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Research article

Differential expression of the virulence gene *nleB* among Shiga toxin-producing *Escherichia coli* strains

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic foodborne pathogen associated with hemolytic uremic syndrome (HUS) that vary in their ability to cause disease in humans. STEC represents a serious problem for public health and Argentina is the country with the highest HUS incidence worldwide. Non-LEE effector (*nle*) genes, present on pathogenicity islands (PAIs), encode translocated substrates of the type III secretion system (T3SS), which could have an important role in STEC virulence. Particularly, *nleB* is one of the main effector genes proposed as a virulence marker that is involved in the action of T3SS during the STEC infection. NleB inhibits the inflammatory response of the host cell allowing the bacteria to persist in the first stage of the infection. In order to identify the potential risk of STEC strains for public health, the aim of this study was to evaluate and compare basal *nleB* transcription of 24 STEC strains belonging to 10 serotypes isolated from cattle, food and patients. The results showed differences in *nleB* transcription among strains. Some non-O157:H7 strains presented transcription levels above the control, an O157:H7 HUS-producing strain. On the other hand, no significant differences were found in basal transcription levels associated with origin or serotype but differences were found between HUS and non-HUS strains. These differences in *nleB* transcription may be of importance in STEC pathogenesis and could help to differentiate high and low virulence STEC strains.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen associated with foodborne diseases, which can colonize the gastrointestinal tract of animals and humans, causing severe infectious diseases in humans, such as diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Karmali et al., 2010). In Argentina, post-diarrheal HUS is endemic and its prevalence is the highest worldwide (Rivas et al., 2016).

The genetic variability of STEC increases the difficulty of identifying potential pathogens, especially among non-O157 strains. However, the evaluation of virulence genes in pathogenicity islands (PAIs) is a tool to determine the potential risk of these strains in public health considering these PAIs play an important role in the pathogenicity of STEC (Karmali et al., 2003; Coombes et al., 2008; Ju et al., 2013). In particular, the pathogenicity of some STEC strains can be attributed in part to the presence of the LEE locus, which encodes the type III secretion system (T3SS). Some pathogens use T3SS to translocate a wide repertoire of different effectors (virulence proteins) into the host cell in order to subvert cell-signaling cascades and promote bacterial colonization and survival. The non-LEE effector genes (*nle*) encode part of these translocated effector proteins (García-Angulo et al., 2012). Different studies suggest that the detection of a high number of these effector genes, in STEC strains belonging to O157:H7 and non-O157 serotypes causing disease in humans, is an advantage of virulence in these strains, since a lower prevalence was found in strains that do not cause disease (Wickham et al., 2006; Mingle et al., 2012).

Currently, more than 30 different types of effectors have been identified (Coombes et al., 2008; Yen et al., 2016). Some of these proteins interfere in the host immune response during the infection with STEC, being key in the virulence in humans after the zoonotic transmission. Genetic screening of effectors secreted by T3SS (Nle) indicates that some of them contribute to the colonization and persistence of STEC in cattle, increasing the possibilities of zoonotic and environmental transmission (Dziva et al., 2004; van Diemen et al., 2005; Coombes et al., 2008). In particular, NleB is a glycosyltransferase that modifies host proteins with *N*-acetyl-d-glucosamine to inhibit antibacterial and inflammatory host responses (Gao et al., 2013). Wickham

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et al. (2006) and Kelly et al. (2006) demonstrated that mutated bacteria of *Citrobacter rodentium* in which *nleB* had been deleted produced reduced colonization by simple infections and decreased colonic hyperplasia in mice, proposing NleB as an essential virulence factor of this pathogen. In addition, they hypothesized that NleB could be one of the molecules that contributes to the low infective dose of STEC (Wickham et al., 2006). More recently, other investigations also demonstrated that NleA, NleB and NleF effectors are important for colonization and pathogenicity in the model of *C. rodentium* infection, through *in-vivo* studies (Yen et al., 2016).

Regarding the role of these effectors in the virulence of STEC, the pathogens that produce the A/E lesion, mediated by LEE, use the T3SS-dependent effectors to alter detection by the host and the subsequent activation of inflammatory responses, thus avoiding the elimination of the host pathogen. The effectors NleB, NleC, NleE, NleH1 and Tir are suppressors of the NF-kB route, intervening in the host's proinflammatory responses. NleB, specifically, inhibits the inflammatory response of the host cell by preventing the translocation of the immune regulator NF-kB to the cell nucleus and antagonizes death receptor-induced apoptosis of infected cells (Newton et al., 2010; Pearson et al., 2013; Stevens and Frankel, 2014; Yen et al., 2016).

Considering the role that these effectors could have in the pathogenicity of STEC, it is important not only to detect the presence of the coding genes, but also to evaluate their transcription levels. In the last decade, it has been demonstrated that the variability of gene expression, due to different mechanisms, plays an important role in pathogenicity, particularly in clonal populations (de Sablet et al., 2008; de Gouw et al., 2014; Staley and Harwood, 2014; Aas et al., 2018).

In this context, and taking into account that *nleB* is one of the main effector genes proposed as a virulence marker, which is involved in the action of T3SS during the pathogenesis of STEC, our aim was to evaluate and compare the basal *nleB* transcription in STEC strains isolated from cattle, humans, and food in relation to serotype and origin.

2. Materials and methods

2.1. Bacterial strains

Twenty-four *nleB*-positive STEC isolates belonging to 10 serotypes available in our laboratory (Cadona et al., 2018) were analyzed in this study (Table 1). The strains belong to a collection of Immunochemistry and Biotechnology Laboratory (CIVETAN, FCV-UNCPBA) and were isolated in Argentina from different sources, meat food (n = 1), cattle (n = 11) and human (n = 12) and stored at -70 °C with 20% v/v glycerol. All strains have been previously analyzed by PCR for the presence of genes encoding for Shiga toxin 1 and 2 (vtx_1 and vtx_2) and intimin (*eae*), and in relation to seropathotype and virulence genes found in plasmids harboured by disease-associated strains (*ehxA*, *saa*, *subA*, *katP*, *espP*, *stcE*, *epeA*) (Parma et al., 2000; Padola et al., 2004; Rivero et al., 2010; Sanso et al., 2015).

2.2. Bacterial growth and RNA isolation

Bacterial cultures were grown overnight at 37 °C in Luria-Bertani broth (LB) with shaking. Each overnight culture was diluted 50-fold in DMEM (Dulbecco's Modified Eagle's Medium) and re-cultivated at 37 °C,

Table 1. Strain features and fold change results.

Strain	Serotype	Origin (and clinical manifestation in human[‡])	Isolation period	Virulence profile[§]	Fold change[*]	Cluster number[**]
224.2.2	**O26:H11**	human (HC)	2002–2009	vtx_1; *eae*; *ehxA*	**0.52654**	**1**
323.5.1	**O26:H11**	human (HUS)	2002–2009	vtx_1; *eae*; *ehxA*	**0.95740**	**2**
73.2.2	**O111:H2**	human (D)	2002–2009	vtx_1; *eae*; *ehxA*	**0.65709**	**1**
306.10.3	**O118:H2**	human (D)	2002–2009	vtx_1; *eae*; *ehxA*	**0.23397**	**0**
299	**O121:H19**	human (HC)	2002–2009	vtx_2; *eae*; *ehxA*	**0.03043**	-
430	**O128:NM**	human (AC)	2002–2009	vtx_2; *eae*; *ehxA*	**NE**	-
355	**O145:NM**	human (D)	2002–2009	vtx_2; *eae*; *ehxA*	**0.00187**	-
74.2	**O145:NM**	human (HUS)	2002–2009	vtx_2; *eae*; *ehxA*	**1.14935**	**2**
FB 78	**O145:NM**	cattle	2000	vtx_1; *eae*	**0.63552**	**1**
FB 79	**O145:NM**	cattle	2000	vtx_1; *eae*; *ehxA*	**0.53219**	**1**
FB 91	**O145:NM**	cattle	2000	vtx_2; *eae*; *ehxA*	**1.56132**	**2**
3.1.2	**O145:NM**	human (HC)	2002–2009	vtx_2; *eae*; *ehxA*	**0.33437**	**0**
506.1.5.2	**O145:NM**	human (HC)	2002–2009	vtx_2; *eae*; *ehxA*	**0.62980**	**1**
FB 67e	**O145:NM**	cattle	2000	vtx_1; *eae*; *ehxA*	**0.36245**	**0**
FB 87	**O145:NM**	cattle	2000	vtx_2; *eae*; *ehxA*	**0.24746**	**0**
FB 92	**O145:NM**	cattle	2000	vtx_2; *eae*; *ehxA*	**0.27122**	**0**
FB 81e	**O145:NM**	cattle	2000	vtx_2; *eae*	**0.69398**	**1**
FB 12	**O146:H21**	cattle	2000	vtx_2; *eae*	**1.08203**	**2**
34[†]	**O157:H7**	human (HUS)	2003	vtx_2; *eae*; *ehxA*	**1**	R
GAL 26	**O157:H7**	human (HUS)	1996–1997	vtx_2; *eae*; *ehxA*	**1.10093**	**2**
HT 2-15	**O157:H7**	food	2000	vtx_2; *eae*; *ehxA*	**0.00192**	-
T 83-1	**O165:NM**	cattle	1995–1996	vtx_2; *eae*; *ehxA*	**0.50530**	**1**
FO 127-3	**O177:NM**	cattle	2001	vtx_2; *eae*; *ehxA*	**0.34384**	**0**
FB 15	**O177:NM**	cattle	2000	vtx_2; *eae*; *ehxA*	**0.34540**	**0**

[†] Control strain: R.

[‡] Human strain: HUS, strain isolated from a patient with hemolytic uremic syndrome; HC, strain isolated from a patient with hemorrhagic colitis; D, strain isolated from a patient with diarrhea; AC, strain isolated from an asymptomatic carrier.

[§] Presence of genes encoding for Shiga toxin 1 and 2 (vtx_1 and vtx_2), intimin (*eae*) and enterohemolysin (*ehxA*).

[*] Fold change values obtained by the ΔΔCT method (Pfaffl, 2001). NE: no detectable transcription.

[**] Clusters based on K-means algorithm according to fold change values of each isolate. K-means analysis was performed for three clusters ($k = 3$). Isolates "299", "355" and "HT 2–15" were not included in ANOVA.

with 5% CO_2 and shaking during 90 min until OD_{600} 0.6. DMEM is a widely used medium in expression assays (Roe et al., 2007; Kudva et al., 2012, 2015; Amigo et al., 2016). After this growth, total RNA was extracted using the SV Total RNA Isolation System kit (Promega, Madison, WI) as per the manufacturer's instructions. The RNA concentration was measured using a ND-2000 Spectrophotometer (NanoDrop), and RNA integrity was evaluated by agarose gel electrophoresis. Additional treatment with DNase I (Roche Diagnostics GmbH) was performed to eliminate genomic DNA contamination before reverse transcription. For this, 1 μg of RNA was incubated with 20 U of DNase I for 1 h at 37 °C followed by 12 min at 72 °C for inactivation. Then, cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Controls without reverse transcriptase were carried out for each sample.

2.3. Quantitative real-time PCR analysis

The transcription levels of the *nleB* gene were evaluated by quantitative real-time PCR assays. The primers used to detect transcripts encoding the target gene and the reference gene, *tufA*, were taken from Bugarel et al. (2010) and de Sablet et al. (2008), respectively. Relative quantification reactions were performed on OneStep Plus Real-Time PCR System (Applied Biosystems). Each 20 μL qPCR reaction contained 4 μl of 1/10 diluted cDNA template, 10 μl of 2X SYBR Green master mix (Fast-Start Universal SYBR Green Master, Roche) and 300 nM of each primer. No-template control was included in each run to assess for reagent contamination and all reactions were carried out in triplicate and included 40 cycles consisting of 95 °C for 15 s followed by 60 °C for 1 min. The relative quantification was performed in comparison to the basal *nleB* transcription of an O157:H7 strain ("34" strain). This strain was chosen as control since O157:H7 is the serotype most involved in severe disease and the isolate was obtained from a child with HUS. Finally, the transcription levels relative to control strain were calculated by the ΔΔCT method (Pfaffl, 2001) using the efficiency corresponding to each gene, which were obtained from the relative standard curves and were determined using Relative Expression Software Tool (REST 2009) (Pfaffl et al., 2002).

2.4. Statistical analysis

Statistical analysis was performance using InfoStat software (v2016). Differences in transcription levels were evaluated using one-way analysis of variance (ANOVA) and Duncan's test was implemented when the analysis involved multiple comparisons.

ANOVA was carried out to compare the transcription levels according to the origin of the isolates (cattle and human; HUS and non-HUS), the serotype (in the case of those serotypes that were represented by more than one isolate) and three clusters according to the values of fold change obtained: cluster 0, 1 and 2. This clustering was performed using K-means algorithm to classify the fold change dataset into three groups ($k = 3$). All data were checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). Datasets were logarithmically transformed and differences with p-values ≤ 0.05 were considered statistically significant.

3. Results and discussion

The association between the presence of certain genes encoding virulence factors and a greater ability of strains to infect or remain in the colonization sites has been studied in numerous investigations. In particular, genes located within PAI have been used as diagnostic markers for the identification of pathogens and for the differentiation of pathogenic strains from those closely related but not pathogenic (Schmidt and Hensel, 2004). Many STEC strains have the potential to produce effectors, encoded on PAI, in order to colonize the host. However, it is likely that they do not always express such genes. In our study, real-time PCR was used to determine the transcription fold changes of the target gene, *nleB*, located in PAI OI (O-island)-122, in 24 STEC strains isolated from humans, cattle and food, compared with an O157:H7 strain isolated from a child who developed HUS.

According to the transcription analysis of *nleB*, heterogeneous transcription levels were detected among the strains studied. Transcription levels of each isolate, expressed as fold change values, relative to the control strain, are shown in Table 1. As for the normalization of the relative quantification reactions, from the construction of the standard curves for the target gene and the endogenous gene, the amplification efficiencies of each gene were 98.8% (*nleB*) and 93.6% (*tufA*). All strains showed detectable transcription levels except one, an O128:NM strain isolated from human ("430"), whose basal transcription was not detected under the experimental conditions tested. Some strains showed considerably low transcription levels, with 10-fold lower transcription than the control strain, such as two from human, O121:H19 ("299") and O145:NM ("355"), and one O157:H7 strain from food ("HT 2–15"). On the other hand, the transcription levels of three non-O157 strains isolated from human and cattle, belonging to O145:NM and O146:H21 serotypes ("74.2", "FB91" and "FB12"), were higher than basal transcription observed in the control, an O157:H7 HUS-producing strain (Figure 1).

The obtained results show that some strains that had the *nleB* gene did not express or expressed it at a low level, at least in basal conditions such as tested here. This does not mean that in the presence of some external signals, already within the eukaryotic cell, strains cannot express this gene. The regulation of T3SS is energetically costly and Enterohemorrhagic *E. coli* (EHEC) is versatile in the ability to sense a wide variety of signals and metabolites derived from the microbiota and microenvironment of the intestine to efficiently use this secretion system (Carlson-Banning and Sperandio, 2016).

Transcription levels were compared according to the origin of the strains, the serotype, and a K-means clustering based on the fold change values obtained for each strain, by an analysis of variance (ANOVA). The three isolates that presented the lowest transcription levels, equivalent to fold change <0.1, were not included in ANOVA to dataset comply with the statistical assumptions of normality and homogeneity of variances. Regarding strains origin, only two groups, cattle and human, were considered because only a single isolate from food (O157:H7) was available. The results showed that no significant differences were observed between the means of the fold change values of cattle and human strains; therefore, no association was established between basal *nleB* transcription and origin of strains (Figure 2a).

The variability of gene expression occurs due to different mechanisms ranging from variations inherent in the biochemical process of gene expression to individual responses to variations in the microenvironment that may be responsible for different phenotypes in a clonal population, a phenomenon called phenotypic plasticity (Roberfroid et al., 2016). Roe et al. (2007) evaluated the expression and regulation of different Nle, particularly NleA, in O157:H7 bacteria grown in DMEM and MEM-HEPES, a medium known as inducer of the secretion pathway of T3SS in EHEC. These authors used a DNA microarray and found that many strains showed transcript levels from 2 to 9 times higher when the culture was carried out in MEM-HEPES. In the present study, we choose DMEM, a widely used medium in expression assays, in order to evaluate the differences in basal *nleB* transcription between STEC strains isolated from different serotype and origin and identified heterogeneous transcription levels. According to the serotype of the strains studied, the results obtained between the means of transcription values of four serotypes are shown in Figure 2b. For this analysis, only those serotypes that were represented by more than one isolate were taken into account, O26:H11, O145:NM, O157:H7 and O177:NM. The results also showed that no significant differences were found between the different serotypes and, therefore, no association was determined between basal transcription values and serotype. Although the means of the fold change values for O26:H11 and O157:H7 serotypes were higher than the other serotypes

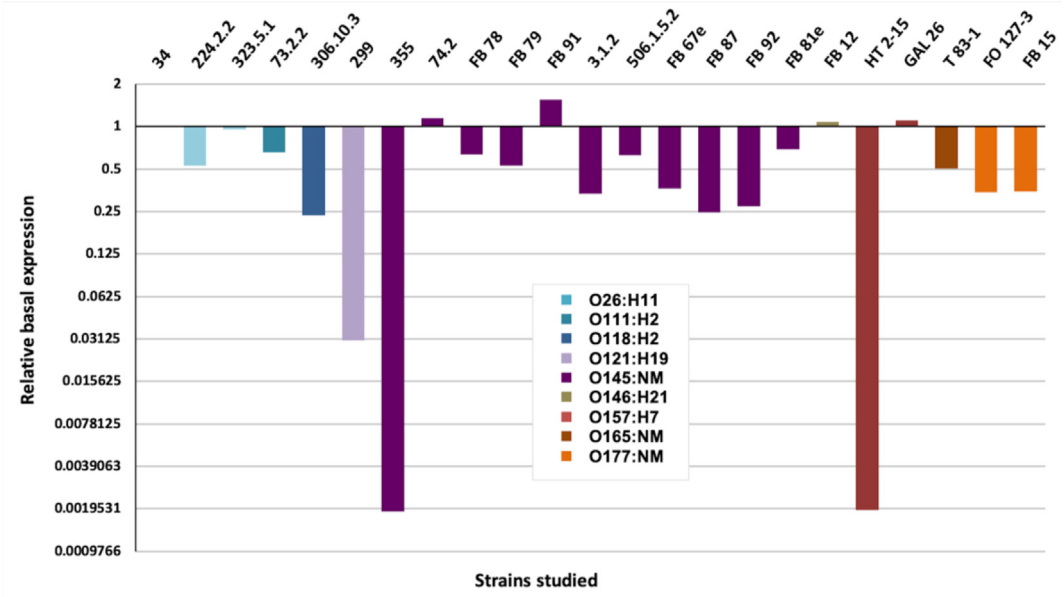

Figure 1. Relative basal *nleB* transcription of STEC strains. Strain "34" (serotype O157:H7) was used as control strain. Those strains with fold change values > 1 have higher transcription levels than control and, those with fold change values < 1, lower transcription levels. Strain of O128:NM serotype ("430") was not included in the figure because no transcription was detected under the experimental conditions tested.

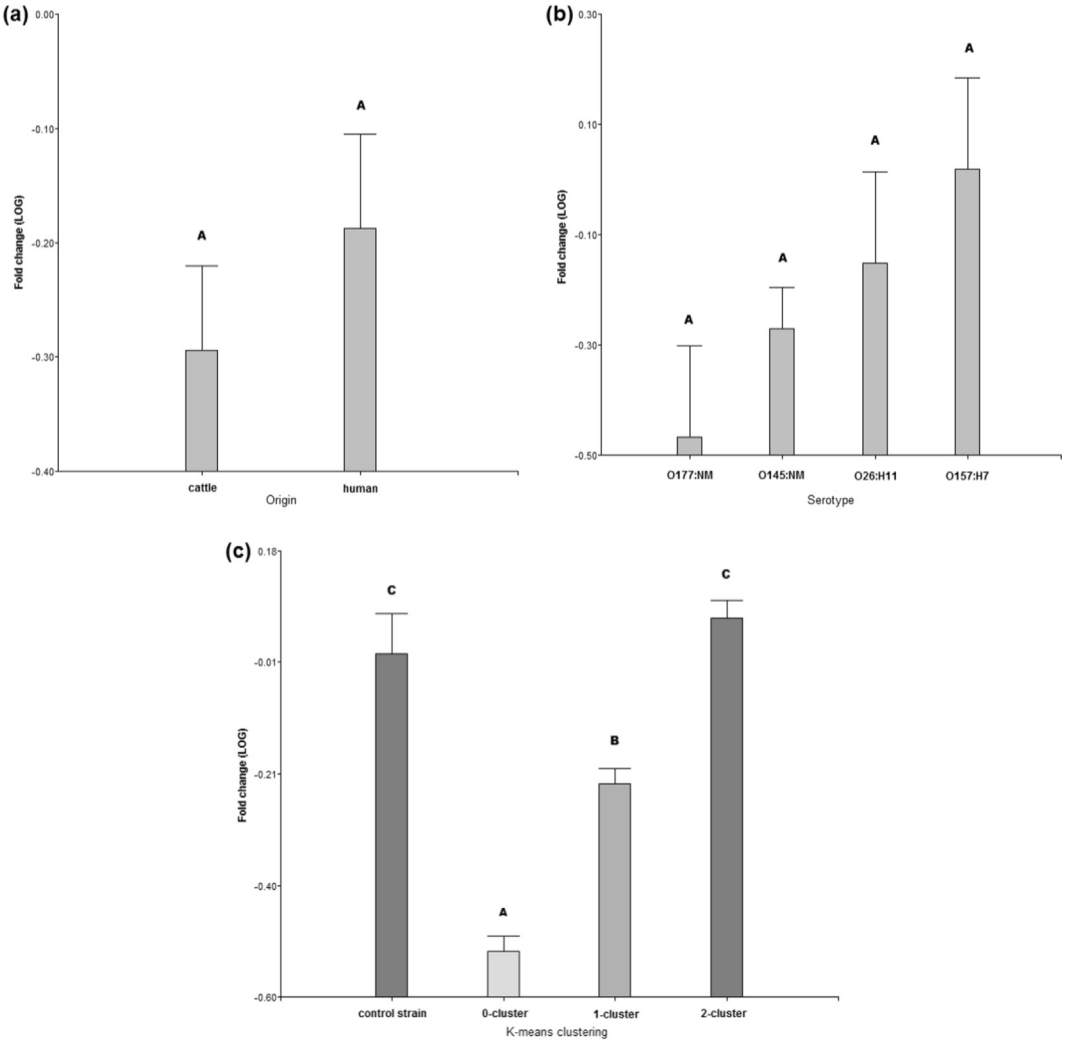

Figure 2. (a–c) Comparison of basal transcription levels of *nleB* according to: origin (a); serotype (b); and K-means clustering based on fold change values (c). The figures show the means for each group and *y-axes* show basal *nleB* expression relative to the control strain expression, an O157:H7 isolated from a clinical sample (HUS), expressed as fold change values. Error bars indicate standard error. Different letters indicate significant differences between groups (p-value ≤ 0.05) and means with the same letter are not statistically different.

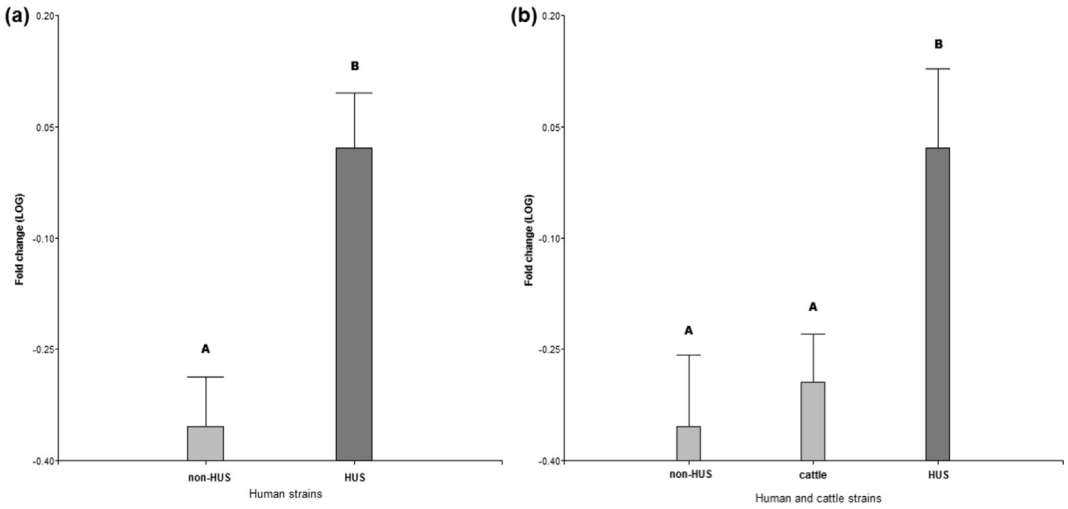

Figure 3. (a,b) Comparison of basal transcription levels of *nleB* according to the origin of the strains: HUS and non-HUS human isolates (a); HUS, non-HUS and cattle isolates (b). The figures show the means for each group and *y-axes* show basal *nleB* transcription relative to the control strain transcription, an O157:H7 isolated from a clinical sample (HUS), expressed as fold change values. Error bars indicate standard error. Different letters indicate significant differences between groups (p-value $\leq$ 0.05) and means with the same letter are not statistically different.

(O145:NM and O177:NM), these differences were not statistically significant.

According to the comparison between groups formed by strains with similar basal transcription levels (K-means clustering), the results showed significant differences between the three defined clusters: 2-cluster (cluster center = 1.17021), that included O26:H11, O145:NM, O146:H21 and O157:H7 strains; 1-cluster (cluster center = 0.59720), that included O26:H11, O111:H2, O145:NM and O165:NM strains; and 0-cluster (cluster center = 0.30553), that included O118:H2, O145:NM and O177:NM strains. The three clusters contained both cattle and human strains. On the other hand, the results showed that 0-cluster had transcription levels significantly lower than the other two clusters, while the transcription levels of the clusters 1 and 2 were highest with significant differences between each other. In the case of the 0-cluster and 1-cluster, they also presented significantly different expression levels than the control strain (Figure 2c).

The similar transcription levels of *nleB* found between the groups of bovine and human isolates (regardless of whether or not it was obtained from a case of HUS) and among those of different serotypes would show that assigning a certain pathogenic potential to a STEC strain according its origin or serotype, at least for this effector gene, would not be useful. In our study, it was found strains (clusters 1 and 2) with *nleB* transcription levels similar or higher than the value of control strain (isolated from a patient with HUS). Particularly noteworthy is an O145:NM strain isolated from cattle which expressed approximately twice the value of the control.

On the other hand, and according to clinical manifestation that caused human strains studied, we did find differential *nleB* transcription between HUS-associated isolates and those isolated from mild disease patients or cattle. An analysis of variance between HUS strains and those isolated from mild disease (non-HUS) revealed that there were significant differences between the HUS and non-HUS isolates (Figure 3a). In addition, the results showed that non-HUS and cattle strains were not statistically different, but transcription levels between cattle and HUS strains showed significant differences (Figure 3b).

Our results are coincident with those from Aas et al. (2018), who compared transcriptomes of clinical STEC strains that caused severe disease like HUS and transcriptomes from non-pathogenic (or mild disease-producing) strains in non-induced condition. They identified several genes that were differentially expressed between HUS-producing STEC (higher level) and non-HUS STEC, including *nleB*. A number of factors have been postulated to account for differential expression between isolates, including differences in DNA sequence, in the number of gene copies and in specific transcriptional regulators (de Gouw et al., 2014; Roberfroid et al., 2016; King et al., 2019).

In conclusion, the role in disease of the wide collection of Nle proteins, either individually or together, may depend, as discussed above, on at least two different aspects: the genetic repertoire of each strain and the characteristics of the host. The study of basal gene transcription levels, such as that carried out here, is a first evaluation that allows to know whether the transcription in a bacterial group is homogeneous or, on the contrary, it varies according to origin or serotype. On the one hand, strains obtained from human and bovine, presented similar transcription levels, but on the other side, we detected significant differences between HUS and non-HUS isolates groups, which would indicate that strains causing severe disease express the NleB effector at a higher basal level. These differences in gene expression may be of importance in the STEC pathogenesis and this finding could contribute to differentiate high and low-virulence STEC strains. While beyond the scope of the current study, further studies involving complete sequences of each isolate genome, particularly promoter regions sequences, will clarify whether the differential transcription levels of the *nleB* gene are associated or not with specific polymorphisms of each isolate.

Declarations

Author contribution statement

Jimena S. Cadona: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Julia Burgán: Performed the experiments; Analyzed and interpreted the data.

Juliana González: Analyzed and interpreted the data.

Ana V. Bustamante: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

A. Mariel Sanso: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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