')-Ib-cr* genes have been reported in faecal samples of diarrhoeic rabbit farms (Zhao *et al.*, 2018; Qing *et al.*, 2006). This difference might be the result of less frequent use of fluoroquinolone in our country, and the detection of quinolone resistance genes in this study might be related to other sources such as contaminated water and food. Indeed, in Tunisia, different reports described the contamination of wastewater treatment plants and vegetables with β -lactam- and quinolone-resistant bacteria (Ben Said *et al.*, 2015).

In this report, Class I integron were detected in 15 *E. coli* isolates out of 29 sulphonamide-resistant strains (37.5%), which was similar to the percentage (30.91% and 31.5%) of previous studies conducted by Zhao *et al.* (2018) and by Hai *et al.* (2014). In addition, Class I integrons are often associated with MDR *E. coli* isolates of animal origin, with diverse resistance genes in their gene cassettes (Allocatti *et al.*, 2013).

Different combinations of the *sul1*, *sul2*, and *sul3* genes were found in the 29 sulphonamide-resistant isolates; most of these isolates (20.69 %) harboured more than one *sul* gene. These findings are in agreement with the high prevalence of these genes in *E. coli* isolates from farms and wild rabbits or other animals in other studies (Zhoa *et al.*, 2018., Alonso *et al.*, 2017).

All the strains isolated from diarrhoeic rabbit contained at least three or four of the nine virulence genes tested and belonged to phylogenetic groups B2 or D. These phylogroups are associated with extra-intestinal infections and virulent strains (Clermont *et al.*, 2008). It is interesting to report that the virulence factor most commonly found was *eae*, characterising the enteropathogenic *E. coli* strain (EPEC). In addition, among these strains only four harboured the *hly* virulence gene.

The identification of this gene in diarrhoeic animals could testify that these *E. coli* strains were responsible for the disease. In fact, this gene encodes intimin, a protein characteristic in EPEC strains, involved in induction of attaching and effacing lesions in the intestine that cause diarrhoea in rabbits. The presence of *E. coli* isolates harbouring *eae* gene recovered from diarrhoeic rabbit was described in a study by Sweenes *et al.* (2012). In addition, pathogenicity of some strains may be enhanced by the presence of virulence genes AF/R1 and AF/R2, and a precise evaluation of the distribution of these genes in *E. coli* population is required to comprehend the ability to induce severe forms of enteric disease (Camarda *et al.*, 2004).

On other hand, the *cnf1*, *fimA* and *aer* genes which encode to cytotoxic necrotising factor, to fimbriae and to aerobactin iron uptake system, respectively, were detected in all *eae* positive strains; these genes, known for their adhesive role, were identified in previous studies in pathogenic *E. coli* strains isolated from food and animals (Ben Sallem *et al.*,

2013 et Jouini *et al.*, 2009). The absence of genes for toxins was expected, as these virulence factors have not been associated with *E. coli* isolated from rabbits in other countries (Blanco *et al.*, 1997).

It has been previously indicated that commensal *E. coli*, as is the case of our strains isolated from healthy rabbits, are more frequently ascribed to phylogroups A and B1 and harboured one or two virulence factors. In addition, these isolates present the same multi-resistance profile to the antibiotics and contain different resistance genes and integron detected in strain isolated from diarrhoeic rabbits. This finding could give idea of the transfer of genetic elements containing resistance genes between *E. coli* isolates according to the health status of rabbits and their environment.

To the best of our knowledge, there are no published data concerning antibiotic resistance and virulence genes from rabbit breeding in Tunisia; moreover, this type of data is also scarce in other African countries.

CONCLUSION

In this report, we detected enteropathogenic *E. coli* isolates in diarrhoeic rabbit with different resistance genes and integrons. Resistance to antibiotics was observable in all the isolates, and, moreover, there were high percentages of resistance to some of the drugs tested, with multiple resistance patterns being frequently observable. Our study indicates that gastrointestinal *E. coli* from rabbits destined for human consumption are resistant to widely prescribed antibiotics in medicine, and constitute a reservoir of antimicrobial resistance and virulence genes. The fact that multiple antimicrobial resistance patterns are highly common in the bacterial population might have critical consequences in farm management and hinder control of the disease. In addition, the onset and spread of antimicrobial resistance may represent a serious problem for public health, as animals could play a role of reservoirs of EPEC for humans and may transmit novel resistance-associated genes to human pathogens.

Conflict of Interest: Authors declare that there are no conflicts of interest.

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Ethical approval: All applicable national and institutional guidelines for the care and use of animals were followed.

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