

Brief Original Article

Characterization of commensal *Escherichia coli* isolates from slaughtered sheep in Mexico

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Abstract

Introduction: Commensal *Escherichia coli* is defined as bacteria without known virulence factors that could be playing a specific role in some diseases; however, they could be responsible to disseminate antimicrobial resistance genes to other microorganisms. This study aimed to characterize the commensal *E. coli* isolates obtained from slaughtered sheep in the central region of Mexico.

Methodology: Isolates were classified as commensal *E. coli* when distinctive genes related to diarrheagenic pathotypes (*stx1*, *stx2*, *eae*, *bfp*, *LT*, *stp*, *ipaH*, and *aggR*) were discarded by PCR. Identification of serotype, phylogenetic group, and antimicrobial resistance was also performed.

Results: A total of 41 isolates were characterized. The phylogenetic groups found were B1 in 37 isolates (90.2%), A in 2 (4.8%), and 1 isolate (2.4%) for C and D groups. Serotypes associated with diarrhea in humans (O104:H2 and O154:NM) and hemolytic uremic syndrome (O8:NM) were detected. Thirty-three isolates (80%) were resistant to ceftazidime, 23 (56%), to tetracycline 8 (19.5%) to ampicillin, and 1 to amikacin. Six isolates (14.6%) were multidrug-resistant.

Conclusions: This study provides new information about commensal *E. coli* in slaughtered sheep, high percentages of resistance to antibiotics, and different profiles of antimicrobial resistance were found, their dissemination constitute a risk factor towards the consuming population.

Key words: commensal *E. coli*; slaughtered; sheep; Mexico.

J Infect Dev Ctries 2021; 15(11):1755-1760. doi:10.3855/jidc.14001

(Received 25 September 2020 – Accepted 08 April 2021)

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Introduction

Commensal *Escherichia coli* (CEC) has colonized and adapted to the mammalian gastrointestinal tract, however, they can be suddenly challenged by the selective pressure originated throughout antimicrobial therapy stewardship. Consequently, CEC acquires different antimicrobial resistance (AMR) genes to withstand this challenge and preserve the microbial homeostasis in the lower intestinal tract. The acquisition of numerous antimicrobial resistance mechanisms is an adaptation of several microorganisms that concede the ability to survive in different environments [1].

Unlike pathogenic strains, information of CEC is overlooked due to their reduced clinic relevance. CEC strains are isolated from healthy animals with no known virulence factors (toxins, adhesins, and proteins). However, the possibility to switch to a pathogenic

lifestyle due to horizontal gene transfer should not be underrated [2].

The high prevalence of some antimicrobial resistance phenotypes and the intrinsic mechanisms like efflux pumps and enzymatic inactivation demonstrate the consequences of indiscriminate abuse of antimicrobial drugs in antibiotic therapy and livestock production. [3] When CEC strains acquire different genes involved in antimicrobial resistance they act as reservoirs. Eventually multi-drug resistance (MDR) could be observed and threaten antimicrobial therapy. Thereby, CEC is a suitable indicator to determine the mobilization of antimicrobial resistance genes in the gastrointestinal tract of their hosts [2,4].

The present work aimed to characterize isolates of CEC from sheep slaughtered in abattoirs from a federal state of Mexico through serotype identification, phylogenetic group, and AMR profile.

Methodology

Sample collection and bacterial isolates

A convenience sampling was performed in a slaughterhouse with the largest number of slaughtered sheep in the central region of Mexico (approximately 900 sheep weekly). The sample size was estimated with a prevalence of 12.3% [5] and a 95% confidence level through sample size determination for finite populations [6]. A non-destructive method employing a swab in 0.1% peptone + NaCl (0.85%) according to the European Union was used [7]. From a total of 321 samples, 159 rectal swabs were taken before evisceration and 162 swab samples were taken from carcasses after final washing and before refrigeration. Finally, swabs were stored in sterile tubes with 25 mL of peptone water (1%) and transported to Center for Research and Advanced Studies in Animal Health - Universidad Autónoma del Estado de México (CIESA-UAEMex).

Samples were streaked onto MacConkey Agar (MAC, Beckton Dickinson, USA), after 24 hours of incubation at 37 °C, with typical *E. coli* morphology, two colonies were selected to identified by biochemical tests (triple sugar iron, sulfide indole motility, methyl-red Voges-Proskauer, urea, malonate, phenylalanine, gluconate, citrate, and sorbitol) [8].

DNA extraction

Isolates were grown in Luria-Bertani (LB) broth (Difco) and incubated at 37 °C for 18 hours. With a micropipette, 1.0 mL of culture was taken in a 1.5 mL vial and centrifuged for 5 min at 9,000 g (Eppendorf Centrifuge 5415D., USA).

The supernatant was discarded and the pellet washed in 400 µL of distilled water, vortexed for 30 seconds and centrifuged for 5 minutes at 9,000 g; again, the supernatant was discarded and the pellet suspended in 100 µL of distilled water, then boiled (95 °C for 15 minutes) followed by a centrifugation step. Finally, the supernatant was stored at -20 °C [9].

Molecular identification of virulence genes

To discard the presence of diarrheagenic *E. coli* (DEC) pathotypes, isolates were screened by PCR using eight virulence genes targeted for distinct DEC pathotypes. The genes used for categorization DEC were: *stx1* and *stx2* genes for Shiga-toxin producing *E. coli* (STEC) [10]; *eae* and *bfp* genes for enteropathogenic *E. coli* (EPEC) [11,12], *lt* and *stx* genes for enterotoxigenic *E. coli* (ETEC) [13], *ipaH* gene for enteroinvasive *E. coli* (EIEC) [14], and *aggR* gene for enteroaggregative *E. coli* (EAEC) [15] (Table 1).

Table 1. List of primers.

Gene	Description of target	Oligonucleotide sequence (5' – 3')	PCR Product (pb)	Reference
<i>stx1</i>	Verocytotoxintype 1	GTACGGGGATGCAGATAAATCGC AGCAGTCATTACATAAGAACGYCCACT GGCACTGTCTGAAACTGCTCCTGT	209 627	[10]
<i>stx2</i>	Verocytotoxintype 2	ATTAAACTGCACTTCAGCAAATCC CGCTGTCTGAGGCATCTCCGCT TAAACTTCACCTGGGCAAAGCC	625	[10]
<i>eae</i>	Intimin	TCAATGCAGTTCGGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG	482	[11]
<i>bfp</i>	bundle-forming pilus	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	300	[12]
<i>lt</i>	Heat-labile toxins	ACGGCGTTACTATCCTCTC TGGTCTCGGTCAGATATGTG	273	[13]
<i>stx</i>	Heat-stable toxins	TCTTTCCCTCTTTTAGTCAG ACAGGCAGGATTACAACAAAG	166	[13]
<i>ipaH</i>	Invasion plasmid antigen	TGGAAAAACTCAGTGCCTCT CCAGTCCGTAATTCATTCT CTAATTGTACAATCGATGTA	423	[14]
<i>aggR</i>	Transcriptional activator of AAFs	ATGAAGTAATTCTTGAAT ATGGTACCGGACGAACCAAC	308	[15]
<i>chuA</i>	Outer membrane hemin receptor ChuA	TGCCGCCAGTACCAAAGACA CAAACGTGAAGTGTCAAGGAG	288	[17]
<i>yjaA</i>	Uncharacterized protein YjaA	AATGCGTTCCTCAACCTGTG CACTATTCGTAAGGTCATCC	211	[17]
<i>TspE4.C2</i>	putative gene for a lipase	AGTTTATCGCTGCGGGTCGC AACGCTATTCGCCAGCTTGC	152	[17]
<i>arpA</i>	Ankyrin repeat protein A	TCTCCCCATACCGTACGCTA	400	[17]

The PCR condition for each gene was similar to described and summarized in Table 1. The reactions were run in thermocycler Multigene TM Mini Personal (Labnet International Inc, USA). Negative and positive PCR controls were included.

Serotyping

Serotyping was worked out through agglutination assay using 96-well microplates. Specific rabbit sera against 188 *E. coli* O antigens and 56 H antigens (SERUNAM®, Mexico) were used following the procedure described by Orskov and Orskov [16].

Table 3. Serotypes of 41 *E. coli* isolates from ovine slaughtered in México.

Serotype	Sample	Strain
O187:H20		1
O73:NM		1
O84:H21		1
O53:H20		1
O120:H25	rectal swab	1
O(NT):H49		1
O65:H38		1
O112ac:H28		1
O3:H8		1
O34:O145H45		1
O104:H2 †	carcass	1
O(NT):H48		1
O142:H10		1
O100:O145H45		1
O100:H21		1
O8:H8		1
Boydii18:H7 [36]		1
O8:H20	rectal swab	1
O84:H25		1
O84:H14		1
O112ac:H12		1
O185:H20		1
O154:NM†		2
O93:H8	carcass	1
O185:H2	rectal swab	1
O98:H2		1
O8:NM ±	carcass	2
O25:H34		1
O37:H10	rectal swab	1
O8:H7		1
O18 ac:NM		1
O8:H7	carcass	1
O(NT):H53		1
O98:H2	rectal swab	1
O32:H7		1
O84:H14		1
O163:H19	Carcass	1
O153:H21	rectal swab	1
O(NT):H8		1
Total		41

†: Serotypes associated with diarrhea in Mexico; ±: Serotypes associated with hemolytic uremic syndrome (HUS); NT: Non - typeable; NM: Not mobile.

Table 2. Phylogenetic groups of 41 *E. coli* isolates from ovine slaughtered in México.

Phylogroup	Number of isolates (%)
A	2 (4.8%)
B1	37 (90.4%)
C	1 (2.4%)
D	1 (2.4%)
	41 (100%)

Determination of *E. coli* phylogroups

Phylogenetic groups (A, B1, B2, C, D, E, and F) were identified with a quadruplex PCR using conditions described by Clermont *et al.*, [17] (Table 1). Phylogenetic groups A, B1, and C are composed of CEC isolates of low virulence, in contrast, phylogroups B2, D, E, and F encompass extra-intestinal isolates. The reactions were run in thermocycler Multigene TM Mini Personal (Labnet International Inc, USA).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was tested using a disk diffusion method according to CLSI [18]. *E. coli* ATCC 25922 and ATCC 35218 were used as quality control strains. Commercial discs of ampicillin 10 µg (AMP), ceftriaxone 30 µg (CRO), ceftazidime 30 µg (CAZ), cefoxitin 30 µg (FOX), amikacin 30 µg (AMK), gentamicin 10 µg (GEN), and tetracycline 30 µg (TET) (BBL™Sensi-Disc™Becton Dickinson, USA) were used.

Statistical analysis

The results of serotyping, antimicrobial resistance and phylogroups were analyzed using descriptive statistics and displayed in a table.

Results

Overall, 321 samples were collected: 160 from rectal swabs and 161 from carcasses. A total of 90 *E. coli* isolates were obtained and confirmed by biochemical test and serotyping, 18 of them from carcasses and 72 from feces, giving a frequency of 28%. It was observed that 49 *E. coli* isolates (54%) expressed at least one virulence factor included in this study (isolates that are not the object of study of this work), whereas the remaining 41 isolates (46%) did not express any virulence factor. The distribution of phylogroups showed that 37/41 (90.2%) belonged to phylogroup B1, 2/41 (4.8%) to phylogroup A, and only 1 isolate (2.4%) for phylogroup C and D (Table 2), the latter considered as extra-intestinal.

Regarding serotyping, serogroup O8 (12.1%), O84 (9.7%), O100 (4.8%), and O112ac (4.8%) were the more predominant. Moreover, serotypes associated

with diarrhea in the Mexican population O154:NM (4.8%) and O104:H2 (2.4%); and a serotype implicated in hemolytic uremic syndrome, O8:NM (4.8%) were also found (Table 3).

It was observed that 85.4% (35/41) of CEC isolates were resistant to some antibiotics, while only 14.6% (6/41) exhibited 100% of susceptibility to the antimicrobials used in this study. The highest percentage of antimicrobial resistance was 80.5% (33/41) against ceftazidime; The second antimicrobial agent with high level of AMR was tetracycline with 56% (23/41). Ampicillin and amikacin resistance were observed in 19.5% and 2.4% of isolates, respectively (Table 4).

Discussion

CEC is defined as bacteria isolated from healthy animals without known virulence factors (toxins, adhesins, and proteins). In the present work, the isolates lacked the expression of the main virulence factors typical of the diarrheagenic pathotypes of *E. coli* [1, 2].

In the present research work we report 46% of CEC isolates, a similar figure is reported in Portugal [19] 38.8%, in Azarbaijan report 40%, [20] both studies carried out with slaughtered sheep. It is worth mentioning that of these isolates, seven came from the canal and thirty-four from rectal swab it is important to note that the isolates detected on the carcass represent a more important risk factor for possible contamination of the product [21]

The strains that may belong to a particular phylogenetic group may be associated with the source of the isolation, the presence of point mutations and horizontal gene transfer, as well as a possible influence of various geographical conditions [18] Wang *et al.*, [22] mentions phylogroup B1 as the predominate in ruminants, this data is consistent with that reported in Portugal with sheep slaughtered [19] and this work.

Several serotypes have been linked throughout history with different types of diarrhea including more serious conditions such as hemolytic uremic syndrome or hemorrhagic colitis [23].

It was possible to detect isolates with serotypes that have been associated with diarrhea in the human population in Mexico: O154: NM and O104: H2 [24] and HUS: O8: NM [23], however absence of related virulence genes were noted, this could be explained due to the relatively fast bacterial replication where horizontal gene transfer and genetic deletions could generate clones with differences in virulence [25].

The percentage of AMR against ceftazidime was 80.5%; in Brazil found 22% of resistance against

Table 4. Antimicrobial resistance of 41 *E. coli* isolates from ovine slaughtered in México.

Antibiotic	Number of isolates (%)
Amikacin (AMK)	1 (2.4%)
Ampicilin (AMP)	8 (19.5%)
Ceftriaxone (CRO)	0
Ceftazidime (CAZ)	33 (80%)
Cefoxitin (FOX)	0
Gentamicin (GEN)	0
Tetracycline (TET)	23 (56%)

ceftriaxone and 72% against cephalothin in sheep slaughtered [26], while in México [27] reported 75% of resistance against cephalothin in bovine carcasses, these results could indicate overuse of cephalosporins in Veterinary Medicine in different countries [1]

Regarding the high percentage of resistance for some β -lactam antibiotics and the low percentage of resistance to penicillins, the present work shares similar figures with those reported in Iran [28] with isolates of commensal *E. coli* in faeces of live sheep that found 85.7% resistance for cefixime and 58% resistance for ampicillin and in Brazil [29] with faeces of live sheep feces they report 36.8% resistance for cefazolin and 7.9% resistance for ampicillin.

This resistance pattern could be due because a single microorganism usually harbors many factors that modulate the expression of β -lactamases and that depending on the environmental conditions can activate or repress the expression of different genes involved in resistance to these antibiotic [30-33]

The levels of AMR against tetracycline were 56% similar results were found in sheep slaughtered in abattoirs in Portugal 52.1% [19] and Azarbaijan [20] with 60%. In this study resistance to AMP was 19.5%, however, in Azarbaijan [20], the percentage was higher, 52.5%. The AMR against amikacin was low (2.4%) which is similar to other studies in Portugal 2.7% [19] and Brazil 4% [20].

Transmission of AMR by mobile genetic elements depicts a threat where CEC of gut microbiota in sheep can harbor these genes and finally reach the food chain [1].

Some genes conferring resistance against aminoglycosides, sulphonamides, and cephalosporins are located in the same plasmid or transposons which explain the relatively easy transmission of AMR [1,2].

The level of AMR has reached critical levels in the last years, while a controversial application of antimicrobials as growth promoters in small ruminants like sheep and goats continues in some countries [19,20,26,27].

One of the most polemic applications of antimicrobials is their use to promote growth in small ruminants such as sheep and goats; which has raised concerns about its contribution to the presence of resistant bacteria in humans and there is substantial evidence that the use of antimicrobial agents for cattle could exert selection pressure for resistance to occur in CEC [3,4]

The report of CEC isolates with serotypes associated with diarrhea and hemolytic uremic syndrome and with considerable percentages of resistance to antibiotics could show that CEC isolates are not exclusive to any animal species or human; and that an adequate characterization of *E. coli* strains must be complementary to the identification of phylogenetic groups and virulence factors in order to determine whether these isolates are potentially pathogenic or not. [34,35]

Finally, a limitation in this research work would be the need to monitor the presence of mobile genetic elements involved in antimicrobial resistance for a better understanding of the phenomenon in commensal bacteria.

Conclusions

This study is one of the few in Mexico to describe CEC isolates from sheep; furthermore, the abattoir sampled is the most important in the country that supplies the region most populated including Mexico City and the metropolitan area. The high levels of AMR observed revealed that sheep is an important reservoir of CEC isolates that have obtained resistance genes and could be a health risk when they are transferred via mobile genetic elements to others CEC isolates altering the genetic background of gut microbiota.

Acknowledgements

We thank: TLQ Luis Antonio León Alamilla, Gabriel Pérez Soto, Biol. Delia Licona Moreno, Biol. José Luis Méndez Sánchez, Dra. Gabriela Delgado Sapien from Faculty of Medicine UNAM and TLC Carlos Martín de la Luz Moreno for their technical assistance in the laboratory.

This work was supported by a grant from Universidad Autónoma del Estado de México (Registration No. 3998/2016R ED) and is part of the Doctor studies of Edgar Enriquez-Gómez who received CONACyT scholarship (No. 394480).

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Conflict of interests: No conflict of interests is declared.