

## Resistance profiles of Shiga Toxigenic *Escherichia coli* strains isolated from the foodshops in Guerrero, Mexico

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### Research papers

#### Abstract:

**Aim.** Determine resistance profiles of Shiga Toxigenic *Escherichia coli* strains isolated from the foodshops in Guerrero, Mexico. **Material and methods.** 180 samples of Mexican fast food from different corner shops in Guerrero., Mexico, were studied and analyzed on the phenotypic and genotypic basis for the resistance profile of seventy isolated strains. **Results.** Samples included chicken, cheese and beef. The most frequently isolated bacteria; Enterobacteriaceae (71%) that set included *Enterobacter sp* (40%), *Klebsiella sp* (26%), *Salmonella sp* (4%), *Escherichia coli* (22%), and other gram-negative bacillus (8%). Notably, 81.8% of the *E. coli* strains were recovered from cheese-containing food and all were positive in the amplification of the *stx1* and *stx2* genes by PCR; however, only 54% of STEC were *eaeA* gene positive. In concordance with the *stx* gene profiles, 57% of STEC strains were tetracycline resistant. **Conclusions.** The results suggest that *Escherichia coli* [STEC] appear to be pathogenic for humans. Finally, the data evidenced cheese as a principal food vehicle of pathogenic STEC, suggesting ‘there is need to be more strict in the application of the public policy on food safety

**Keywords:** Food contamination, *Escherichia coli*, STEC strains, Cheese, microbial resistant.

#### Introduction

Annually in The United States Shiga toxigenic *E. coli* (STEC) causes more than 96,000 cases of diarrheal illness and 3,200 infection-attributable hospitalization<sup>1</sup>. The largest outbreak caused by STEC occurred in Japan in 1996<sup>2, 3</sup>. Argentina ranks as one of the countries with detection frequencies and non-O157 STEC O157 close to 30%. In this country, HUS causes acute renal failure and chronic renal insufficiency in children, and is responsible for 20% of kidney transplants in children and adolescents.<sup>4</sup>. Among the

serotypes of *E. coli* belonging the STEC group, enterohemorrhagic serotype O157: H7 has been the one that has received the most attention because of its particular virulence that is reflected by the high number of people affected in each of the outbreaks<sup>5</sup>.

STEC strains of human, animal or food sources can produce one or more cytotoxins which are part of the family of shiga toxins (STXS). These cytotoxins (*stx1* and *stx2*), are the main mechanism of pathogenicity of enterohaemorrhagic *E. coli* O157: H7 with other virulence factors<sup>6,7</sup>.

The largest outbreak caused by STEC occurred in Japan in 1996<sup>2,3</sup>. Epidemiological studies from different parts of the world point to STEC as a principal cause of bloody diarrhea, hemolytic uremic syndrome and hemorrhagic colitis<sup>8-11</sup>. Hemolytic uremic syndrome predominantly affects children under 5 years of age, whereas hemorrhagic colitis is greatest in persons above 65 years of age<sup>9</sup>. Worldwide, beef and milk products from different sources are the most common vehicles carrying STEC<sup>9,12-15</sup>. However, human infections with STEC are caused not only by milk and beef products, but also by the consumption of vegetables, dairy products and drinking water containing STEC<sup>15-19</sup>.

Although several routes exist for human infection, beef continues to be a main source; thus, milk and beef cattle are considered reservoirs of different STEC strains<sup>20</sup>.

In a study in Chilean children, strains were identified by PCR 8 from patients with sporadic STEC diarrhea. Two of the strains had the *stx2* gene and 6 were non-O157 and had the *stx1* gene<sup>21</sup>.

STEC strains reside in the gastrointestinal tract, (GT) and the beef carcass contamination occurs during GT removal<sup>22, 23</sup>. This type of contamination subsequently affects prevalence in various beef products.

In addition, cheeses from different sources are emerging as an important vehicle of infection. In a previous work, 125 samples of soft and semi-soft cheeses made with raw cow's milk were studied by multiplex-PCR method for the presence of STEC, in Kerman-Iran. Authors suggested that raw milk cheeses could be considered a risk for food borne STEC contamination. Other similar studies were reported by other groups<sup>24-27</sup>. On this concern, Kousta et al., (2010) set out that the food-borne pathogens originate from direct excretion from animals' infected udders, and thus, in dairy plants they may enter via contaminated raw milk, colonize the environment and consequently contaminate the dairy products.

Some of the commonly isolated pathogens from cheeses are *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella sp*, *Escherichia coli* O157:H7 and *Mycobacterium avium* (Kousta et al., 2010). Listeriosis, caused by *L. monocytogenes* is characterized by a very high fatality rate when compared with those of other foodborne bacteria<sup>28</sup>. *S. aureus* is considered the third most important cause of disease in the world among the food-borne bacteria<sup>29,30</sup>. Moreover, in two interesting cases, it has been reported that salmonellosis was caused by the consumption of

contaminated Cheddar and Mozzarella cheeses<sup>31, 32</sup>. Finally, in spite of the fact that many food-borne outbreaks related to *E. coli* O157:H7 are caused by the consumption of milk, some outbreaks have been linked to the consumption of cheeses in France and Canada<sup>24, 25, 27</sup>.

The importance of food-borne STEC strains lies in part on their antimicrobial resistance profiles. A work from Mora and co-workers (2005) showed a differential antimicrobial resistance profile for *E. coli* recovered from humans, cattle, ovines and foods in Spain. In that study, the antimicrobial resistance profile was associated with serotypes, phage types and virulence genes. The highest prevalence of antimicrobial resistance in non-O157 STEC was found in beef meat and human patient isolates, whereas multiresistant STEC O157:H7 was recovered mainly from bovine and beef meat. Streptomycin, sulfisoxazole and tetracycline were the antimicrobial agents most often observed in the multiple resistance patterns<sup>33</sup>. The aim of our study was to determine the occurrence of pathogenic *E. coli* strains and their resistance profiles from Mexican fast food issued in corner shops in Chilpancingo, Guerrero, Mexico.

#### **Methods. Isolation and identification**

One hundred and eighty samples containing chicken meat, pork meat, beef cattle and cow cheese food were collected from vendors, shops and markets in Chilpancingo, Guerrero., Mexico, from February to August 2014, according to the Mexican norm NOM-109-SSA1-1994. Ten milliliters or 10 g of food sample were placed in a sterile plastic bag, and then 90 ml of diluent were added. Solid samples were homogenized in a blender at maximum speed for 1.5 minutes or until complete homogenization, following the suitable protocol for each type of sample.

Large particles were precipitated by sedimentation, and 1 ml of the upper suspension was taken to make 1:1000 and 1:10000 dilutions. One ml from dilutions was inoculated into MacConkey agar and salt and manitol agar. For aerobic microorganism counting, only a minimum of 25-250 colonies were considered as a positive results. For identification, samples were inoculated in MacConkey and MacConkey/sorbitol agar and incubated to 37°C during 24 hours.

The typically phenotyped Enterobacteriaceae colonies were identified by means of biochemical tests and verified by Vitek2 using the ID-GNB panel and the ID-GPB panel for *Staphylococcus sp* strains.

#### **DNA purifications, PCR and electrophoresis**

Total DNA was purified from only *E. coli* isolates<sup>34</sup>, following the method used by Rey et al., (2006). For stx1, stx2 and eaeA gene amplification, the PCR reaction was assembled in a final volume of 50 µl using 20 pM of each oligonucleotide. (for stx1, forward 5'ATAAATCGCCATTCGTTGACTAC 3' and reverse 5'AGAACGCCCACTGAGATCATC-3'; for stx2, forward 5'GGCACTGT CTGA AACT GC TCC 3' and reverse 5'-TCGCCAGTTATCTGACATTCTG-3'; forward 5'GACCCGG CACA AGCA TAAGC-3' and reverse 5'-CCACCTGCAGCAACAAGAGG-3'), 200 mM of each dNTP, 2.5 µl of reaction

buffer (10X), 2.5 mM of MgCl<sub>2</sub>, 2 U of DNA polymerase, and 300 ng of DNA template. The protocol for amplification was taken from Bandyopadhyay et al., (2012)<sup>35</sup>. As a negative control, DNA from *E. coli* ATCC 25922 strain was used. All reactions were performed in a gradient Mastercycler®Ep. Products were electrophoreted in 1.5% agarose gels and stained with ethidium bromide

### Resistance profiles

The resistance profiles were determined using the Vitek2® using the ID-GNB panel according to the Clinical and Laboratory Standards Institute Guidelines. In addition, the Concentration Minime Inhibitory (CMI) was determined for amikacin, ampicillin, ceftriaxone, ceftazidime, cefuroxime, cefalotin, cefazolin, ciprofloxacin, gentamicin, imipenem, levofloxacin, piperacillin, tetracycline and tobramycin.

### *In silico* identification of genes *stx1*, *stx2* and *eaeA*

Then, predicted proteins were characterized *in silico* using the software deposited in the ExPasy Bioinformatics Resource Portal <<http://expasy.org>> and the NCBI Home Page <<http://www.ncbi.nlm.nih.gov>>. Phylogenetic analysis was performed using the MEGA 5.05 software package.

### Phylogenetic analysis

In order to analyze the phylogenetic relation between *stx1*, *stx2* and *eaeA*, the obtained NCBI gene sequences were used: *stx1* A subunit [*Escherichia coli*] GenBank: BAA77751.1, *stx2* [*Escherichia coli* O104: H4 str. C227-11] GenBank: EGT68086.1, *stx2* [*Escherichia coli* O104: H4 str. C227-11] GenBank: EGT68086.1, *stx2*, partial [*Escherichia coli*] GenBank: ADK02660.1, *stx2*, partial [synthetic construct] GenBank: AIC54351.1, *eaeA*, partial [*Escherichia coli*] GenBank: AHB39871.1, *eaeA*, partial [*Escherichia coli*] GenBank: AFO70074.1 and intimine *eaeA* [*Escherichia coli* O127: H6 str. E2348 / 69].

### Results and discussion

The major types of foods analyzed in this study included those containing chicken, beef and cheese. The main groups of isolated microorganisms from cheese were Enterobacteriaceae (71%) and *Staphylococcus sp* (29%) (Figure 1A). Due to the importance of *E. coli* and other coliforms as fecal contamination markers, the analysis of this research was focused on the enterobacteria group. Interestingly, *Enterobacter sp* (40%), *Klebsiella sp* (26%) and *E. coli* (22%) were the most frequently isolated microorganisms, whereas *Salmonella sp* represented only 4% of strains (Figure 1B).

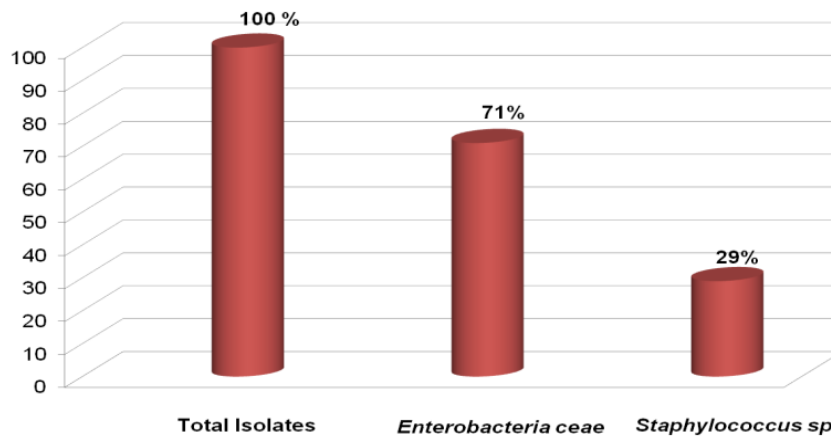


Figure 1A. Groups and Genus of bacteria isolated from different fast food sources.

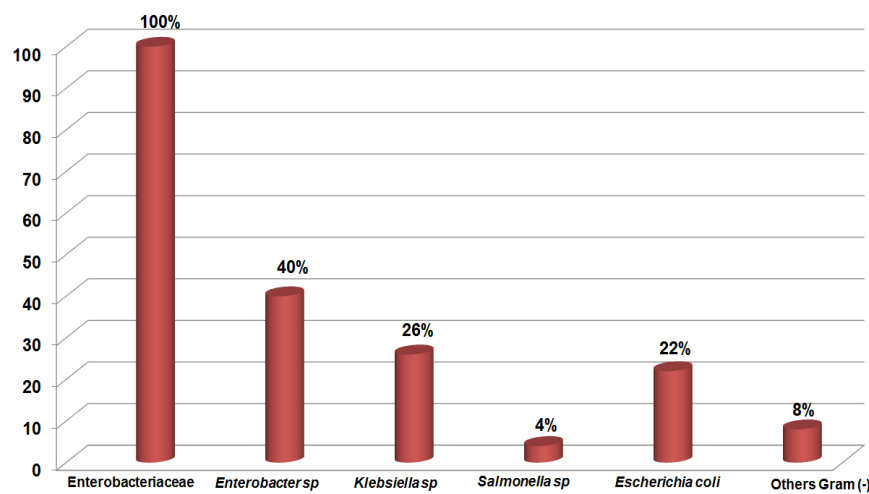


Figure 1B. Groups and Genus of bacteria isolated from different fast food sources.

In Mexico the scant reports of STEC came from Gallegos et al (2009)<sup>36</sup> and Srinivasan et al (2007)<sup>37</sup>, who separately isolated STEC harboring the *stx1*, *stx2* and *eaeA* genes from ovine and bovine meats. Results showed that 6.1% (11/180) of food samples contained STEC, characterized by harboring *stx1*, *stx2* and *eaeA* genes. Interestingly, 81.8% (8/11) of STEC strains were isolated from cheese-containing foods. 57% of STEC strains were tetracycline resistant, and 43% were ampicillin resistant (Table 1).

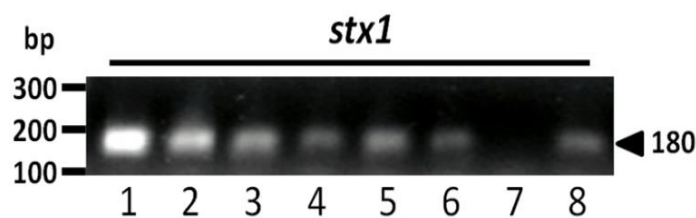
**Table 1. Antimicrobial resistance profile of STEC strains isolated from food in Chilpancingo, Mexico.**

Isolates	Resistance profile of STEC strains													
	TE	CF	CAZ	PI	AM	LVX	CAX	CP	GM	TO	CFT	CFZ	AK	IMP
M3J	R	R		R	R			R						
M40	R		R	R	R			R						
MS3	R	R	R					R	R	R				
M05	R	R	R					R	R	R				
M12-3	R	R	R					R	R	R				
MT3	R	R	R	R	R	R	R							
M15-3	R	R	R	R	R	R	R	R						
ME11	R	R	R	R	R	R	R	R						
M11	R	R	R	R	R	R	R	R						
M16	R	R	R	R	R	R	R	R						
M21	R	R	R	R	R	R	R							

**Abbreviations:** amikacin (AK), ampicillin (AM), ceftriaxone (CAX), ceftazidime (CAZ), cefuroxime (CF), cefalotina (CFT), cefazolin (CFZ), ciprofloxacin (CP), gentamicin (GM), imipenem (IMP), levofloxacin (LVX), piperacillin (PI), tetracycline (TE) and tobramycin (TO).

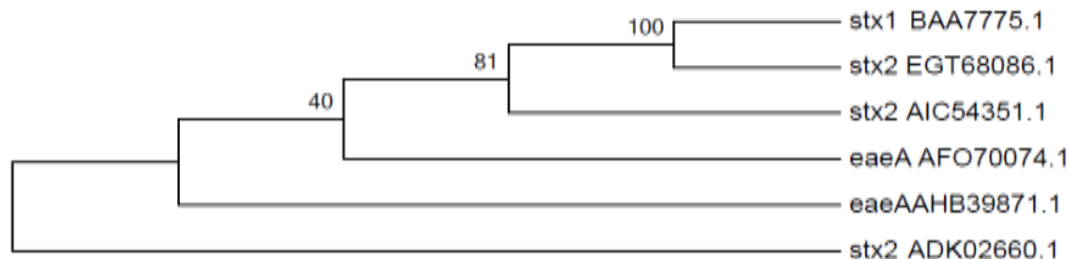
Additionally, a large set of other Enterobacteriaceae and *Staphylococcus sp* was isolated, thus evidencing the matter of the shortage of regulations in food safety.

Further testing of PCR with several primer pairs revealed that *stx1*, *stx2* and *eaeA*, and mainly *stx1* genes are located in different fast food in Chilpancingo, Guerrero: cows' milk cheese, chicken meat and beef cattle. This was demonstrated by PCR amplification of *stx1* (Figure 2), *stx2* and *eaeA*; although, the fragment sequences would be interesting in order to demonstrate their presence. Also, ELISA experiments using specific antibodies against toxins would be necessary. We noticed that the frequency (12.1%) of STEC isolated from bovine feces<sup>38</sup> was similar to our results (15.7%), pointing to cattle as a possible contamination source. In addition, preliminary results from our group have shown significant frequency of STEC in ambulatory patients (data not shown). Therefore, further analyses are necessary in order to determine the relationship between the strains isolated from cheese and those from patients, and to evaluate if the resistance profile shown in this study would support the treatment schemes.



**Figure 2. *E. coli* strains harboring *stx1* genes.** A 180 bp corresponding to *stx1* gene that was amplified (lanes 1-6, and 8) in different *E. coli* strains. Lane 7 corresponds to a *stx1* negative *E. coli* strain

The phylogenetic analysis of *stx1* and *eaeA* genes suggests that these genes, which have high a homology of 50%, are present in various foods and also can produce one or more cytotoxins and are part of the family of shiga toxin (STXS). These cytotoxins (*stx1* and *stx2*), are the main mechanism of pathogenicity of enterohaemorrhagic *E. coli* O157: H7 with other virulence factors<sup>6</sup> (Figure 3).



**Figure 3. Identification and phylogenetic analysis of *stx1*, *stx2* and *eaeA* genes.**

In order to analyze the phylogenetic relationship of these genes, the first alignment with the MEGA 6.0 program was performed, the sequences were located in the GenBank database. Each gene has a corresponding access number for the *stx1* gene, only one complete sequence was found, while the *stx2* gene leg three sequences were located and finally for the gene *eaeA* two sequences were obtained.

In this paper we propose that these genes may be present in Fast Food from Chilpancingo, Guerrero., Mexico, given the pharmacological, molecular and bioinformatic results.

### Conclusions

Therefore, we suggest studying these genes to identify different types of bacteria such as *E. coli*, *Salmonella sp*, *Klebsiella sp* and others will help to provide a better prevention in fast food establishments in Chilpancingo and also helps enforce better hygiene in preparing food.

The results suggest that *Escherichia coli* [STEC] appears to be pathogenic for humans. Given the importance of these genes it will be interesting to use them as potential biomarkers for detecting these bacteria in fast food. Finally, the data evidenced cheese as a principal food vehicle of pathogenic STEC, suggesting that the public policy on food safety must be applied. We suggest that these genes may be present in the Fast Food Chilpancingo, Guerrero., Mexico, given the pharmacological, molecular and bioinformatic results.

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