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Phenotypic and Genotypic Determination of Biofilm Formation in Shiga Toxin-Producing *Escherichia coli*

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Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this article.

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is the most prevalent serotype associated with severe diseases worldwide. Biofilms by STEC O157:H7 constitute a high risk to public health and the food industry since they allow cross-contamination of surfaces and the consequent transmission to humans. This study aimed to detect the presence of adhesins genotypically and determine the ability to form biofilm and the curli expression in a collection of 30 O157:H7 strains from healthy cattle and human cases. The *efa1*, *iha*, *fimCD*, *ehaA*, *lpfA1-3*, and *lpfA2-2* genes were detected in all strains; *cah* was frequently detected in strains isolated from humans (16/20), and *agn43* was the least prevalent gene (3/30). All strains could form a biofilm, although those isolated from cattle were the most biofilm-formers. The curli-negative phenotype was the most prevalent phenotype observed at 37 °C and room temperature. The association between curli production and biofilm formation could not be determined, but the highest proportion of curli-positive strains at room temperature were strong biofilm-formers. These results highlight the possibility of the persistence of STEC O157:H7 in environmental conditions and food processing facilities, increasing the risk of contamination or infection.

Keywords: Biofilm Formation, Curli Fimbriae, Hemolytic Uremic Syndrome, Shiga Toxin-Producing *Escherichia coli*, Sources.

INTRODUCTION

Infections with Shiga toxin-producing *Escherichia coli* (STEC) are associated with severe diseases, including acute diarrhea (AD), bloody diarrhea (BD), and hemolytic uremic syndrome (HUS) [1]. HUS has no specific treatment, and antimicrobial therapy is not recommended [2].

Ruminants, especially cattle, are the main reservoirs of STEC and shed the bacteria in their feces, spreading these pathogens throughout the environment [3, 4]. The ingestion of contaminated, undercooked food, e.g., cattle-derived products like meat or milk, water, and direct contact with animals or person-to-person transmission are common ways to acquire STEC infections [5, 6]. STEC O157:H7 is the most prevalent serotype associated with large outbreaks and sporadic cases of BD and HUS. This serotype has been isolated from different sources as well as healthy cattle, the water of sanitary lagoons and pens from feedlots [4, 7], and from human cases, e.g., bloody diarrhea and HUS [1, 8].

The main virulence factors of STEC are two variants of Shiga toxins (Stx), named Stx1 and Stx2, and their respective subtypes [9]. However, the adherence and colonization of the gut are also important and constitute a necessary step in the pathogenesis of STEC [10]. STEC can harbor several adhesins and fimbriae, which play an important role in adherence to the host, abiotic surfaces, and biofilm formation. Intimin (*eae*), the long polar fimbriae (*lpf*), Antigen 43 (*agn43*), the Calcium-binding antigen 43 homolog (*cah*), an adherence-

conferring protein homolog to IrgA (*iha*), an enterohemorrhagic *E. coli* factor of adherence (*efa1*), type 1 fimbriae (*fim*) and the EHEC autotransporter EhaA (*ehaA*) are some of the proteins involved in STEC adherence and colonization [11, 12, 13].

Biofilms are communities of sessile cells that grow embedded in a polymeric organic matrix on biotic and abiotic surfaces. Curli and cellulose are the major constituents of the extracellular matrix of biofilms of *E. coli*. Bacteria growing within the biofilm have differences in the growth rate and gene transcription for their planktonic counterparts [14]. This lifestyle contributes to increasing the survival of bacteria in environmentally adverse conditions, disinfection procedures, and antibiotic therapies [15, 16]. Biofilms formed by bacterial pathogens as STEC O157:H7 constitute a high risk to public health and the food industry since they allow cross-contamination of surfaces and the consequent transmission to humans [17].

Considering the high incidence rates of STEC infections in Argentina and the importance of biofilm formation for the transmission of this pathogen, this study aimed to evaluate the ability to form biofilms of STEC O157:H7 strains isolated from healthy cattle and human cases. The genotypic characterization of adherence factors and curli production was also determined.

MATERIALS AND METHODS

Bacterial strains

Thirty O157:H7 STEC strains isolated from healthy cattle (n=10) and humans with STEC infections (n=20) from a collection of STEC belonging to the Laboratory of Immunochemistry and Biotechnology of the University of Center of Buenos Aires Province were studied (Table 1). All the strains were previously serotyped and characterized in their classic virulence factors -*stx1*, *stx2*, *eae*, *ehxA*, *rfbO157*- [18, 19, 20, 21, 22, 23] (Table 1).

Table 1: Sequences of PCR primers and size of amplification products used in this study.

Primers*	Sequence 5'-3'	PCR amplicon (bp)	References
efa1 R (88T9) *efa1 F (88T14)*	GGTATTGTTGCATGTTCAG GAGACTGCCAGAGAAAG	479	[24]
iha R *iha F*	CAGGTCGGGGTTACCAAGT CAAATGGCTCTCTTCCGTCAATGC	925	[10]
ehaA F *ehaA R*	AGGCATGAGACACGATC AAGTCGTGCCATTGAGC	500	[13]
fimCD F *fimCD R*	TCGTTAGGATTCAACAGGAACAGGACAGTGAG TGCCAGAAGCTTGCAGGGAACAATCCCTTGTT	1154	[25]
lpfA1c (1-3) F *lpfA1c (1-3) R*	GGTTGGTGACAAATCCCCG CGTCTGGCCTTTACTCAGA	244	[11]
lpfA2c (2-2) F *lpfA2c (2-2) R*	CTACAGGCGGCTGATGGAACA GCTAATACCAGCGGCAGCATCGT	297	[11]
agn43_F *agn43_R*	GGGGGACTTCAGCACGATAA CCGGCGGGCAATGGGTACA	1803	[12]

* Primers *agn43 Fw* and *Rew* amplify for both *agn43* and *cah* genes.

PCR assay

PCR assay was used to detect several genes encoding for adherence and colonization factors such as *efa1* [24], *iha* [10], *fimCD* [25], *ehaA* [13], *agn43*, *cah* [26], *lpfA1-3* and *lpfA2-2* [11]. Primers and the size of PCR products are shown in Table 1. *E. coli* O157:H7 EDL933 (*efa1*, *iha*, *fimCD*, *ehaA*, *cah*, *agn43*, *lpfA1-3*, *lpfA2-2*) was used as positive control.

Two monoplex PCR were made for *efa1* and *iha* genes; the reactions were performed in a 25 µl volume containing 1X PCR buffer (50 mM KCl, 10 mM Tris pH 9, 0.1% Triton X-100), 2 mM Mg2Cl2, 200 µM of each dNTP, 10 µM of each primer, 1 U of DNA Taq polymerase (InBio, Highway) and 2.5 µl of DNA. The same conditions were used to perform a multiplex PCR for *fimCD* and *ehaA* genes. For the *lpfA1-3*, *lpfA2-2*, *agn43*/*cah* genes the similar conditions of reaction were used, except for the concentration of primers which were 2.5 pM of each primer *lpfA1c (1-3)* and *lpfA2c (2-2)* and 0.4 µM of each primer of *agn43*/*cah*. Monoplex PCR for *efa* and *iha* consisted of an initial denaturation of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 ºC (denaturation), 1 min at 55ºC (annealing), 1 min at 72 ºC (extension), and a final extension cycle of 5 min at 72 ºC. Multiplex PCR for *fimCD* and *ehaA* consisted of a first denaturation step for 2 min at 95 ºC, followed by 30 cycles of 45 s at 95 ºC (denaturation), 45 s at 55 ºC (annealing), 1 min 30 s at 72 ºC, and a final extension

of 7 min at 72 °C. Multiplex PCR for *lpfA1c (1-3)* and *lpfA2c (2-2)* consisted of an initial denaturation of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C (denaturation), 30 s at 57 °C (annealing), 30 s at 72 °C (extension) and a final extension of 10 min at 72 °C. On the other hand, the reaction corresponding to *agn43*/*cah* consisted of a first denaturation cycle of 5 min at 94 °C, 30 cycles of 45 s at 94 °C (denaturation), 30 s at 58 °C (annealing), 1 min 50 s at 72 °C (extension) and a final extension cycle of 5 min at 72 ° C. All PCR reactions were realized in the thermocycler T-18 (IVEMA Desarrollos, Argentina).
PCR products were separated by electrophoresis in agarose gel (2%) stained with 0.8 µg/ml of ethidium bromide and were visualized by UV transillumination.

Table 2: Genotypic characterization of O157:H7 STEC strains isolated from healthy cattle and human cases (n = 30).

STEC strain	Sample[a]	Virulence profile	PCR: Adherence factors							
			efa1	***iha***	***fimCD***	***ehaA***	***lpfA1-3***	***lpfA2-2***	***agn43***	***cah***
C1	FC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
C2	FC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
C3	FC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
C4	WC	*stx2, eae, ehxA*	+	+	+	+	+	+	+	+
C5	WC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
C6	RC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
C7	RC	*stx2, eae, ehxA*	+	+	+	+	+	+	+	+
C8	RC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
C9	MC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
C10	MC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
INE#A	BD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
INE#B	HUS-AHC	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
INE#C	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
INE#D	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	-
INE#E	HUS	*stx1, stx2, eae, ehxA*	+	+	+	+	+	+	-	+
INE#F	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
INE#G	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
INE#H	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
INE#I	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
INE#J	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#1	WD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#2	WD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#3	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#4	WD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#5	BD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#6	WD	*stx2, eae, ehxA*	+	+	+	+	+	+	+	+
CH#7	BD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#8	WD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#9	WD	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+
CH#10	HUS	*stx2, eae, ehxA*	+	+	+	+	+	+	-	+

a Strains isolated from: FC, feedlot cattle; WC, weaning calves; RC, rearing calves; MC, milk cows; BD, bloody diarrhea; HUS-AHC, asymptomatic household contact of a patient with HUS; HUS, hemolytic uremic syndrome; WD, watery diarrhea.

Biofilm formation

The ability to form biofilm was estimated by culture on polystyrene microplates using the Crystal Violet (CV) technique, according to Cáceres [27]. Briefly, an aliquot of each strain culture grown in Luria Bertani broth (Britania) supplemented with glucose 0.25 % were seeded in 96 well polystyrene plates and was incubated statically for 48 h at 37 °C. The plates were stained with CV (0.1%), and the remaining dye was eluted with 200 µl of 96% ethanol. The biofilm formation was estimated by measuring of OD570 using a microplate reader (Labsystem Multiscan EX, I.C.T, Instrumentación Científica Técnica S.L).The experiments were performed for triplicate in 3 independent events. The OD obtained for each strain was averaged and corrected by a cutting OD (sum of the OD average of the control wells -3 wells with non-inoculated sterile medium- and three times their standard deviation) [28]. According to the corrected OD (ODc), the strains were classified into four categories: non-biofilm former (NBF) (ODc < cutting OD); weak biofilm former (WBF)

(cutting OD ≤ ODc < 2 cutting OD); moderate biofilm former (MBF) (2 cutting OD ≤ ODc < 4 cutting OD); strong biofilm former (SBF) (ODc > 4 cutting OD).

Curli production assay

The production of curli fimbria was examined by Congo red test according to Bokranz [29] with modifications. Briefly, STEC O157:H7 isolates were streaked on LB agar plates supplemented with Congo red (40 mg/L; Biopack) and Coomassie brilliant blue (20 mg/L; Merck, R250) -hereinafter CR plates- and incubated for 24 h at 37 °C. Then, the plates were incubated at room temperature (20 °C approximately), and colonies were observed two times (24 and 72 h). The colonies were classified according to the phenotype as saw (curli-negative, "soft and white"), sar (curli-positive, "soft and red"), or rdar (curli-positive, "red, dry and rough"). In some cases, a double phenotype was observed (saw-sar).

RESULTS

Search results

Adherence and colonization factors

The *efa*, *iha*, *fimCD*, *ehaA*, *lpfA1-3*, and *lpfA2-2* genes were detected in all STEC strains. The *cah* gene was present in most of the strains isolated from humans (16/20) and cattle (7/10); *agn43* was the least prevalent (3/30). Three genotypic profiles were identified according to the presence of genes related to adherence (Table 2): *efa1*, *iha*, *fimCD*, *ehaA*, *lpfA1-3*, *lpfA2-2*, *cah* (20/30; 66.7%); *efa1*, *iha*, *fimCD*, *ehaA*, *lpfA1-3*, *lpfA2-2* (7/30; 23.3%) and *efa1*, *iha*, *fimCD*, *ehaA*, *lpfA1-3*, *lpfA2-2*, *cah*, *agn43* (3/30; 10%).

Biofilm formation

All strains were able to form biofilm. Most of the strains, all isolated from human cases, were classified as WBF and MBF (14/30, 46.7% and 4/30, 13.3% respectively) (Figure 1), while 12/30, (40%) were considered SBF, all isolates from cattle and two isolates from humans (Table 3, Figure 1). The most prevalent genotypic profile among each category of biofilm formation was *efa1*, *iha*, *fimCD*, *ehaA*, *lpfA1-3*, *lpfA2-2*, *cah*.

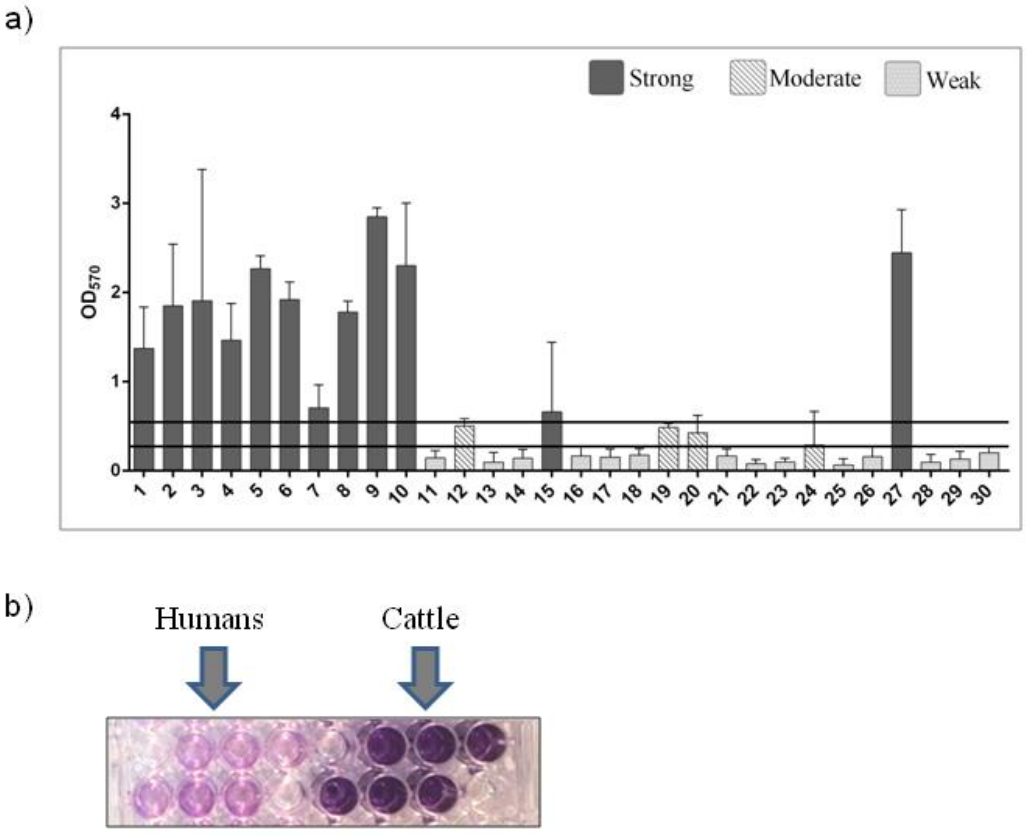

Figure 1: Biofilm formation by STEC O157:H7 from different sources. Classification of strains according to a cutting OD = 0.137 into strong, moderate, and weak biofilm-formers (a); representative image of crystal violet microplate staining (b). Lines represent the 2OD and 4OD values corresponding to the change of biofilm formation categories.

Table 3: Biofilm formation and curli production by STEC O157:H7 from cattle and humans (n = 30).

N°	STEC strain	Biofilm formation	Curli production		
			37 °C 24 h	20 °C 24 h	20 °C 72 h
1	C1	+++	saw	saw	saw
2	C2	+++	saw	saw-sar	saw-sar
3	C3	+++	saw	saw	saw
4	C4	+++	saw-sar	saw-sar	saw-rdar
5	C5	+++	sar	saw-sar	sar
6	C6	+++	sar	saw-sar	saw-sar
7	C7	+++	sar	saw-sar	saw
8	C8	+++	saw	sar	rdar
9	C9	+++	saw	sar	sar
10	C10	+++	saw	saw-sar	saw-sar
11	INE#A	+	saw	saw	saw
12	INE#B	++	saw-sar	saw	saw
13	INE#C	+	saw-sar	saw-sar	saw
14	INE#D	+	saw	saw	saw
15	INE#E	+++	sar	sar	sar
16	INE#F	+	saw-sar	saw	saw
17	INE#G	+	saw	saw-sar	saw
18	INE#H	+	saw-sar	saw-sar	saw
19	INE#I	++	sar	saw	sar
20	INE#J	++	sar	saw	saw
21	CH#1	+	sar	saw	saw
22	CH#2	+	sar	saw	saw
23	CH#3	+	sar	saw	sar
24	CH#4	++	sar	saw	saw
25	CH#5	+	saw-sar	saw	saw
26	CH#6	+	sar	saw	sar
27	CH#7	+++	sar	saw	saw
28	CH#8	+	saw	saw	saw
29	CH#9	+	saw	saw	saw
30	CH#10	+	saw	saw	saw

b Classification of the ability to form biofilms in: weak biofilm former (+); moderate biofilm former (++); strong biofilm former (+++), according to a cutting OD = 0.137.
c Curli phenotypes: “saw” (curli-negative, soft and white); “sar” (curli-positive, soft and red); “saw-sar” (double phenotype); “rdar” (curli-positive, red, dry, and rough).

Curli production

The curli production was evaluated at two temperatures, 37 °C and room temperature (≈ 20 °C), to observe the behavior and stability of STEC O157:H7 strains. The different phenotypes of curli expression are shown in Figure 2.

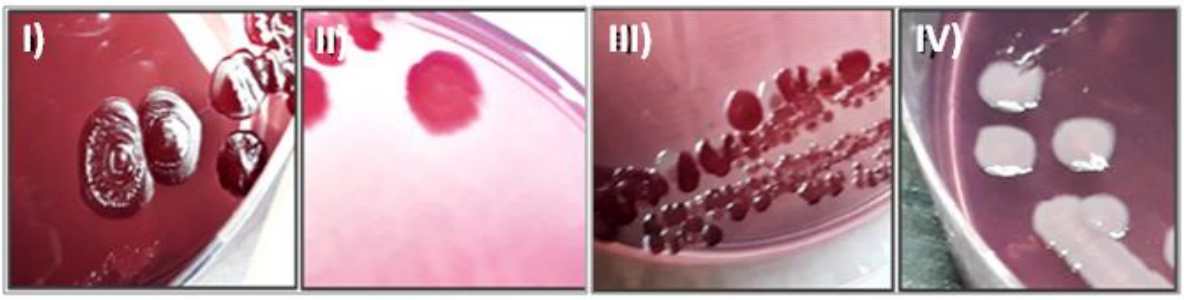

Figure 2: Representative image of the different curli phenotypes observed on CR plates: curli-positive rdar (I) y sar (II); double phenotype saw-sar (III) and curli-negative saw (IV).

Most strains showed curli-negative -saw- (12/30, 40%) or curli-positive phenotypes -sar- (12/30, 40%), while 6/30 (20%) had a double phenotype (saw-sar) at 37 °C (Table 3). After incubation at room temperature, the saw phenotype became the most prevalent among all strains at 24 and 72 h of incubation (18/30 and 19/30, respectively). The double phenotype

(saw-sar/saw-rdar) was observed in 9/30 (30%) and 3/30 (10%) at 24, and 72 h, respectively. The curli-positive phenotypes (sar/rdar) were the least prevalent and they were observed in 3/30 (10%) and 7/30 (23.3%) strains at 24 and 72 h of room temperature incubation, respectively (Table 3).

It was noticeable that the saw phenotype remained the most prevalent among the weak and moderate biofilm-former strains when the temperature of incubation decreased. While the double and sar phenotypes increased among the strong biofilm formers strains after temperature change (Table 3).

DISCUSSION

STEC O157:H7 is the main agent involved in cases of diarrhea and HUS worldwide. Biofilms allow bacteria to survive harsh environmental conditions such as disinfection procedures or antibiotic treatment, so they become difficult to remove, favoring colonization and bacterial transmission [30]. Therefore, this study focused on evaluating the ability to form biofilms, to detect adherence factors genotypically, and to determine the curli expression of O157:H7 STEC strains isolated from cattle and humans.

In this study, all O157:H7 strains harbored *efa1*, *iha*, *fimCD*, *ehaA*, *lpfA1-3* and *lpfA2-2*. Previous reports have related the distribution of adherence genes with the source of isolation [20]. The *efa1* and *lpf*O157 genes were closely related to *eae*-positive strains, including O157:H7 serotypes isolated from humans in Chile [20]. The *efa1* gene was also found in a high number of STEC *eae*-positive strains, including O157:H7 serotypes, isolated from young cattle of Argentina [31], suggesting that this adhesin may have an important role in the attachment of STEC both to the bovine and human gut. The *iha* gene was widely detected in human and bovine STEC strains [10] mainly. Several STEC strains were isolated from cattle regardless the age [31]. The *fimCD* and *ehaA* genes have been detected widely in several STEC strain isolated from cattle and human cases [13, 25], but only serotypes non-O157 expressed type 1 fimbria and could form biofilm on abiotic surfaces [25, 32]. Most of the strains isolated from humans and cattle carried *cah*, but only three strains (two from cattle and one from humans) harbored *agn43*. Both *agn43* and *cah* encode for proteins involved in adherence and cellular autoaggregation necessary to form biofilms [33]. In addition, it has been observed that Agn43 is synthesized during human disease, suggesting that it plays an important role in the development of HUS [12, 26]. In this study, the association presence/absence of genes and biofilm formation of each strain could not be determined. Other authors have concluded that the presence or absence of a genotype or phenotype cannot explain the differences observed between biofilm formation and that biofilm formation was highly dependent on strain rather than gene expression, serotype, or fimbriae production [34, 35]. However, genotypic characterization of STEC isolates constitutes a key rule for the identification and investigation of this pathogen worldwide [1].

On the other hand, all strains analyzed were biofilm-forming with different intensities, especially those isolated from humans. Although most of the STEC strains harbored almost all adherence factors studied, more strains were weak and moderate biofilm-former among those isolated from humans. In contrast, all strains isolated from cattle were classified as strong-biofilm formers. In agreement with these findings, other authors found that only strains isolated from cattle and water formed biofilm versus human strains, which could not form biofilms [32]. Conversely, Vogeleer [36] has found that O157:H7 serotype isolated from food and humans had a greater ability to form biofilms than other serotypes. Biofilm formation in cattle feces or the environment contributes to STEC O157:H7 evolution by horizontal transfer of genetic elements that could enhance pathogenicity and increase variety [37]. Since cattle are persistent shedders of STEC, the ability to form a biofilm of this pathogen could favor the contamination of beef, dairy products, and water systems with a consequent infect human [38].

The fimbria curli plays an important role during the adhesion stage to the surfaces and maturation of the biofilm, favoring cellular aggregation and interaction with cellulose [39]. Curli has been studied in the biofilm formation of *E. coli*, especially in STEC O157:H7, on surfaces commonly used in the food industry, such as stainless steel, polystyrene, glass, and rubber [40]. The production of this fimbria is favored by temperatures lower than 30 °C and varies not only according to the serotype but also within the same serotype [32]. In this study, the curli production at both 37 °C and environment temperature (≈ 20 °C) was variable between all strains, being saw the most prevalent phenotype observed. Incubation at room

[1] World Health Organization & Food and Agriculture Organization of the United Nations (2018). Shiga toxin-producing Escherichia coli (STEC) and food: attribution, characterization, and monitoring: report (online]. Website https://apps.who.int/iris/handle/10665/272871 (accessed 12 November 2020].

temperature notably reduced the proportion of curli-positive strains, while the proportion of curli-negative strains increased even after 72 h. However, the proportions of positive, negative, or double curli phenotypes between 24 and 72 h at 20 °C did not vary greatly. These results slightly differ from those found by other authors who have observed an increase of curli expression at temperatures less than 37 °C but at more than 24 h of incubation [35]. Cattle strains showed more proportion of curli-positive phenotypes at room temperature, while human strains notably increased curli-negative phenotypes. On the other hand, most of the weak biofilm-former strains were curli-negative both at 37 °C and at room temperature, and most of them were isolated from humans. These data suggest that it could be a correlation between curli production, biofilm formation, and the source of STEC O157:H7.

CONCLUSION

In conclusion, this study showed that STEC O157:H7 strains from different sources formed biofilm under the given conditions. Although the virulence and adherence genes profiles were similar, the biofilm formation of the strains isolated from cattle was higher than those isolated from human clinical cases. The association of curli production and biofilm formation could not be strongly determined, but it was observed that the highest proportion of curli-positive strains at room temperature were strong biofilm-formers. These results highlight the possibility of the persistence of STEC O157:H7 in environmental conditions and food processing facilities, increasing the risk of contamination or infection.

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