Trop Anim Health Prod (2015) 47:975–981
DOI 10.1007/s11250-015-0818-3

REGULAR ARTICLES

Determination of extended spectrum β-lactamases/AmpC β-lactamases and plasmid-mediated quinolone resistance in *Escherichia coli* isolates obtained from bovine carcasses in Mexico

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Received: 2 October 2014 / Accepted: 31 March 2015 / Published online: 17 April 2015

Abstract Food-borne bacterial infections have worldwide importance, and a great variety of antibiotic resistance mechanisms, mainly of the chromosome type, have rapidly developed. Antimicrobial resistance was determined in this study in terms of the presence of extended-spectrum β-lactamases (ESBLs), plasmid AmpC β-lactamases (pAmpC), and plasmid-mediated quinolone resistance (PMQR) from 155 *Escherichia coli* isolates obtained from bovine carcasses from two states in Mexico (states of Mexico and Jalisco). Isolates were challenged with β-lactam antimicrobials (ampicillin, ceftazidime, and cefotaxime) and quinolones (nalidixic acid and ciprofloxacin). The presence of the bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, bla_{CMY}, bla_{MOX}, bla_{LAT}, bla_{BIL}, *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* genes was examined by PCR. Clonal relationship was determined using pulsed-field gel electrophoresis (PFGE). The highest resistance was found to be to nalidixic acid (64 %), followed by ampicillin (32 %), ciprofloxacin (10 %), and ceftazidime and cefotaxime (both 1.3 %). bla_{CMY} (n=1), bla_{TEM} (n=24), *qnrB* (n=9), and *qnrS* (n=7) genes were detected. PFGE analysis showed that the majority of isolates had a different genotypic profile. To our knowledge, this is the first report of the presence of the *qnrB*, *qnrS*, and bla_{CMY} genes in *E. coli* isolated from bovine meat in Mexico.

Keywords *Escherichia coli* · Antibiotic resistance · β-Lactams · Quinolones

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Introduction

Escherichia coli causes intestinal and extraintestinal infections due to harboring of various adherence and virulence factors (Bok et al. 2015). A large part of these infections is treated with β-lactam and quinolone antimicrobials, which are considered critical in human medicine by the World Health Organization. Nevertheless, their widespread use in the health sector, as well as their inadequate use in veterinary medicine, has put their usefulness at risk due to the spread of microorganisms that are resistant to these antibiotics (Collignon et al. 2009; Acar and Moulin 2013).

The presence of infections caused by *E. coli* resistant to β-lactam antimicrobials such as extended-spectrum cephalosporins and quinolones has reached very high levels (Poirel et al. 2012; Seiffert et al. 2013). The main resistance mechanism to these antimicrobials is generated through β-lactamase enzymes of which the extended-spectrum β-lactamases (ESBLs) of the TEM, SHV, and CTX-M families, as well as plasmid AmpC β-lactamases, are noteworthy. Both types of β-lactamases provide resistance to cephalosporins such as ceftazidime and cefotaxime, albeit they are different in that ESBLs are inactivated by β-lactamase inhibitors such as clavulanate, sulbactam, or tazobactam while plasmid AmpC

β-lactamases (pAmpC) are resistant to these inhibitors (Seiffert et al. 2013).

Furthermore, resistance to quinolones has increased during the last two decades in *E. coli* isolates obtained from human patients and hospital facilities, as well as from animal-origin isolates. Although the main mechanisms of resistance to quinolones are coded in the chromosome, plasmid-mediated quinolone resistance (PMQR) has been reported since 1998. These include the Qnr proteins, acetyltransferase aminoglycoside enzyme (AAC(6′)-Ib-cr), or the QepA efflux pump, which, although they provide low-level resistance to quinolones, they are a favorable start for the selection of additional resistance mechanisms. The increase in resistance to β-lactam and quinolone antimicrobials is possibly due to their widespread use in human and veterinary medicine (Poirel et al. 2012).

Apart from the acquisition of resistance genes, the interchange of *E. coli* isolates between humans and animals destined for human consumption is very important because the latter can act as reservoirs of isolates resistant to antimicrobials and cause disease in humans (Egervärn et al. 2014; Fortini et al. 2011).

In Mexico, the presence of the bla_{SHV} and $bla_{CTX\text{-}M}$ ESBL coding genes has been reported from hospital infections. Furthermore, the *qnr A*, *B*, and *S*; *aac(6')-Ib-cr*; and *qepA* genes have also been reported. All these genes for antimicrobial resistance have been identified in various genera of the *Enterobacteriaceae* family isolated mainly in hospitals for humans throughout the country (Garza-González et al. 2011; Silva-Sánchez et al. 2013).

In contrast, little is known in Mexico on the presence of these resistance genes in animal-origin or meat product isolates. Only a single study has demonstrated the presence of pAmpC $bla_{CMY\text{-}2}$ in animal-origin *Salmonella enterica* serovar Typhimurium isolates (Zaidi et al. 2007). The objective of this study was to examine the presence of ESBL/pAmpC and PMQR determinants in *E. coli* isolates from bovine meat of two states in Mexico.

Materials and methods

Bacterial isolates A total of 155 *E. coli* isolates were collected from two Republic Mexican states, 75 isolates originated from the State of Mexico: 56 were from bovine carcasses in municipal slaughterhouses, 12 from food processing plants, and 7 from minced meat in butcher's shops (3-month sampling duration, once each month). Eighty bovine carcass isolates in municipal slaughterhouses in Jalisco (10-month duration) were collected. All samples collected from 2009 to 2012 came from fattened beef calves reared in each state. Carcass and colon samples were taken; approximately 1 g of feces was taken directly from the colon and was placed in an assay tube with 9 ml of 1 % peptonate water. Carcass samples were taken after carcass washing and before refrigeration using a sterile cotton swab moistened with 0.1 % NaCl at 0.85 %; afterwards, each swab was placed in a Falcon-type tube with 25 ml of 1 % peptonate water (European Commission 2001). Samples were transported under refrigeration until their arrival to the laboratory. All samples were incubated for 24 h at 37 °C.

Isolates were inoculated into MacConkey agar plates; a single colony from each plate was selected, subjected to conventional biochemical tests, and confirmed by PCR using primers UAL1939b 5′-ATGGAATTTCGCCGATTTTGC-3′ and UAL2105b 5′-ATTGTTTGCCTCCCTGCTGC-3′ to identify the *uidA* gene that codes for the β-glucuronidase enzyme (Heijnen and Medema 2006).

Antimicrobial susceptibility testing The test was carried out using the disk diffusion method on Mueller-Hinton agar, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2012a). The following antimicrobials were used: ampicillin 10 μg (AMP), ceftazidime 30 μg (CAZ), cefotaxime 30 μg (CTX), nalidixic acid 30 μg (NAL), and ciprofloxacin 5 μg (CIP). Isolates resistant to β-lactam antimicrobials were subjected to a ESBL confirmatory test using cefotaxime and ceftazidime on their own and in association with 10 μg clavulanic acid (CLA), as described by CLSI (2012b). The ATCC 25922 *E. coli* strain was used as control.

Detection of antimicrobial resistance genes All isolates were tested using PCR in order to detect the bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{CMY}, bla_{MOX}, bla_{LAT}, and bla_{BIL} genes using the previously mentioned primers (Table 1) (Dallenne et al. 2010). In order to determine the presence of the *qnrA*, *qnrB*, and *qnrS* genes, a multiplex PCR was used followed by an end-point PCR. The *aac(6')-Ib-cr* and *qepA* genes were analyzed using primers specific to each gene (Table 1) (Cattoir et al. 2007; Park et al. 2006; Yamane et al. 2007).

Pulsed-field gel electrophoresis The clonal relationship of the strains which were resistant to CAZ, CTX, and CIP was carried out using pulsed-field gel electrophoresis (PFGE) of samples digested with the XbaI restriction enzyme in agarose following the standardized protocol of the Centers for Disease Control and Prevention, Atlanta, GA (Hunter et al. 2005). Electrophoresis was carried out using a CHEF-DRII chamber. Band patterns were analyzed using the NTSYSpc version 2.2 software while the relationships among genotypes were determined using the unweighted pair group method with arithmetic mean (UPGMA) and band similitude was calculated using Dice's coefficient.

Statistical analysis The significant differences ($P<0.05$) in the gene presence and the sample origin were assessed by

Table 1 Primers used for detecting the β-lactam and quinolone resistance genes

Primer	Sequence (5′→3′)	Gene	Amplicon size (bp)
MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	bla_{TEM}	800
MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC		
MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	bla_{SHV}	713
MultiTSO-S_rev	ATCCCGCAGATAAATCACCAC		
MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	bla_{OXA}	564
MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG		
MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA[a]	bla_{CTX-M} Grupo 1	688
MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT[a]		
MultiCTXMGp2_for	CGTTAACGGCACGATGAC	bla_{CTX-M} Grupo 2	404
MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT[a]		
MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	bla_{CTX-M} Grupo 9	561
MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG		
MultiCaseMOX_for	GCAACAACGACAATCCATCCT	bla_{MOX}, bla_{CMY}	895
MultiCaseMOX_rev	GGGATAGGCGTAACTCTCCCAA		
MultiCaseCIT_for	CGAAGAGGCAATGACCAGAC	bla_{LAT}, bla_{BIL}, bla_{CMY}	538
MultiCaseCIT_rev	ACGGACAGGGTTAGGATAGY[a]		
QnrAm-F	AGAGGATTTCTCACGCCAGG	*qnrA*	580
QnrAm-R	TGCCAGGCACAGATCTTGAC		
QnrBm-F	GGMATHGAAATTCGCCACTG[a]	*qnrB*	264
QnrBm-R	TTTGCYGYYCGCCAGTCGAA[a]		
QnrSm-F	GCAAGTTCATTGAACAGGGT	*qnrS*	428
QnrSm-R	TCTAAACCGTCGAGTTCGGCG		
AAC(6′)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	*aac(6')-Ib-cr*	482
AAC(6′)-Ib-cr	CTCGAATGCCTGGCGTGTTT		
QEPA-F	GCAGGTCCAGCAGCGGGTAG	*QepA*	199
QEPA-R	CTTCCTGCCCGAGTATCGTG		

[a] Y=T or C, R=A or G, S=G or C, D=A or G or T, H=A or C or T

the chi-square test and Yates's correction using SPSS for Windows ver. 20.0 (SPSS, Chicago, IL).

Results

Of the 155 *E. coli* isolates confirmed by biochemical tests and the presence of the *uidA* gene, 64 % (99/155) were resistant, 6 % (9/155) were intermediately resistant, and 30 % (47/155) were susceptible to NAL. Nevertheless, in the case of CIP, 88 % (135/155) of isolates were susceptible, 2 % (3/155) were intermediately resistant, and 10 % (16/155) were resistant. In terms of AMP, 65 % (100/155) were susceptible, 3 % (5/155) were intermediately resistant, and 32 % (50/155) were resistant. Only two isolates (1.3 %) showed resistance to CAZ and CTX, while the rest (98.7 %, 153) were susceptible. The confirmatory test for determining ESBLs in these two isolates showed a different pattern to that established by CLSI since they were resistant to CAZ, CTX, and their combination with CLA.

The bla_{TEM} gene was present in 48 % (24/50) of the isolates resistant to AMP. Of the two isolates suspected of producing the AmpC β-lactamase, only one had the bla_{CMY} gene while for the other, the gene that provided β-lactam antibiotic resistance, could not be determined (Table 2). Up to 5 % (5/100) of the isolates phenotypically susceptible to AMP had the bla_{TEM} gene, while none of the intermediately resistant isolates had this gene. All isolates that had the bla_{TEM} gene were phenotypically susceptible to CAZ and CTX.

In terms of resistance to quinolones, we found that 10 % (16/155) of the isolates had some of the *qnr* genes, 9 isolates had the *qnrB* gene, and 7 had the *qnrS* gene, while no isolate had the *qnrA* gene. Up to 87.5 % (14/16) of the isolates that had a *qnr* gene were phenotypically susceptible to CIP, and only 12.5 % (2/16) of the isolates that also had the *qnrS* gene were resistant to this antimicrobial. Furthermore, only 31 % (5/16) of the isolates had both the *qnr* and the bla_{TEM} gene. The *aac(6')-Ib-cr* and *qepA* genes were not detected (Table 2).

Isolates showed an almost equal distribution of the bla_{TEM} gene among both states, 52 % (15/29) of the isolates that carried the gene came from the State of Mexico, while the remaining 48 % (14/29) came from Jalisco. Also, 55 % (5/9) of isolates carrying the *qnrB* gene were from the State of Mexico, while 44 % (4/9) came from Jalisco. In contrast, 86 % of the isolates positive to the *qnrS* gene came from the State of Mexico and only 14 % (1/7) from Jalisco. The single

Table 2 Characterization of the antimicrobial resistance genes and their phenotypic sensitivity pattern

Resistance pattern	Number of isolates	β-Lactamase/*qnr* (number of carrier isolates)
AM, CAZ, CTX, NA, CIP[a]	1	bla_{CMY} (1)
AM, NA, CIP	7	bla_{TEM} (7)
AM, NA, CIP[a]	3	bla_{TEM} (3)
AM, NA	9	bla_{TEM} (5), *qnrB* (3), *qnrS* (1)[b]
AM	7	bla_{TEM} (7), *qnrB* (1), *qnrS* (1)[b]
AM, NA[c]	2	bla_{TEM} (2), *qnrB* (1)[d]
–	2	*qnrB* (1), *qnrS* (2) [e]
NA, CIP	2	bla_{TEM} (2)[f], *qnrS* (2)
NA	2	bla_{TEM} (2)[f], *qnrS* (1)[g]
NA[c]	3	*qnrB* (3)
NA, CIP[a]	3	bla_{TEM} (1)[f]

AM ampicillin, *CAZ* ceftazidime, *CTX* cefotaxime, *NA* nalidixic acid, *CIP* ciprofloxacin

[a] Intermediate susceptibility to CIP

[b] A single isolate had the bla_{TEM}+*qnrB*+*qnrS* genes

[c] Intermediate susceptibility to NA

[d] A single isolate has the bla_{TEM}+*qnrB* genes

[e] A single isolate has the *qnrB*+*qnrS* genes

[f] A single isolate has the bla_{TEM}+*qnrS* genes

[g] Isolates that have the bla_{TEM} gene but are sensitive to AM

isolate with the bla_{CMY} gene came from the State of Jalisco (Table 3).

PFGE A total of 18 isolates with intermediate sensitivity and resistant to CIP, CAZ, and CTX were selected from the 155 isolates in order to obtain their genetic profiles using the PFGE technique from which a dendrogram was constructed (Fig. 1) that shows the phylogenetic relationship among isolates. The presence of two distinct clades with a <70 % similitude between them can be observed in Fig. 1, one composed of a single isolate (isolate 147) from Jalisco and the other encompassing the remaining isolates (17) from the states of Mexico and Jalisco. Within the largest clade, two isolates were found to be closely related between them (isolates 4 and 15 from municipal slaughterhouses in the State of Mexico) that were resistant to CIP and had a 100 % similitude coefficient, which suggests that they had clonal relationship.

Discussion

In this study, resistance of up to 64 % to NAL and 10 % to CIP was found, which contrasts with the results obtained in a Polish study in which they found resistance of up to 1.3 for norfloxacin in *E. coli* isolates from cattle. This study also found resistance to AMP of up to 32 %, while for CAZ and CTX, it reached 1.3 %. The aforementioned authors reported resistance of 3.9 % for AMP and 3.4 % for CAZ (Mazurek et al. 2013). Another study in the USA, which included *E. coli* isolates obtained from beef, reported resistance levels to AMP of 6.0 %, CIP of 0 %, and NAL of 0.8 % (Zhao et al. 2012). A

Table 3 Distribution of resistance genes from different origin sites

Number of isolates n=155	Resistance genes				
	bla_{TEM}	bla_{CMY}	*qnrB*	*qnrS*	bla_{TEM}/*qnr*
State of Mexico (75)					
Municipal slaughterhouses (56)	9				
TIF-type slaughterhouses (12)	2		3	1	
Butcher's shops (7)	4		2	5	4
Jalisco (80)					
Municipal slaughterhouses (80)	14	1	4	1	1
Total	29	1	9	7	5

P<0.05

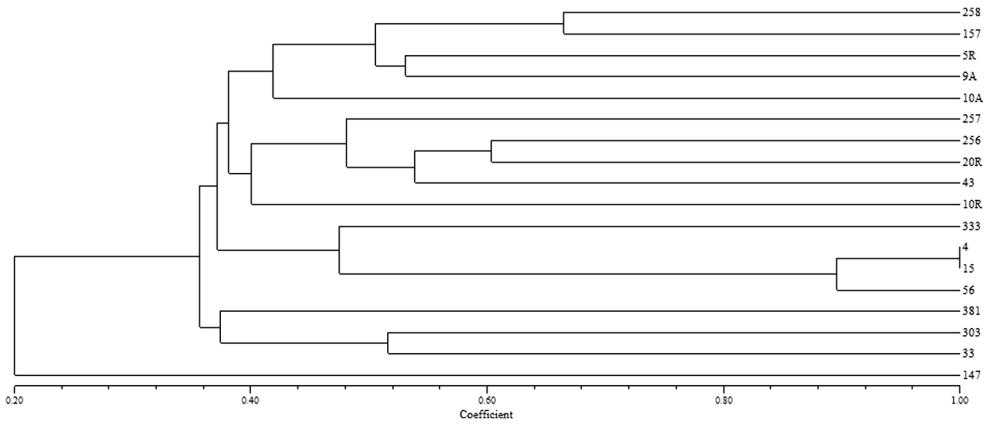

Fig. 1 Dendrogram constructed from PFGE showing the genotypic relationship between *E. coli* isolates

German study found that isolates obtained from bull calves showed up to 59 % resistance to AMP, 3 % to CAZ, 1.4 % to CTX, 10.5 % to NAL, and 13.3 % to CIP (Kaesbohrer et al. 2012). Lastly, a study carried out in France found resistance levels of up to 61 % to amoxicillin, 1.0 % to CTX, 0 % to CAZ, 14.7 % to NAL, and 5.4 % to enrofloxacin (Haenni et al. 2014). In general, the results in this study showed resistance levels to antimicrobials that were similar, and sometimes higher for some of them, when compared to those found in the aforementioned countries. Such increase in resistance has also been observed in studies carried out with human-origin *E. coli* isolates in Mexico. Amábile-Cuevas (2010) reported resistance levels of up to 74 % for AMP, 33 % for CIP, and 8 % for CAZ, which could suggest the spread of clones or antimicrobial resistance mechanisms between the human and animal populations in our country.

To our knowledge, this is the first report of *E. coli* isolates collected from bovine meat samples that have the bla_{CMY} gene, as well as the first report of the presence of the *qnrB* and *qnrS* genes in animal-origin isolates in Mexico. This finding highlights the possibility that isolates that are carriers of these resistance mechanisms decrease the efficacy of antimicrobials used in humans as they become less effective for fighting infections placing public health at risk.

The presence of the bla_{CMY} gene agrees with a study carried out in Mexico with *Salmonella* Typhimurium that found that it was the only gene present that provided resistance to cephalosporins, which in turn suggests the wide distribution of the gene within isolates of different origins in our country (Zaidi et al. 2007). In North America, the presence of the bla_{CMY} gene, specifically the $bla_{CMY\text{-}2}$ variant, is relatively common, although the plasmid AmpC β-lactamase is more widespread as it is found in isolates from samples of poultry, pig, and cattle (Doi et al. 2010; Glenn et al. 2012; Mollenkopf et al. 2011).

The bla_{TEM} gene was present in almost half of the isolates resistant to AMP. Nevertheless, 52 % (26/50) of the isolates resistant to AMP did not have this gene. This suggests that there is another gene that is responsible for phenotypic resistance, such as the $bla_{PSE\text{-}1}$ gene, whose presence has been reported in *Salmonella* spp. isolates in Mexico (Varela-Guerrero et al. 2013).

This study showed a relatively low frequency of bovine-origin isolates that contained the bla_{CMY} gene when compared to other animal species (Zaidi et al. 2007). This could be related to animal production practices, as well as to the quantity and class of antimicrobials used in comparison to swine and poultry farms (EMA 2012). Nevertheless, the fact that an *E. coli* isolate that had the bla_{CMY} gene was found suggests that such gene could spread in the following years within animal populations and meat products in a similar fashion as what is observed in isolates from *Salmonella* spp. in Mexico (Zaidi et al. 2007).

In regard to the resistance to quinolones, 16 isolates were found that had *qnr* genes. Studies carried out in other countries also using *E. coli* which were obtained from cattle show a lower number of positive isolates. This could reflect that in Mexico, there is a wider spread of this gene in bovine-origin isolates. For example, two studies carried out in Europe with *E. coli* isolates from cattle just found one positive *qnrS* gene in each, respectively (Kirchner et al. 2011; Veldman et al. 2011). Notwithstanding, 87.5 % (14/16) of the isolate carriers of a *qnr* gene were susceptible to ciprofloxacin, confirming that these genes only slightly increase their resistance to such antibiotics, and they are classified as susceptible under the CLSI criteria making them pass unnoticed. Nevertheless, it has been reported that the presence of *qnr* genes favors the acquisition of other mechanisms of resistance to quinolones (Poirel et al. 2012).

The PFGE analysis showed that the majority of the isolates had differing genotypic profiles. Both clades formed did not show particular similitudes which would group isolates by state of origin, with the exception of the two isolates that had similar banding patterns suggesting the same clonal origin at the local level. Nevertheless, cross-contamination during processing in an abattoir cannot be ruled out.

This study evidences the fact that the detection of these antimicrobial resistance genes in animal-origin isolates constitutes a warning of the risk and the lack of limiting and control of antimicrobial treatments used in veterinary medicine represents, as well as their possible transmission through the food chain, especially in meat products. In Mexico, restrictions have been placed upon the sale of antimicrobials. Nevertheless, such measures only apply to pharmaceuticals for human use excluding those used in veterinary medicine. As such, their prophylactic application or as growth promoters could contribute to an increase in antimicrobial resistance levels as years go by. Furthermore, it is evident that further epidemiological studies are needed on the presence of β-lactamase and PMQR determinants in animal-origin isolates in order to determine if these resistance mechanisms are spreading, as well as allow the establishment of mechanisms that counter antimicrobial resistance.

Conflict of interest The authors declare that they have no conflict of interest.

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