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ORIGINAL ARTICLE

Characterization of six Shiga toxin-producing *Escherichia coli* (STEC) strains carrying Stx2-phages from Colombia

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Abstract

Shiga toxin producing *Escherichia coli* (STEC) is a bacterial pathogen that causes diarrhea and severe human diseases like HUS; its main virulence factor are the Shiga toxins (Stx1 and Stx2). Some Stx2 subtypes have been identified and associated with the risk of developing severe illness. Stx toxins are encoded in temperate bacteriophages which control their expression through the lytic cycle that is regulated by the late genes and Q anti-terminator protein. The aim of this work was to characterize six STEC strains carrying Stx2-phages to provide preliminary information and understanding about Stx2 strains from Colombia regarding the Stx production, lytic cycle induction and subtyping of Stx2. Two highlighted strains with higher levels of Stx production and lytic cycle's induction were observed. All the evaluated strains carried either the Stx2a, Stx2c, or Stx2d subtypes. In addition, most of the strains carried the *qO111* allele, and only one strain showed differences in the *ninG* region. Differences in the evaluated characteristics of the strains were observed, which could indicate the variability of these six STEC strains carrying Stx2-phages.

Keywords: STEC; Bacteriophages; Shiga toxin; Cattle; HUS

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), is an emerging pathogen involved in food-borne infections which causes diarrheal disease outbreaks, like hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The HUS is one of the most severe human diseases caused by STEC, characterized by producing thrombocytopenia and severe kidney failure, mainly affecting children under 5 years old and older adults [1]. In this sense, cattle is considered the main reservoir of pathogenic STEC for humans and its transmission may occur through the consumption of food contamination by the cattle feces, such as meat, cheese, and unpasteurized milk. Likewise, other ways of transmission are possible by the intake of contaminated water, fruits, and vegetables, causing the STEC infection outbreaks [2].

The main STEC virulence factor associated with HUS is Shiga toxins (Stx). The Shiga toxins belong to the AB_5 family of protein toxins, with an enzymatically active A moiety and a nontoxic B moiety responsible for binding to cellular receptors on the target cells, such as kidney cells, the gastrointestinal tract and central nervous system cells. There are two types of Stx: Stx1 and Stx2, which share approximately 56 % of the homology in their amino acid sequence. However, Stx2

has been epidemiologically linked to the development of severe diseases, like HUS [3]. Studies on the human brain microvascular endothelial cells (HBMC) have shown to be up to 1000 times more susceptible to Stx2 than to Stx1 [4], in this sense Stx2 has received greater research attention.

Seven subtypes of Stx2 have been identified: Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g. The most frequent subtypes identified so far in strains of human and bovine origin are Stx2a, Stx2c and Stx2d; these three subtypes have shown high toxicity *in vitro* and are often associated with development of hemorrhagic colitis (HC) and HUS. Unlike the Stx2b, Stx2e, Stx2f and Stx2g subtypes that are rarely associated with severe diseases in humans, and are frequently isolated from animal reservoirs different from cattle. [5, 6]. The amino acid sequences of the Stx2a, Stx2c, and Stx2d subtypes showed to be closely related, and shared between 97 % and 99 % identity; however, due to their biological differences and toxicity, they are classified as different subtypes [7].

Shiga toxin genes (*stx*) are encoded by temperate bacteriophages inserted in the bacterial chromosome, phages encoding *stx* (Stx-phages) can be induced by DNA damaging agents such as mitomycin C. The Stx expression is linked to the lytic cycle of the bacteriophage and its release depends on the lysis of the bacteria. As a result of the induction process, Stx-phages play an important role in STEC pathogenesis due to the regulation of the Stx expression and their contribution to the propagation of the *stx* genes in other bacteria [8]. The *stx* genes are located in the late region of the Stx-phages where genes implicated in the lytic cycle are found. Likewise, late genes are regulated by a transcription anti-terminator protein Q. In absence of the Q protein, the phages cannot carry out their lytic cycle, which implies they will not produce Stx [9]. Three allelic variants: *q933*, *q21*, and *qO111,* have been identified for the Q protein gene. which are related with differences on the levels of expression of the Stx and the virulence of the strains that carry them [10, 11].

In Colombia, the knowledge about STEC is still limited. The few studies have focused on the detection of the O157:H7 serotype from different sources without the use of molecular techniques [12, 13]; in addition, a characterization for the isolated native strains is not currently existing. The aim of this study is to characterize six STEC strains carrying Stx2-phages to provide preliminary information and understanding about Stx2 production, lytic cycle induction and subtyping of Stx2 in STEC strains from Colombia.

2. Materials and methods

2.1. Bacterial strains

This study was descriptive cross-sectional. Six cattle STEC strains (102, 10610, 600 5052, 615, and N108) carrying Stx2-phages were selected, belonging to the Center of Biomedical Research strains collection, Universidad del Quindío. Quiguanas et al. [14] characterized previously these strains according to the presence-absence of STEC virulence genes (*stx*2, *eae*, *saa*, and *hly*A). Briefly, fecal samples were collected by rectal swabbing from cattle in the farm El Cofre, located in Ulloa-Valle del Cauca, Colombia. Each sample was transported in test tubes with peptone water at room temperature to the Biomedical Research Center (CIBM) of the Universidad del Quindío for processing. By multiplex PCR, *stx1*, *stx2* or both genes were detected. Positive samples for one or both toxin genes were considered STEC positive. Strains were isolated in MacConkey agar. Individual colonies were characterized by multiplex PCR for detection of genes, encoding different virulence factors (*eae*, *saa*, and *hly*A). However, the serotype of these strains is still unknown (**Table 1**). For this study, we selected only STEC strains carrying Stx2-phages due to

Table 1. Genetic characteristics and virulence genes of the STEC strains.

Strain	Virulence genes			*q* gene alleles		*ninG*
	Sub Stx2	*saa*	*hlyA*	*q933*	*qO111*	
102	Stx2d	+	+	−+[a]	++[b]	+
10610	Stx2a	+	+	−+[a]	++[b]	+
5052	Stx2c	+	+	−+[a]	++[b]	+
600	Stx2c	+	+	−+[a]	−	+
615	Stx2d	+	+	−+[a]	++[b]	+
N108	Stx2c	+	−	−+[a]	++[b]	+[c]

[a] Positive for primers QATG and Q3 and negative for primers Q-stx-f and 595
[b] Positive for primers SF1-F and SF1-R, and SF1-R and 595
[c] Size of amplified 1200 pb

its epidemiological importance worldwide, since it is the predominant type in STEC infections in humans, and it is associated with a greater severity of disease. The STEC reference strain EDL933 was used as positive control and the *E. coli* DH5α strain was used as negative control for its great capacity to host Stx-phages.

2.2. Induction of the lytic cycle

The six strains were grown individually in LB Broth Base (Lennox, powder, L Broth Base) overnight at 37 °C, 100 rpm. These cultures were refreshed in LB medium at 37 °C, 180 rpm, when the culture reached the exponential growth phase (optical density $OD_{600} = 0.2$ nm to 0.3 nm); it was divided into two subcultures and Mitomycin C (MMC) was added to a fraction at a final concentration of 0.5 µg ml^{-1} (inducer of the lytic cycle) [15]. The OD_{600} of the cultures both with and without MMC were monitored at different times (0 h, 1 h, 2 h, 3 h, 4 h, and 5 h) to construct growth curves/lysis; the growth was measured with a microplate spectrophotometer Epoch™ (BioTek). All assays were done at least three times and independently for each strain.

2.3. Stx2 production

To compare the titers of Stx2, after overnight incubation (37 °C, 180 rpm) the cultures with and without MMC were centrifuged at 11.500 rpm, 4 °C during 10 min and the supernatants were evaluated for the presence of Stx2 by using an enzyme immunoassay (Ridascreen® Verotoxin, R-Biopharm) according to the manufacturer's instructions. The ELISA plates were read in the microplate spectrophotometer Epoch™ (BioTek) at OD_{650}. However, when the OD_{650} values exceeded the limit permitted by the Epoch™ (BioTek), serial dilutions were conducted to obtain a value within the equipment's reading range. The toxin titers were expressed as the result of the multiplication between the OD_{650} and the reciprocal of the dilution factor; all the assays were carried out at least three times.

2.4. Analysis of the late region of Stx2-phages

The upstream region of the *stx*$_2$ gene was analyzed by PCR in Veriti 96-Well Thermal Cycler (Applied Biosystems™). The proximity of the *ninG* gene with *stx*$_2$ was evaluated using NinG/526 and 595 *primers* [16], the variability in this gene was evaluated by comparing the sizes of the

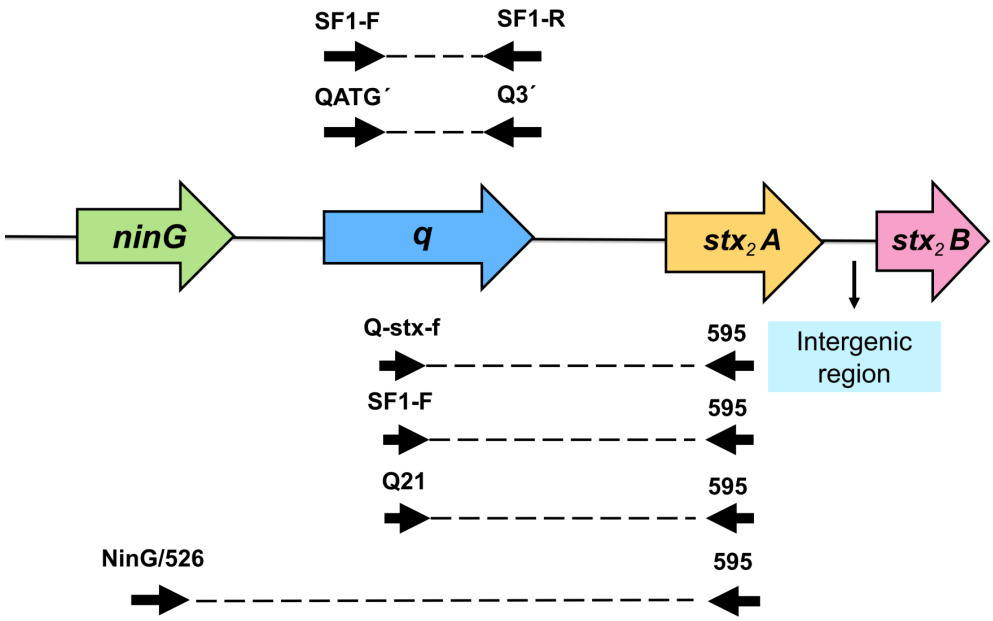

Figure 1. Scheme of the late region of Stx-phages. Colored arrows show the genes evaluated. Dotted lines show the region amplified by the *primers*. Taken and adapted from: Burgan et al. (39).

amplified fragments. The presence of the different alleles of *q* gene (*q933*, *qO111*, and *q21*) was evaluated in all of the strains as well as its closeness with the *stx*$_2$ gene. To detect the *q933* allele, the QATG5' and Q3' *primers* were used [17], and the closeness with the *stx*$_2$ gene was evaluated using the Q-stx-f and 595 *primers* [16]. The presence of the *qO111* allele was evaluated with the SF1-F and SF1-R *primers* [11] and its closeness with the *stx*$_2$ gene was evaluated with the SF1-F and 595 *primers*. The *q21* allele and its closeness with the *stx*$_2$ gene was evaluated with the Q21 and 595 *primers* [18] (**Figure 1**). All the *primers* used here are shown in **Table 2**. The conditions for the PCR were the following: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 3 min, ending with 72 °C for 10 min. For the QATG5' and Q3 *primers* annealing temperature was 53 °C.

2.5. Identification of Stx2 subtypes

The identification of Stx2 subtypes was performed through analysis of sequences with the method proposed by Persson et al. [19] and Scheutz et al. [7]. A partial sequence of *stx*$_2$ gene was obtained by using the F4 and R1 sequencing *primers* (Table 2), these *primers* amplifying a 491 bp fragment which was translated into 159 amino acids, covering 95 residues from the C-terminal region of the A subunit and 64 residues from the N-terminal region of the B subunit, where the sequences of the toxins have greater variability. The PCRs were performed with the Platinum™ Taq DNA Polymerase High Fidelity kit (Invitrogen™) with the following conditions: 95 °C for 15 min, followed by 35 cycles at 94 °C for 50 s, 56 °C for 40 s, and 72 °C for 1 min, and an ending temperature of 72 °C for 3 min. The amplicons obtained with forward and reverse PCR primers were sequenced by the Sanger method in an ABI3500 (Applied Biosystems™). The DNA used for the different PCRs were purified by using the Wizard® Genomics Kit (Promega).

Table 2. *Primers* used to evaluate the late region and sequencing the *stx*$_2$ gene of Stx-phages.

Primer	**Sequence**	**Reference**
NinG/526 595	CACAAGCAATGCGTGGTGTGC CCGAAGAAAAACCCAGTAACAG	Unkmeir and Schmidt, 2000
QATG5' Q3'	ATGTTCTTATGGTTCACCG TTACGATCGTAAACTATTTTT	Smith et al. 2007
Q-stx-f	CGGAGGGGATTGTTGAAGGC	Unkmeir and Schmidt, 2000
SF1-F SF1-R	ATACCGTGGCATTTGAAGAGAAGT TTTTTAGCAGCCAGTCGTCCA	Haugum et al. 2012
Q21	GAAATCCTCAATGCCTCGTTG	Lejeune et al. 2004
F4 R1	GGCACTGTCTGAAACTGCTCCTGT ATTAAACTGCACTTCAGCAAATCC	Scheutz et al. 2012

The consensus sequences were obtained with the forward and reverse chromatograms using the Uniprot software UGENE RRID: SCR_005579. To identify the subtypes, the intergenic regions between A and B subunits of *stx*$_2$ sequences of all strains were compared. The nucleotide sequences were translated into amino acids with the ORF established for Stx2 (excluding the intergenic region). A multiple alignment with the amino acid sequences obtained and reference sequences for Stx2 subtypes was carried out by employing the MEGA7 software RRID:SCR_-000667 and the Muscle algorithm. Conserved positions described in the literature [7, 19] for each subtype were examined in the multiple alignment; the accession codes for the reference sequences used in the alignment were: Stx2a (GenBank ID: Z37725) Stx2c (GenBank ID: L11079) Stx2d (GenBank ID: DQ059012).

The partial sequences obtained were used to construct a dendrogram by using the UPGMA algorithm (bootstrap of 1000) with various reference sequences of Stx2 subtypes register in the GenBank database: X07865; Z37725; AF524944; AF461173; AY633471; AY633472; EF441599; EF441609; AY443052; AY443057; EF441613; Z50754; DQ344636; FM998856; EF441618; M59432; AB015057; DQ235774; L11079; AY633473; AY443045; AY633467; AY633453; AF291819; EF441604; AY739670; AY739671; AY443044; AY443043; AY443049; AB071845; FM998860; FM177471; EU086525; AF479828; AF479829; AY095209; X61283; DQ235775; AF500190; AF500189; AF500191; AY443047; AY443048; DQ059012; AF329817; AF500192; FM998855; FM998840; EF441605; M21534; AJ313016; M29153; AB472687; AY286000; AB048227; X65949. Although the sequences of the reference strains were complete sequences, in order to perform the analysis, these sequences were cut in the region shown in the alignment (**Figure 4**A). The sequences obtained in this study were deposited in the GenBank with the following access numbers: MT680394; MT680395; MT680396; MT680397; MT680398; MT680399.

3. Results

3.1. Induction of the lytic cycle

Analysis of the growth curves showed that all the cultures without MMC had an exponential growth with OD_{600} values of approximately 1.4; the same behavior was observed with the positive and negative control cultures: EDL933 and DH5α. However, upon inducing the phages with MMC, we observed different kinetics in the induction of the stx2 phages (**Figure 2**). Two strains (10610 and 600) showed a high induction with OD_{600} values lower than 0.2 (5 h after MMC induction) and the others (102, 5052, 615 and N108) showed a more reduced induction with OD_{600} values of approximately 0.7 (5 h after MMC induction).

3.2. Shiga toxin production

All the strains evaluated showed Stx2 production; nevertheless, the titers obtained were different among each other. The 10610-strain showed higher Stx production, presenting similar titers to the positive control strain. The 5052, 600, and N108 strains showed lower titers than the control strain, but with moderate Stx production. The 102 and 615 strains produced the lowest titers compared with the other strains (**Figure 3**). In general, all the strains revealed an increase in the toxin titers when the cultures were induced with MMC. We observed that Stx was also detected under non-induction conditions, but always with low titers, demonstrating a basal toxin production in all the strains. The results of absorbance and dilutions carried out are shown in supplementary material: **Table S1**.

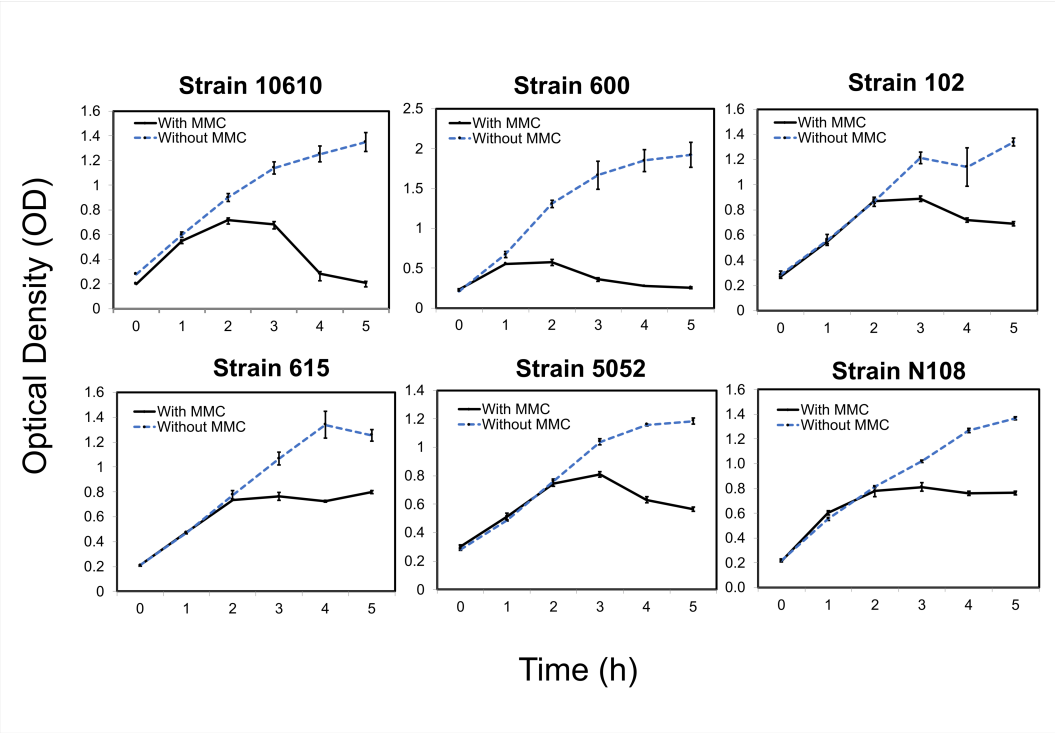

Figure 2. Growth curves/lysis of the evaluated strains in presence/absence of Mitomycin C (MMC).

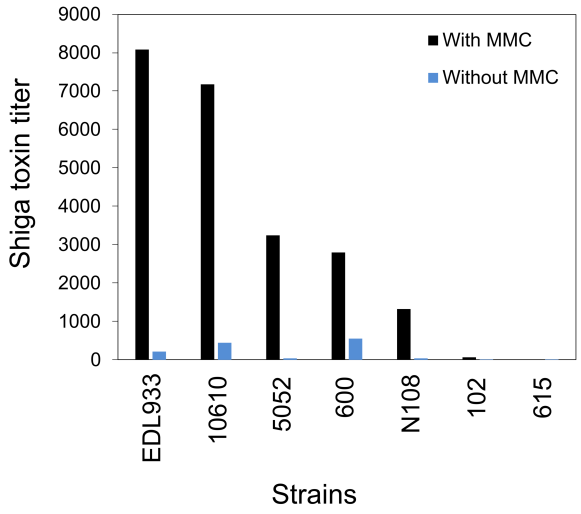

Figure 3. Shiga toxin production of evaluated STEC strains in presence/absence of Mitomycin C (MMC).

3.3. Analysis of the late region of Stx2-phages

The proximity of the *ninG* gene with the stx_2 gene was confirmed in all the strains, where an amplified of 1700 bp fragment was observed, except for the N108 strain that obtained a fragment of 1200 bp, suggesting that there are differences in the regulating region of these phages. Regarding the *q* gene alleles, the *q933* allele was detected in all the strains; however, upon evaluating its closeness with the stx_2 gene, the amplicon was not obtained. As for the *qO111* allele, its presence and closeness with the stx_2 gene were confirmed in five strains. None of the strains studied carried the *q21* allele, the results are shown in Table 1.

3.4. Identification of Stx2 subtypes

In some strains, the chromatograms showed double peaks in specific positions that corresponded to different nucleotides, in these cases, the IUPAC nucleotide nomenclature was used to solve these ambiguities. Once the consensus sequence was obtained and translating it into amino acids, we observed that in most cases the ambiguities did not alter the amino acid sequences (synonymous mutations); only in two strains (10610 and 600) we observed a codon change in the position 137 and it could be translated into two different amino acids: Alanine or Aspartic acid (Figure 4A).

Analysis of the intergenic region between the A and B subunit genes of Stx2, showed that all the strains had the same sequence (AGGAGTTAAGT); this sequence has been reported for the Stx2a, Stx2c, and Stx2d subtypes. Analyzing the alignment, we observed that all subtype sequences were highly conserved and only differ in some positions (Figure 4A). The combination of these positions was used to classify the subtypes, obtaining the following results: Stx2a subtype; one strain, Stx2c subtype; three strains and Stx2d subtype; two strains. Specific changes in amino acid residues were observed in some sequences; however, these are not strictly restricted to a subtype. In the dendrogram both the studied and the reference sequences formed separate groups, confirming what was observed in the alignment (Figure 4B).

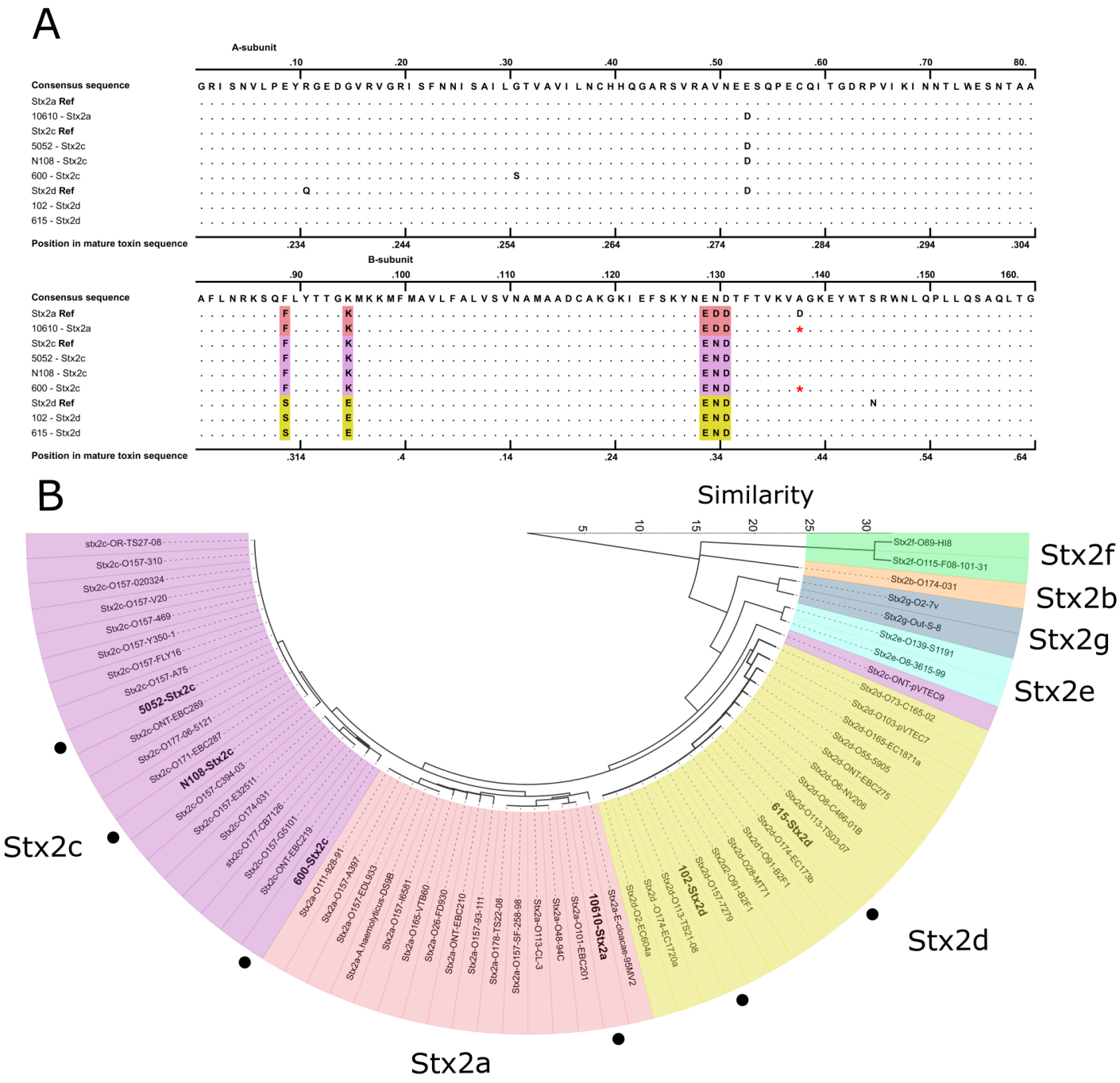

Figure 4. A) Multiple alignment with partial reference sequences of Stx2 subtypes and sequences of Stx2 obtained in this study. The colors show conserved sites for each subtype. * In that position, both Aspartic acid or Alanine may be present. B) The dendrogram shows the groups formed by the reference sequences and the sequences of Stx2 obtained in this study.

4. Discussion

Presence of inducible phages in the studied strains was evaluated through the analysis of the growth curves/lysis; two strains (10610 and 600) showed bacteriolysis by phage induction with MMC. Likewise, their cultures had the highest Stx titers together with the 5052 strain under induced conditions. Demonstrating that these strains display inducible phages capable of producing Stx2. Various studies showed that STEC strains with inducible Stx-phages increase substantially the Stx production, when the cultures were treated with MMC, evidencing a direct relationship between induction and Stx production [20, 21]. Additionally, Ogura et al. [22] have proposed that variability in Stx production among STEC strains may be related with the genetic characteristics of the phages that carry them.

Analyzing the bacterial growth/lysis curves constructed, two strains (102 and 615) showed a different behavior; both had low response to induction and low Stx titers, even under induced conditions. Indeed, there are studies demonstrating that bacteria can have a high frequency of defective prophages [20, 21, 22, 23, 24], which are not capable of carrying out its lytic cycle, limiting its capacity to kill the host bacterial cell; therefore, this would avoid (in the case of Stx-phages) Stx production. Even though Johansen et al. [25] suggest that the level of Stx production in bacteria carrying apparently defective phages is lower than bacteria carrying inducible phages. In this sense, the low Stx production in the 102 and 615 strains may be explained by the lack of Stx2-phage induction observed through the OD_{600} kinetics, which could be due to the presence of a defective Stx2-phage.

In this study we detected Stx production in cultures without MMC, suggesting that stx2 genes are carried by prophages with spontaneous induction, leading to high release of Stx independently of the bacterial SOS system, involved in the activation of the lytic cycle [21]. In agreement with our results, Gamage et al. [26] by analyzing pure supernatants, also reported that all stx_2 strains produced high levels of Stx both in presence and absence of inductors of the lytic cycle. Shimizu et al. [27] also reported that some Stx phages have a relatively high spontaneous induction, detecting the presence of Stx2 in the extracellular fraction in absence of any applied induction.

Three alleles encoding Q protein have been described (*q933, qO111* and *q21)*, all of them differ in their activity as anti-terminators [10, 11]. The *qO111* allele was detected in five strains (102, 10610, 5052, 615, and N108), which was described recently in sorbitol-fermenting STEC strains isolated from patients with diarrheic diseases and HUS in several European countries [28, 29, 11] and related with high progression to HUS [30]. In this study it was not possible to demonstrate the closeness of the *q933* allele with the stx_2 genes. Perhaps, Stx2 is encoded in a defective Stx-phage or some phages encoded in these strains do not carry the stx_2 gene. Regarding the *q21* allele, none strains were found carrying this allele, principally identified in STEC strains with low or null Stx production and frequently isolated from beef, animal origin or the environment in Asian countries, where these are widely distributed [31, 32, 33, 10].

Subtyping the STEC strains permitted identifying three Stx2 subtypes: one Stx2a-positive strain, three Stx2c-positive strains and two Stx2d-positive strains. According to the literature, the STEC strains carrying these three subtypes are frequently isolated from patients with HUS [5, 34], which has led to be strongly related with this disease. The most relevant subtypes identified in this study were: the Stx2a; according to the literature, it has demonstrated to be the most potent and active on the endothelial cells [4, 6] and the Stx2d, due to an "activatable tail" that causes an increase of up to 25 times its toxicity on Vero cells when it is previously incubated with elastases [35].

Although the three subtypes share a high percentage of identity regarding their amino acid sequence, differences were found in positions 89, 95 at the end of C-terminal of the A subunit, and in the END or EDD motif in the B subunit. The END motif of the B subunit plays an important role in the interaction of the toxin with its receptor; therefore, mutations in these positions would cause changes in the affinity of the toxin with its receptor [6], which could explain the differences in toxicity of these Stx2 subtypes. Finally, in the evaluated strains, the Stx2b, Stx2e, Stx2g, and Stx2f subtypes were not identified; these subtypes are rarely associated with human diseases and contrary to the other subtypes, they have been isolated from pigs and pigeons. In this study, these subtypes were not detected, since all the strains were isolated from cattle [36, 37].

5. Conclusion

The results obtained in this study show that the six native STEC strains evaluated had differences in the Stx2 titers, induction of the phage's lytic cycle, Stx2 subtypes, and late region of the Stx2-phages. The characteristics of the STEC strains found in this study will help to improve the understanding of STEC from Colombia, since these strains are similar to STEC strains that cause diseases in humans reported in the literature. However, more studies are necessary with broader samples that permit determining their virulence.

6. Acknowledgements

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7. Conflict of interest

The authors declare that there is no conflict of interest.

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Caracterización de seis cepas colombianas de *Escherichia coli* productoras de toxina Shiga (STEC) portadoras de fagos codificantes de Stx2

Resumen: *Escherichia coli* productora de toxinas Shiga (STEC) es un patógeno bacteriano que causa diarrea y enfermedades graves como síndrome urémico hemolítico en los humanos. Su principal factor de virulencia son las toxinas Shiga (Stx1 y Stx2). Algunos subtipos de Stx2 han sido identificados y asociados con el riesgo de desarrollar enfermedades severas. Las toxinas Stx están codificadas por bacteriófagos temperados que controlan su expresión a través del ciclo lítico que a su vez es regulado por los genes tardíos y la proteína antiterminadora Q. El objetivo de este trabajo fue caracterizar seis sepas STEC portadoras de fagos Stx2 para proveer información preliminar y conocer las cepas stx2 de Colombia respecto a su producción de toxina, inducción del ciclo lítico y subtipificación de Stx2. Nuestras observaciones resaltaron dos cepas que producían mayores niveles de Stx y mayor inducción del ciclo lítico. Todas las cepas evaluadas contenían los subtipos Stx2a, Stx2c o Stx2d. Adicionalmente, la mayoría de las cepas portaban el alelo *qO111* y solo una cepa mostró diferencias en la región *ninG*. Las diferencias en las características evaluadas entre las cepas observadas podrían indicar la variabilidad de estas seis cepas de STEC portadoras de fagos Stx2.

Palabras Clave: STEC; Bacteriófagos; Toxina Shiga; Ganado; HUS

Caracterização de seis cepas colombianas de *Escherichia coli* produtoras de toxina Shiga (STEC) portadoras de fagos codificantes de Stx2

Resumo: *Escherichia coli* produtora de toxinas Shiga (STEC) é um patógeno bacteriano que causa diarreia e doenças graves como síndrome hemolítico-urêmica nos humanos. Seu principal fator de virulência são as toxinas Shiga (Stx1 e Stx2). Alguns subtipos de Stx2 têm sido identificados e associados com o risco de desenvolver doença severa. As toxinas Stx estão codificadas por bacteriófagos temperados que controlam sua expressão através do ciclo lítico que é por sua vez regulado pelos genes tardios e a proteína antiterminadora Q. O objetivo deste trabalho foi caracterizar seis cepas STEC portadoras de fagos Stx2 para prover informação preliminar sobre as cepas stx2 da Colômbia respeito a sua produção de toxina, indução do ciclo lítico e subtipificação de Stx2. Nossas observações destacaram duas cepas que produziam maiores níveis de Stx e uma maior indução do ciclo lítico. Todas as cepas avaliadas portavam um destes subtipos: Stx2a, Stx2c ou Stx2d. Adicionalmente, a maioria das cepas portavam o alelo *qO111* e só uma cepa apresentou diferenças na região *ninG*. As diferenças nas características avaliadas entre as cepas observadas poderiam indicar a variabilidade destas seis cepas de STEC portadoras de fagos Stx2.

Palavras-chave: STEC; Bacteriófagos; Toxina Shiga; Gado; HUS

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