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SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN HUMAN, CATTLE AND FOODS. STRATEGIES FOR DETECTION AND CONTROL

Topic Editors
Nora Lía Padola and Analía I. Etcheverría

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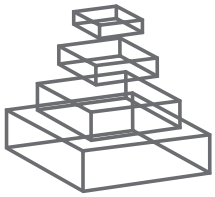

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SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN HUMAN, CATTLE AND FOODS. STRATEGIES FOR DETECTION AND CONTROL

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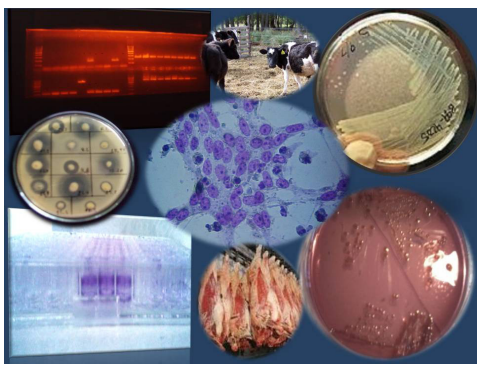

Cover image by Nora Lía Padola and Analía Etcheverría Cytotoxic effect of Shiga toxin-producing *E. coli* on Vero cell culture (40x)

Shiga toxin-producing *Escherichia coli* (STEC) is an important foodborne pathogen associated with both outbreaks and sporadic cases of human disease, ranging from uncomplicated diarrhoea to haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). STEC affects children, elderly and immuno-compromised patients. STEC is capable of producing Shiga toxin type 1 (Stx1), type 2 (Stx2) or both, encoded by *stx1* and *stx2* genes, respectively. These strains are likely to produce putative accessory virulence factors such as intimin (encoded by *eae*), an enterohaemolysin (EhxA) and an autoagglutinating protein commonly associated with eae-negative strains (Saa), both encoded by an enterohaemorrhagic plasmid. Several studies have confirmed that cattle are the principal reservoir of STEC (O157 and non-O157:H7 serotypes) and many of these serotypes have been involved in HUS and HC outbreaks in other countries. Transmission of STEC to humans occurs through the consumption of undercooked meat, vegetables and water contaminated by faeces of carriers and by person-to-person contact. Diagnostic methods have evolved to avoid selective diagnostics, currently using molecular techniques for typing and subtyping of strains. Control is still a challenge, although there are animal vaccines directed against the serotype O157:H7.

Table of Contents

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EDITORIAL
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Shiga toxin-producing *Escherichia coli* in human, cattle, and foods. Strategies for detection and control

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Keywords: STEC, cattle, food, environment, virulence factors

Shiga toxin-producing *E. coli* (STEC) also known as "verocytotoxin-producing *E. coli*," refers to *E. coli* pathotypes capable of producing Shiga toxin type 1 (Stx1), type 2 (Stx2), or both, encoded by *stx1* and stx2 genes, respectively (Paton and Paton, 1998). The genes encoding Stx are carried by temperate bacteriophages insert into bacterial genoma so that Stx production is linked to the induction of the phage lytic cycle (O'Loughlin and Robins-Browne, 2001). STx2 is the toxin type most related to hemolytic uremic syndrome (HUS) and comprise several subtypes which differ in their citotoxicity (Persson et al., 2007). Stx2g is one of those subtypes that were studied by Granobles Velandia et al. (2012) who found several differences among *stx*2g-positive strains. The strains with the highest cytotoxic titer showed higher levels of *stx*2-phages and toxin production by EIA, while the opposite occured for strains that previously showed low cytotoxic titers, confirming that in *stx*2g-positive strains Stx production is phage regulated.

Other typical virulence factor is intimin, which is required for intimate bacterial adhesion to epithelial cells inducing a characteristic lesion defined as "attaching and effacing" (A/E). It is encoded by *eae* gene that presents heterogeneity in their 3′ end and involved in binding to the enterocytes (Guth et al., 2010). Additional virulence-associated markers are a plasmid-encoded enterohemolysin and, in strains lacking *eae*, an autoagglutinating adhesin (Saa) which could be involved in the adhesion of strains (Paton et al., 2001). Strains laking *eae* are named as LEE-negative STEC. Steyert et al. (2012) demonstrate that the overall genome content, phage location, and combination of potential virulence factors are variable in this strains group.

STEC are zoonotic pathogens that cause the vascular endothelial damage observed in patients with hemorrhagic colitis (HC) and HUS. HUS is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia and is a potentially fatal cause of acute renal failure in children (Etcheverría and Padola, 2013). HUS there has not treatment and use of antimicrobial agents is associated with an increased risk of severe sequelae such as HUS. Referred to this, Rahal et al. (2012) dicussed novel modalities and regimen of antimicrobial agent administration in an attempt at decreasing their association with aggravating infection outcomes.

Cattle are the main reservoir of STEC and shed the bacteria through their feces spreading these pathogens among cattle herds and the environment. Nguyen and Sperandio (2012) review about the factors and mechanism utilized by O157:H7 STEC for its survival through the acidic environment of the distal stomach and for its colonization in the recto-anal junction. Fernández et al. (2013) characterized two most prevalent serotypes in argentinian cattle demonstrating the potential pathogenic of this strains. Blanco Crivelli et al. (2012) informed that synanthropic species could play role in the transmissibility of the agent thus being a risk to the susceptible population.

Food, water, milk, and person to person contact commonly participate in transmission, although there is a growing concern about some sporadic cases and outbreaks attributable to direct contact with the animal environment (Duffy, 2003). Brusa et al. (2013) report the prevalence of STEC O157 and non-O157 in commercial ground beef and ambient samples, including meat table, knife, meat mincing machine, and manipulator hands suggesting cross-contamination between meat and the environment. One method for reducing STEC in food could be the use of phages. About this, Tomat et al. (2013) inform the isolation of phages highly specific for virotypes of *E. coli* that could be useful in reducing STEC in meat products.

In order to diagnose STEC (O157 and non-O157) several methods have been implemented in the last years (Padola, 2014). Botkin et al. (2012) investigate a multiplex PCR to differentiate EPEC, STEC, and EHEC strains from other pathogenic *E. coli*, Fratamico and Bagi (2012) use a GeneDisc system to evaluate a new PCR-real time technology based on simultaneous detection of multiple targets, Quiñones et al. (2012) evaluate a DNA microarray targeted 12 virulence factors implicated in produce human disease while Parma et al. (2012) developed a sandwich ELISA for determination of Stx using anti-Stx2 B subunit antibodies showing that could be used in routine diagnosis as a rapid, specific and economic method for detection of STEC. The implementation of Multiple-locus variable-number tandem repeat analysis (MLVA) as subtyping method is review by Bustamante et al. (2012) who have adapted this method for analysis of non-O157 STEC performing an efficient O157:H7 and non-O157 STEC subtyping.

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Differences in Shiga toxin and phage production among stx_{2g}-positive STEC strains

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Shiga toxin-producing *Escherichia coli* (STEC) are characterized by the production of Shiga toxins (Stx) encoded by temperate bacteriophages. Stx production is linked to the induction of the phage lytic cycle. Several *stx* variants have been described and differentially associated with the risk of developing severe illness. The variant named stx_{2g} was first identified in a STEC strain isolated from the faeces of healthy cattle. Analysis of stx_{2g}-positive strains isolated from humans, animals, and environmental sources have shown that they have a close relationship. In this study, stx_{2g}-positive STEC isolated from cattle were analyzed for phage and Stx production, with the aim to relate the results to differences observed in cytotoxicity. The presence of inducible phages was assessed by analyzing the bacterial growth/lysis curves and also by plaque assay. Bacterial growth curves in the absence of induction were similar for all isolates, however, notably differed among induced cultures. The two strains that clearly evidenced bacteriolysis under this condition also showed higher phage titers in plaque assays. However, only the phage plaques produced by one of these strains (FB 62) hybridized with a stx_2-probe. Furthermore, the production of Stx was evaluated by enzyme immunoassay (EIA) and Western immunoblotting in overnight supernatants. By EIA, we detected Stx only in supernatants of FB 62, with a higher signal for induced than uninduced cultures. By immunoblotting, Stx2 could be detected after induction in all stx_{2g}-positive isolates, but with lower amounts of Stx2B subunit in those supernatants where phages could not be detected. Taking into account all the results, several differences could be found among stx_{2g}-positive strains. The strain with the highest cytotoxic titer showed higher levels of stx_2-phages and toxin production by EIA, and the opposite was observed for strains that previously showed low cytotoxic titers, confirming that in stx_{2g}-positive strains Stx production is phage-regulated.

Keywords: cytotoxicity, Stx2g, phage induction, toxin production

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are important pathogens that can cause severe human diseases, including hemorrhagic colitis and hemolytic uremic syndrome (Karmali et al., 1985). STEC comprise a diverse group of *E. coli* strains characterized by the production of Shiga toxins (Stx1 and/or Stx2), which are regarded as their main virulence factors.

The genes encoding Stx are usually carried by bacteriophages. In general, *stx* genes are situated among genes controlled by the phage late promoter suggesting that Stx production is linked to the induction or progression of the phage lytic cycle (Neely and Friedman, 1998; O'Loughlin and Robins-Browne, 2001). Several variants of *stx* genes have been described, and have been differentially associated with the risk of developing severe illness (Friedrich et al., 2002; Beutin et al., 2004; Persson et al., 2007).

A probably emergent variant named Stx2g was identified by Leung et al. (2003) in STEC isolated from faeces of healthy cattle. These authors found that this stx_{2g} variant had high similarity with stx_2 genes associated with human disease, and besides, Stx2g cytotoxicity for HeLa and Vero cells was comparable to that of Stx2EDL933.

Other studies have also described strains carrying stx_{2g} isolated from cattle, wastewater, aquatic environments, and humans (Garcia-Aljaro et al., 2005; Beutin et al., 2006; García-Aljaro et al., 2006; Beutin et al., 2007; Krüger et al., 2007; Persson et al., 2007; Garcia-Aljaro et al., 2009; Nguyen et al., 2011; Prager et al., 2011). Differences have been detected in regard to toxin production, cytotoxic activity, and *stx*-phage release among stx_{2g}-positive strains (Beutin et al., 2006; García-Aljaro et al., 2006; Krüger et al., 2011; Prager et al., 2011). Interestingly, Prager et al. (2011) demonstrated that stx_{2g}-positive strains isolated from humans, animals, and environmental sources have a close phylogenetic relationship, reinforcing the idea of human infections as a potential zoonotic disease. At

present, the role of stx_{2g} in human pathogenicity has not been evaluated.

In this study, stx_{2g}-positive STEC isolated from cattle were analyzed for phage and Stx production, with the aim to relate the results to differences observed in cytotoxicity.

MATERIALS AND METHODS

BACTERIAL STRAINS

The stx_{2g}-positive isolates analyzed in this study (**Table 1**) have been previously described regarding the serotype and other virulence factors (Padola et al., 2004; Krüger et al., 2007; Granobles Velandia et al., 2011). Cytotoxic activity was evaluated in a previous study showing differences among these isolates (Krüger et al., 2011). One of the strains, belonging to O2:H25 serotype had a high basal titer comparable to those obtained from strains carrying the $stx_{2EDL933}$ subtype, but the others showed low basal cytotoxicity. All these stx_{2g}-positive strains showed a low response to mitomycin C induction.

As a positive control of phage lysis the strain *E. coli* EDL933 ($stx_{1EDL933}$/$stx_{2EDL933}$, O157:H7) was used. This strain was kindly provided by Dr. J. Blanco (Laboratorio de Referencia de *E. coli*, Spain). The strain *E. coli* DH5α was used as host strain for phage detection.

BACTERIAL GROWTH/LYSIS CURVES

Bacteria were grown overnight in Luria Bertani (LB) medium at 37°C with shaking at 100 rpm. An aliquot was inoculated into fresh LB medium and incubated at 37°C and 180 rpm up to an optical density at 600 nm (OD_{600}) ≈ 0.2−0.3. In that moment (named 0 h), each culture was subdivided into two flasks and mitomycin C was added to one of them to a final concentration of 0.5 μg/ml. The cultures were incubated overnight and monitored spectrophotometrically every hour for the first 5 h, and when necessary, dilutions of the samples were performed. Bacterial enumeration was also conducted by plating appropriate dilutions in duplicate by using LB agar plates. The assays were done at least three times.

Table 1 | Characteristics of STEC strains.

Strain	Serotype	*stx* genotype	Verotoxicity		
			Uninduced conditions[a]	Induced with mitomycin C[b]	Increase (I/U[c])
FB 62	O2:H25	stx_{2g}	High	I	16
FB 11	O15:H21	stx_{2g}	Low	I	16
FB 40	O175:H8	stx_{2g}	Low	I	8
FB 46	O175:H8	stx_{2g}	Low	I	8

[a] *Mean titers classified in three categories: (low) ≤16; (medium) 32–128; (high) ≥256.*

[b] *Mean titers classified in three categories: (I) ≤4,096; (II) 8,192–65,536; (III) ≥131,072.*

[c] *I/U fold change: mean induced titer/mean uninduced titer.*

EVALUATION OF PHAGE PRODUCTION

To evaluate phage production, we followed the methodology described by Muniesa et al. (2004), with some modifications. At 3 h after mitomycin C induction, an aliquot of each culture was centrifuged for 10 min at 10,000 × g. The supernatants were filtered through low-protein-binding 0.22 μm membrane filters (Millex-GV, Millipore) and tenfold serially diluted. One hundred μl of each dilution were then mixed with 500 μl of an exponential phase culture of *E. coli* DH5α (OD_{600} ≈ 0.6−0.8) and incubated for 30 min at 37°C with shaking (180 rpm). The suspension was then mixed with 3 ml of LB soft agar supplemented with 3.2 mM $CaCl_2$ and 0.5-1 μg/ml ampicillin (Muniesa et al., 2004; Santos et al., 2009), and poured onto LB agar plates. The plates were examined for the presence of lysis plaques following incubation for 18 h at 37°C. The assays were done at least three times.

PLAQUE HYBRIDIZATION

Plaques were transferred onto nylon membranes positively charged (Roche Diagnostics GmbH) according to a standard procedure (Sambrook and Russell, 2001) and hybridized at 68°C with a stx_2 specific probe. The probe was synthesized by PCR using stx_2 generic primers (Paton and Paton, 1998), and labeled by incorporating digoxigenin 11-deoxyuridine triphosphate (Roche Diagnostics, Germany).

EVALUATION OF EXTRACELLULAR SHIGA TOXIN PRODUCTION

Stx production was evaluated in the supernatants of stx_{2g}-positive strains after overnight incubation with or without mitomycin C, by using an enzyme immunoassay (EIA, Ridascreen® Verotoxin, R-Biopharm, Germany). The results were analyzed spectrophotometrically at 450 nm. The supernatant of the *E. coli* DH5α culture was included as negative control besides the negative control of the kit. Test results were recorded as weak positive (1+) if the extinction was >0.1–0.5 above the negative control, moderate (2+) (extinction > 0.5–1.0 above negative control) and strongly positive 3+ (>1.0–2.0) to 4+ (>2.0). The assays were done at least three times.

The supernatants of stx_{2g}-positive strains after overnight incubation with mitomycin C were also evaluated by Western immunoblotting. Briefly, 12 μl of supernatants were separated by 12.5% SDS-PAGE (under reducing conditions) and transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). The membrane was blocked overnight at 4°C with 5% skimmed milk in PBS-Tween 0.1%, and incubated with a 1:500 dilution of anti-Stx2B rabbit IgG in PBS-Tween 0.1% for 1 h at 37°C (Parma et al., 2011). After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) for 1 h at 37°C. Finally, membranes were revealed using DAB/H_2O_2 system (Pierce). As positive controls, recombinant Stx2B protein and the supernatant of an overnight culture of a $stx_{2EDL933}$-positive *E. coli* strain were used.

RESULTS AND DISCUSSION

In this study, stx_{2g}-positive STEC isolates belonging to serotypes O2:H25, O15:H21 and O175:H8, which have previously shown differences in cytotoxicity titers, were analyzed for phage and Stx production, under inducing and non-inducing conditions.

The presence of inducible phages was assessed by analyzing the bacterial growth/lysis curves constructed for each strain and also by plaque assay using *E. coli* DH5α as host strain. The bacterial growth curves in the absence of mitomycin C were similar for all stx_{2g}-positive isolates and also similar to that of *E. coli* EDL933. However, the bacterial growth/lysis curves notably differed when cultures were exposed to mitomycin C (**Figure 1**). Only two of the isolates (FB 62 and FB 11) clearly evidenced bacteriolysis under this condition. The strain FB 62 (serotype O2:H25), which had the highest cytotoxicity titer among stx_{2g}-positive isolates (Krüger et al., 2011), showed an OD_{600} pattern with a maximum of 2.5 at 2 h after mitomycin C induction, followed by a significant decrease typical of host cell lysis, which reached the baseline OD_{600} at 5 h of culture. The FB 11 strain also showed a bacteriolytic pattern, but the maximum OD_{600} value, which occured 2 h after mitomycin C induction, was lower than 2.0. On the contrary, the other stx_{2g}-positive isolates (FB 40 and FB 46) did not show a marked bacteriolytic pattern and their growth/lysis curves were similar to that of the stx_2-negative strain *E. coli* DH5α. These two STEC isolates reached a maximum OD_{600} earlier (1 h after mitomycin C induction) with a lower value (1.0), and along the following 4 h of culture the OD_{600} decreased gradually.

The different patterns were related to differences in the viable bacterial counts. In the FB 62 and FB 11 cultures, the bacterial counts remained stable comparing 0–1 h after mitomycin C induction, and then a drop was observed between 1 and 2 h (a 2 log for FB 62 and a 1.5 log for FB 11). In contrast, bacterial counts diminished earlier in FB 40 and FB 46, reaching a 2 log decrease in the first hour after the addition of mitomycin C.

We could only observe lysis plaques with the supernatants of FB 62 and FB 11 cultures, and the phage titers were higher from induced than from uninduced cultures (pfu increased from 1.0×10^2 to 3.0×10^3 for FB 62 and from 5.0×10^3 to 2.3×10^4 for FB 11). However, only the phages produced by FB 62 strain were stx_{2g}-phages (as these phage plaques hybridized with a stx_2-probe). The production of extracellular Stx was evaluated by

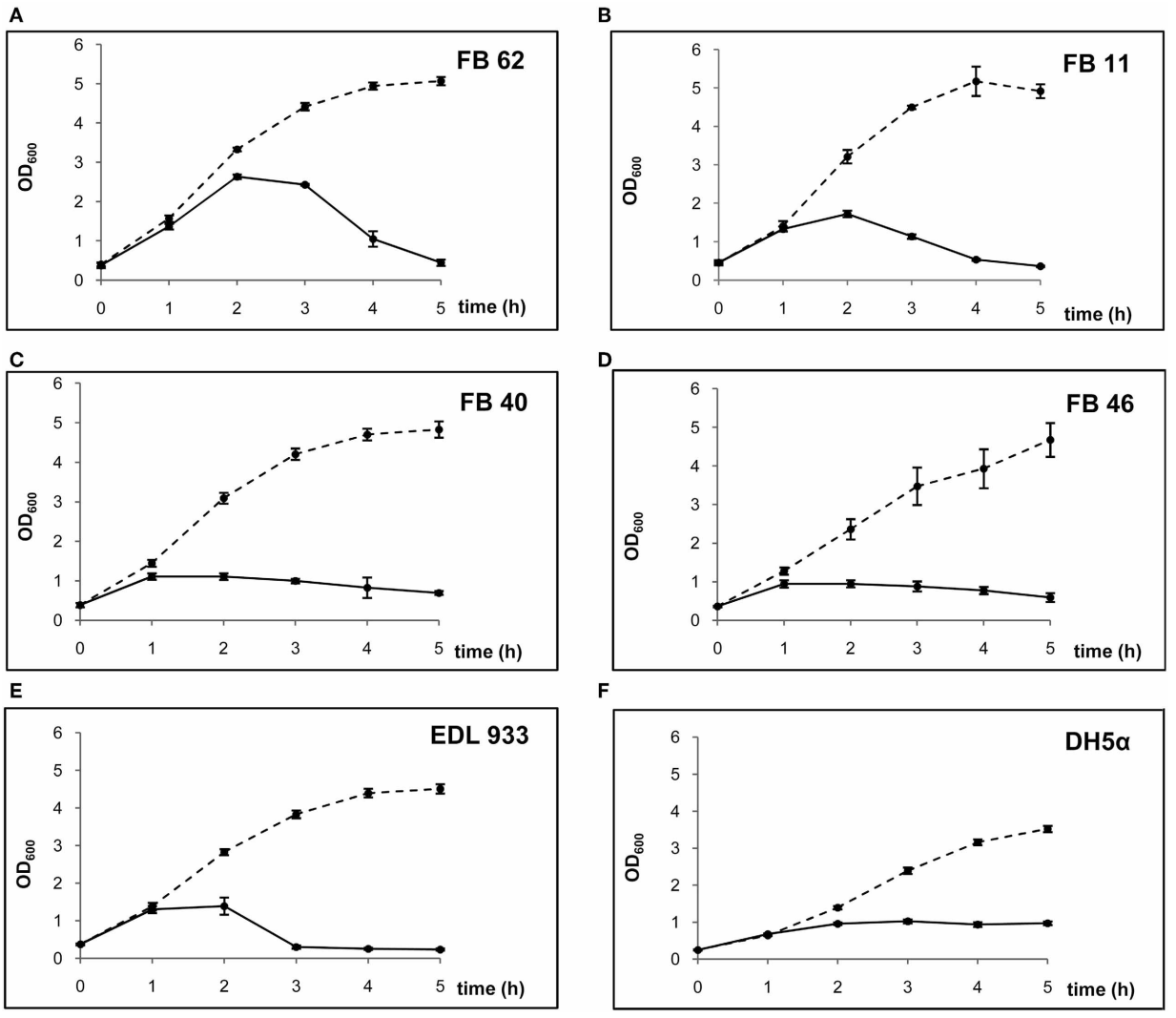

FIGURE 1 | Growth/lysis curves of the isolates studied in presence and absence of mitomycin C (solid and dashed lines, respectively).

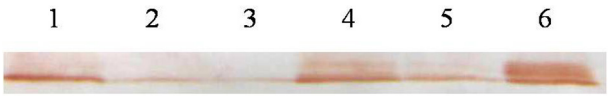

FIGURE 2 | Stx2B detection by Western immunoblotting. Anti-Stx2B IgG was used as first antibody. Lanes 1–5: supernatants of isolates FB 11, FB 40, FB 46, FB 62, and positive control (O26:H11 STEC strain, harboring $stx_{2EDL933}$ subtype), respectively. Lane 6: recombinant Stx2B protein.

EIA and Western immunoblotting in overnight supernatants. By EIA, we detected the toxin only in supernatants of FB 62 (with values of 3+ and 4+ for uninduced and induced cultures, respectively). By Western immunoblotting (using anti-Stx2B subunit antibodies), toxin production after mitomycin C induction was detected in all stx_{2g}-positive isolates (**Figure 2**). Despite the same volume of supernatant form each culture was loaded onto the gel, a faint band was observed in strains FB 40 and FB 46 comparing to strains FB 11 and FB 62, evidencing the presence of lower amounts of toxin (B subunit) in those supernatants.

Taking all the results into account, several differences could be found among the four stx_{2g}-positive strains. The strain with the highest cytotoxic titer (FB 62) presented a bacteriolytic pattern when the growth curve under mitomycin C treatment was analyzed. As we expected, this strain also had high levels of Stx and stx_2-phage production, and both were higher under inducing conditions. Therefore, it can be concluded that FB 62 strain has an inducible stx_2-phage, and produces high amounts of Stx2, biologically active on Vero cells. Noticeably, this strain belongs to the same serotype (O2:H25) as the strain 7v isolated by Leung et al. (2003) from cattle, which is the reference strain for stx_{2g}.

Regarding FB 11 strain, we observed that it carries one or more inducible phages because of both the presence of infective particles in the supernatants and the bacteriolytic pattern observed by monitoring the OD_{600} of the culture. These phages do not seem to encode stx_{2g}, as no signal was obtained when the plaque hybridization assay was performed. Possible explanations could be that stx_{2g} either is not phage encoded in this strain or is encoded in a defective *stx*-phage, or that lytic cycle of the stx_{2g}-phage is repressed by other phage/s. Indeed, there are studies demonstrating that not all stx_2 genes are associated with inducible prophages as well as studies that suggest the existence of regulatory mechanisms when two stx_2-phages are present in a same strain (Teel et al., 2002; Muniesa et al., 2003; Zhang et al., 2005; Karama and Gyles, 2008).

The apparent absence of lytic cycle induction of stx_{2g}-phages in FB 11 strain correlates with the low cytotoxic titer under inducing conditions. It seems it produces a low amount of toxin, which is undetectable by EIA but detectable by Western immunoblotting (Stx2B subunit). The epitopes recognized in the EIA are probably different from the ones detected by the anti-Stx2B antibodies used in the immunoblotting. Besides, limits caused by sensitivity of EIA-Ridascreen to detect low Stx production, such as the case of some stx_{2g}-positive strains, have been reported by Beutin et al. (2006).

The FB 40 and FB 46 isolates, both with low cytotoxic titers on Vero cells and a low increase under inducing conditions, showed a particular behavior in the present study since both strains did not have OD_{600} curves typical of lytic cycle induction. Instead, they seemed to have a bacteriostatic pattern when incubated with mitomycin C, similarly to *E. coli* DH5α strain. Moreover, they showed an earlier decrease in viable bacterial counts than FB 11 and FB 62. Analyzing these isolates, neither phage plaques were obtained nor Stx production was detected by EIA, and the Stx2B subunit was detected by Western immunoblotting with low intensity. In this regard, Johansen et al. (2001) observed that the level of Stx production in bacteria that carry apparently defective phages is lower than in bacteria from which phages can be induced.

Interestingly, Prager et al. (2011), assessing Stx production by EIA-Ridascreen and by Vero cell cytotoxicity assays, detected some stx_{2g}-positive strains that did not produce Stx2, some of which contained stx_{2g} pseudogenes but others presented intact stx_{2g} genes. Other authors have reported strains PCR-positive for stx_{2g} with lack of Stx expression (García-Aljaro et al., 2006; Beutin et al., 2007; Miko et al., 2009).

In accordance with the present work, García-Aljaro et al. (2006) found that only those stx_{2g}-positive strains that carried inducible stx_{2g}-phages showed Stx production, and noticeably, these strains also belonged to O2:H25 serotype as FB 62 strain.

Our results highlight the variability among stx_{2g}-positive strains and show that phage regulation can affect Stx2g production as differences in verocytotoxicity correlated both with differences in lytic cycle induction, and with phage and Stx production.

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Comparative genomics and *stx* phage characterization of LEE-negative Shiga toxin-producing *Escherichia coli*

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Infection by *Escherichia coli* and *Shigella* species are among the leading causes of death due to diarrheal disease in the world. Shiga toxin-producing *E. coli* (STEC) that do not encode the locus of enterocyte effacement (LEE-negative STEC) often possess Shiga toxin gene variants and have been isolated from humans and a variety of animal sources. In this study, we compare the genomes of nine LEE-negative STEC harboring various *stx* alleles with four complete reference LEE-positive STEC isolates. Compared to a representative collection of prototype *E. coli* and *Shigella* isolates representing each of the pathotypes, the whole genome phylogeny demonstrated that these isolates are diverse. Whole genome comparative analysis of the 13 genomes revealed that in addition to the absence of the LEE pathogenicity island, phage-encoded genes including non-LEE encoded effectors, were absent from all nine LEE-negative STEC genomes. Several plasmid-encoded virulence factors reportedly identified in LEE-negative STEC isolates were identified in only a subset of the nine LEE-negative isolates further confirming the diversity of this group. In combination with whole genome analysis, we characterized the lambdoid phages harboring the various *stx* alleles and determined their genomic insertion sites. Although the integrase gene sequence corresponded with genomic location, it was not correlated with *stx* variant, further highlighting the mosaic nature of these phages. The transcription of these phages in different genomic backgrounds was examined. Expression of the Shiga toxin genes, stx_1 and/or stx_2, as well as the *Q* genes, were examined with quantitative reverse transcriptase polymerase chain reaction assays. A wide range of basal and induced toxin induction was observed. Overall, this is a first significant foray into the genome space of this unexplored group of emerging and divergent pathogens.

Keywords: *Escherichia coli*, microbial genomics, Shiga toxin, evolution, phage

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) isolates can colonize the intestinal tract in animals and humans, and in humans are associated with diarrheal symptoms ranging from mild diarrhea to severe hemorrhagic colitis (Kaper et al., 2004; Manning et al., 2008). Hemolytic uremic syndrome (HUS), although arising in only a minority of colonized individuals, is a serious and sometimes fatal complication resulting from elaboration of the Shiga toxins (Stx; Karch et al., 1999; Kaper et al., 2004). Many STEC disease outbreaks have been caused by a subset of STEC isolates, *L*ocus of *E*nterocyte *E*ffacement (LEE)-positive STEC, that harbor the LEE pathogenicity island and one or more *stx* genes (Yoon and Hovde, 2008). These isolates have often been designated enterohemorrhagic *E. coli* (EHEC), but the current study will use a genomic designation of LEE-positive STEC. The genes carried in the LEE pathogenicity island encode a type III secretion system that transports effector molecules into the host cells (Kaper et al., 2004). LEE-positive O157:H7 has been responsible for the majority of STEC disease outbreaks in the United States; however, non-O157 LEE-positive STEC serogroups are prevalent in other countries and are increasingly found associated with outbreaks in the United States (Brooks et al., 2005; Johnson et al., 2006; Gould et al., 2009). Although the LEE pathogenicity island is known to be an important virulence factor, LEE-negative STEC isolates from diverse serogroups have been found to cause the same severe diarrheal symptoms and HUS (Johnson et al., 2006; Mellmann et al., 2008; Newton et al., 2009; Kappeli et al., 2011). With the exception of the recent O104:H4 outbreak that occurred in Germany (Rasko et al., 2011), non-O157 STEC isolates have received much less scrutiny at the whole genome level than their LEE-positive counterparts.

Shiga toxin, the crucial virulence factor attributed to the progression of HUS, can be identified in two major antigenic forms, Stx1 and Stx2, with Stx2 identified as the more potent form (Boerlin et al., 1999; Friedrich et al., 2002). However, stx_1 and stx_2 allele variants have been identified; LEE-negative STEC, in particular, have been determined to often carry these diverse toxin subtypes (Zhang et al., 2002; Burk et al., 2003; Orth et al., 2007; De Sablet

et al., 2008; Slanec et al., 2009). Scant information exists on the potency of the different allelic forms, but one report concluded that both *in vitro* and *in vivo* potencies of Stx2a and Stx2d were greater than Stx2b and Stx2c (Fuller et al., 2011). In addition to the potency of the particular encoded Stx, the amount of Stx produced is thought to play a role in virulence (De Sablet et al., 2008; Neupane et al., 2011). Stx genes are encoded by lambdoid bacteriophages and enhanced levels of *stx* expression has been observed for some isolates in prophage inducing conditions (Zhang et al., 2000; Ritchie et al., 2003). Considerable heterogeneity in both basal and induced levels of stx_2 expression has been reported among LEE-positive O157:H7 isolates (Ritchie et al., 2003; De Sablet et al., 2008; Zhang et al., 2010; Neupane et al., 2011). In comparison, less information is available regarding levels of *stx* expression for LEE-negative STEC isolates.

Qualitatively, lambdoid bacteriophages are composed of non-homologous DNA segments, or modules, that have been exchanged between various prophages, leading to broad genetic diversity even within single isolates (Johansen et al., 2001; Brussow et al., 2004; Casjens, 2005). For example, substantial phage sequence diversity has been noted among the 11 lambdoid prophages within the genome of the LEE-positive O157:H7 Sakai isolate (Brussow et al., 2004), and other LEE-positive O157:H7 isolates (Johansen et al., 2001; Ogura et al., 2006). Although sequence divergence of *stx*-encoding phages has been identified, the gene structure of the *stx* cassettes is less well known, and has been determined for only a few LEE-negative STEC isolates. Along with the assortment of mosaic structures, a variety of chromosomal insertion locations have been identified for *stx*-encoding phages in LEE-positive STEC isolates. These insertion sites include *wrbA*, *yecE*, *torS/T*, *sbcB*, *yehV*, *argW*, *ssrA*, and *prfC* (Ogura et al., 2007). Interestingly, the insertion sites of the *stx* phages in the genomes of the majority of LEE-negative STEC isolates are often different than those determined for LEE-positive STEC isolates, and remain largely unidentified (Garcia-Aljaro et al., 2006, 2009; Prager et al., 2011).

Although production of Shiga toxin is essential for the progression of infection to HUS, STEC utilize many other virulence mechanisms during colonization of the human intestine (Yoon and Hovde, 2008). The tight adherence of the bacterial cell to the colonic epithelium resulting from expression of the *eae* encoded Intimin and Tir proteins encoded by the LEE pathogenicity island is considered an important step in infection. The LEE-positive STEC also utilize other chromosomally encoded adhesins and typically express multiple fimbriae (Toma et al., 2004; Farfan and Torres, 2011). LEE-positive STEC genomes also carry genes encoding autotransporter (AT) proteins that have been associated with virulence (Wells et al., 2010). Many AT proteins expressed by pathogenic *E. coli* have been characterized and determined to either to function as proteases, adhesins, hemagglutinins, or to promote autoaggregation or biofilm formation (Wells et al., 2010). LEE-negative STEC isolates must utilize factors other than the Intimin/Tir complex to adhere, thus the question arises as to whether they only make use of factors already identified in LEE-positive STEC genomes or also use as yet undiscovered chromosomally encoded adherence factors. The long polar fimbrial gene cluster, designated lpf_{O113}, was identified in the LEE-negative STEC O113:H21 isolate EH41 (Doughty et al., 2002), and subsequently identified in other LEE-negative STEC isolates, as well as some non-O157 LEE-positive STEC isolates (Doughty et al., 2002; Toma et al., 2004). Along with chromosomally encoded virulence factors, pathogenic *E. coli* often harbor a large virulence plasmid encoding a variety of additional virulence factors. Although there is heterogeneity between virulence plasmids carried by a particular *E. coli* pathotype, the plasmids display a greater level of similarity within the pathotype than between pathotypes (Johnson and Nolan, 2009). A single LEE-negative STEC O113:H21 isolate, designated EH41, harbors a virulence plasmid of ∼166 kb, designated pO113 (Newton et al., 2009). Both pO157, commonly carried by O157:H7 isolates, and pO113 carry the *ehxA* gene encoding enterohemolysin and an *espP* gene encoding a serine protease autotransporter of *Enterobacteriaceae* (SPATE; Newton et al., 2009; Ogura et al., 2009). The STEC autoagglutinating adhesion, encoded by *saa*, has been suggested to be unique to LEE-negative STEC isolates (Paton et al., 2001; Toma et al., 2004; Cergole-Novella et al., 2007; Wu et al., 2010) and is encoded on pO113. Additional genes carried on pO113, reported to be unique to LEE-negative STEC, are *epeA*, *sab*, and *subAB* (Paton and Paton, 2005; Cergole-Novella et al., 2007; Herold et al., 2009; Newton et al., 2009; Bugarel et al., 2010; Irino et al., 2010; Wu et al., 2010) encoding, respectively, a SPATE exhibiting protease and mucinase activity (Leyton et al., 2003), an AT family protein contributing to adherence and biofilm formation (Herold et al., 2009) and the subtilase cytotoxin; this virulence factor is an AB_5 family toxin that displays cytotoxicity in Vero cell assays and is lethal to mice (Paton et al., 2004).

Multilocus sequence typing (MLST) based on housekeeping genes has demonstrated that LEE-negative STEC isolates are evolutionarily divergent (Tarr et al., 2008; Newton et al., 2009; Steyert et al., 2011). Whereas whole genome comparative analysis has been predominately focused on LEE-positive STEC (Ogura et al., 2007, 2009; Eppinger et al., 2011b). The current study focuses on a diverse set of nine LEE-negative STEC carrying various *stx* alleles, and includes a comparison with four complete reference LEE-positive STEC isolates. The genome-wide comparison allowed for identification of genes located outside the LEE pathogenicity island that are shared in the four LEE-positive STEC genomes, but not in the nine LEE-negative STEC, as well as virulence profile comparisons and identification of sequence regions unique to each isolate. Additionally, we characterized the *stx* phages in the LEE-negative STEC isolates in terms of chromosomal insertion site, genetic sequence, and structure, and levels of basal and induced *stx* expression. Insertion sites not previously reported for *stx*-encoding phages were identified. We were also able to demonstrate that in the more highly virulent of the nine isolates examined, despite carrying different *stx* alleles, the phages share similar Q protein sequences and genetic structure directly upstream of the *stxAB* genes.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

Nine LEE-negative STEC isolates were examined in this study; the isolate names, serotypes, and origins are listed in **Table 1**. These particular isolates were chosen to represent LEE-negative STEC

Table 1 | Characteristics of LEE-negative STEC isolates sequenced in this study.

Isolate	Serotype	*stx* variant(s)	Origin	Reference	Accession number
7V	O2:H25	stx_{2g}	Feces of healthy cattle	Leung et al. (2003)	AEXD00000000
94C	O48:H21	$stx_{1a,\ stx2a}$	Patient with HUS	Paton et al. (1995b)	AFDU00000000
B2F1	O91:H21	$stx_{2d1,\ stx2d2}$	Patient with HUS	Ito et al. (1990)	AFDQ00000000
C165-02	O73:H18	stx_{2d}	Patient with bloody diarrhea	Persson et al. (2007)	AFDR00000000
DG131	O174:H8	$stx_{1c,\ stx2b}$	Sheep	Paton et al. (1995a), Koch et al. (2001)	AFDV00000000
EH250	O118:H12	stx_{2b}	Child with abdominal cramps	Pierard et al. (1998)	AFDW00000000
MHI813	O8:H19	stx_{1d}	Bovine feces	Burk et al. (2003)	AFDZ00000000
031	O174:H21	$stx_{2b,\ stx_{2c}}$	Bowel contents of baby with SIDS	Paton et al. (1992), Paton et al. (1993)	AFDY00000000
S1191	O139:H1	stx_{2e}	Pig with edema disease	Weinstein et al. (1988)	AFEA00000000

with diverse serotypes and *stx* allele variants as part of a Genomic Sequencing Center for Infectious Diseases (GSCID) project[1]. Bacteria were cultured in Luria–Bertani (LB) broth at 37˚C.

GENOMIC DNA EXTRACTION, SEQUENCING, AND ASSEMBLY

Genomic DNA was isolated from an overnight culture using the Sigma GenElute kit (Sigma-Aldrich) and was sequenced at the University of Maryland School of Medicine, Institute for Genome Sciences, Genome Resource Center[2]. The genome sequence was generated using 3 kb insert paired-end libraries on the 454 Titanium FLX (Roche) and the raw paired-end sequence reads were assembled with Celera v. 6.0 (wgs-assembler.sourceforge.net). The raw sequence reads are available for each genome sequenced in this study[3].

PHYLOGENETIC ANALYSIS BASED ON WHOLE GENOME ALIGNMENT

The sequence data for *E. coli/Shigella* genomes (**Table A1** in Appendix) were downloaded from GenBank and combined with sequence data from the nine LEE-negative STEC isolates in this study for a total of 39 genomes. The genome sequences were aligned with Mugsy (Angiuoli and Salzberg, 2011), and the genomic core alignment, which consisted of ∼2.5 Mb, was parsed from the Mugsy output using methods described previously (Sahl et al., 2011). A phylogenetic tree was inferred using FastTree2 (Price et al., 2010) with *E. fergusonii* isolate 35469 as the outgroup.

WHOLE GENOME SEQUENCE COMPARISON

The sequences of the nine LEE-negative STEC genomes were compared in detail to four complete reference LEE-positive STEC genomes (**Table A1** in Appendix). These reference isolates were LEE-positive O157:H7 EDL933 (Perna et al., 2001), O111:H- str. 11128 (Ogura et al., 2009), O26:H11 str. 11368 (Ogura et al., 2009), and O103:H2 str. 12009 (Ogura et al., 2009). The shared genomic sequence regions between the 13 isolates were identified using Mugsy (Angiuoli and Salzberg, 2011) as defined above. Sequence regions uniquely shared by subsets of the 13 genomes, or by a single genome, were identified from the Mugsy output using scripts from bx-python[4] combined with custom python scripts. Putative unique regions were then further characterized using BLAST (Altschul et al., 1997) against the entire sequence set to verify uniqueness of the alignments.

BLAST SCORE RATIO ANALYSIS

BLAST score ratio analysis of selected virulence factors was performed as previously described (Rasko et al., 2005). BLAST score ratio (BSR) analysis identifies the level of relatedness between peptide sequences by dividing the protein query BLAST score by the reference BLAST score. The normalized BSR values were visualized using the MultiExperiment Viewer (Saeed et al., 2003).

PCR SCREENS FOR GENES OF INTEREST

Genomic DNA from two collections of *E. coli* isolates was screened by PCR for the presence of genes of interest. These collections consisted of 73 isolates from the environmental *E. coli* ECOR set (all *stx*-negative, Ochman and Selander, 1984) and the diarrheagenic DECA set containing 79 isolates[5]. The gDNA was interrogated for the genes *saa*, *perC1*, and a gene coding for a hypothetical protein (ECO103_2361 from O103:H2 isolate 12009) using primer pairs saa1, perC1, and hyp, respectively (**Table 2**). These primers were designed to anneal to conserved regions of the genes after examining MUSCLE alignments for regions with no polymorphism. In addition, the LEE-negative STEC isolate 87-1714 was included in the PCR screen as a control (Tarr et al., 2008; Newton et al., 2009; Steyert et al., 2011). Each 20 μL reaction included 30 cycles consisting of 95˚C for 30 s, 53˚C for 30 s, and 72˚C for 40 s. The *E. coli* K12 isolate MG1655 was employed as a negative control, and STEC O48:H21 94C and LEE-positive O157:H7 EDL933 were used as positive controls for *saa* and the other two genes, respectively.

SHIGA TOXIN CONTAINING PHAGE SEQUENCES AND INSERTION SITES

The insertion sites of the phages carrying the Shiga toxin genes were bioinformatically determined for each isolate. The *stx* genes were located in the assembled contigs and the adjacent sequence surrounding the *stx* genes was extracted and subjected to coding sequence (CDS) analysis[6]. The phage integrase gene and genes adjacent to the integrase gene were identified using BLASTp where possible. The gene adjacent to the integrase was designated as the phage insertion site. The *stx* phage sequences were compared using

[1]http://gscid.igs.umaryland.edu/
[2]http://www.igs.umaryland.edu/
[3]http://gscid.igs.umaryland.edu/wp.php?wp=emerging_diarrheal_pathogens
[4]http://bitbucket.org/james_taylor/bx-python/wiki/Home
[5]http://www.shigatox.net/
[6]http://www.ncbi.nlm.nig.gov

Table 2 | Oligonucleotide primers used in this study.

Primer set	Amplicon size (bp)	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
stx1RT	115	ACCACGTTACAGCGTGTTG	ACTGCGTCAGTGAGGTTCC
stx2RT	104	CAACGGTTTCCATGACAACG	TGAAACCAGTGAGTGACGACTG
rpoART	57	GCGCTCATCTTCTTCCGAAT	CGCGGTCGTGGTTATGTG
saa1	548	GGGAAGCAACTTGACATAAGTAAAGC	ACCACCAATTATGCGAGTTTCTCC
perC1	249	AGGACTGTACCGGAGAGCAG	GACGTATTCTGTTCTCCTGTCC
hyp	214	TATCAGAGCGGTAACTAAGCG	TCTTGCCCAGAATGTGGTG
RTQ1	133	CATCTGCCACTAAACCACG	CAGTCTTTTTGATATTCGCAAC
RTQ2	104	GGCTGCTTCAGACAATAGC	CGTCATCATCACACTGAATCC
RTQ3	98	GACTGATCCCCGAAAAAGTA	CAACCAGCAAGTCATGCAG
RTQ4	104	TTGAAGGTCTGCTCAATTACG	GGCAAAATTCACAAGGTAAGG
RTQ5	154	GACATCATCATGGCGACG	TTTTCTGGTACCGGATTGAG
RTQ6a	100	GGTTAATACCGTCGAAGGTG	ATCCACCAGTAGATCATGCTG
RTQ6b	106	GGATTGATCCCGACTAAAGTG	AATAATCTACCAACAAATCGTGC

Mauve (Darling et al., 2010). In some cases contigs were bioinformatically linked where appropriate to obtain complete phage sequences. Although the integration site was determined for all *stx* phages, the 3′ end of the phage could not be conclusively identified in three cases.

INTEGRASE, Q, AND SHIGA TOXIN GENE PHYLOGENY

Phylogenetic analysis was performed on *stx* gene sequences extracted from the LEE-negative STEC genomes and the four reference LEE-positive genomes. *Q* genes carried by the *stx* phages were identified by BLASTp and were aligned with MUSCLE (Edgar, 2004), to the *Q* gene sequence identified in the STEC EDL933 isolate stx_1- and stx_2-encoding phages. Integrase gene sequences were identified from reference genomes and the LEE-negative STEC genomes in this study. Sequence surrounding BLAST alignments was extracted and integration sites of insertion elements were determined as described above in an iterative process to provide the most complete dataset. For analysis of each of the *stx*, *Q*, and integrase gene phylogenies, the sequences were aligned using MUSCLE (Edgar, 2004) and a phylogeny was inferred with FastTree (Price et al., 2009).

MITOMYCIN C PHAGE INDUCTION

Overnight cultures of each STEC isolate were diluted 1:500 into fresh LB broth and grown to an OD_{600} of ∼0.35, then divided into separate cultures of equal volume. Mitomycin C at a final concentration of 0.5 μg/mL was added to one of the cultures. The induced and control cultures for each isolate were incubated at 37°C with shaking for 2 h, followed immediately by RNA extraction. The experiment was performed in triplicate for each isolate.

RNA ISOLATION AND QUANTITATIVE RT-PCR

Total RNA was extracted from 8 mL cultures using the RiboPure Bacteria Kit (Ambion) and treated with DNaseI (Ambion). The RNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop). SuperScript III Reverse Transcriptase (Invitrogen) with random hexamers was used to prepare cDNA from 1 μg total RNA for each sample. The resulting cDNA, diluted 1:50, was used in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) reactions performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Each 10 μL qRT-PCR reaction contained 2.5 μL cDNA template, 2X SYBR Green mix, and gene specific primers at a concentration of 0.2 μM each. All qRT-PCR reactions were carried out in triplicate for each of the three biological replicates for each condition, and included 40 cycles consisting of 95°C for 15 s followed by 60°C for 1 min. Fluorescence was monitored in a dissociation stage as products were heated from 60 to 95°C to verify primer specificity by melting curve analysis. Transcripts encoding the target genes $stxA_1$, $stxA_2$, and *Q*, along with the reference gene, *rpoA*, were detected using primer pairs listed in **Table 2**. Efficiencies for qPCR reactions were determined using LinRegPCR (Ramakers et al., 2003), and relative expression levels of the target genes in induced versus control cultures for each isolate were calculated from C_t results and efficiencies using the Pfaffl method (Pfaffl, 2001). Basal level target gene expression for each isolate relative to EDL933 were also calculated from results obtained from control cultures.

Notably, the primers annealing to the A subunit of the Shiga toxin genes were designed to be specific for either stx_1 or stx_2; this was verified by examining isolates carrying stx_1 or stx_2 or in combination. The *Q* gene primers were designed to be specific for a particular cluster of *Q* gene sequences as described below; however, there are cases where two *Q* genes with similar sequence are present in a single genome. For example, there is a similar *Q* gene associated with the stx_1 and stx_2 genes in EDL933, thus measured transcript abundance cannot distinguish between *Q* mRNA from the two phages. This is also true for isolates EH250 and 7V.

RESULTS

ISOLATE DIVERSITY

The nine LEE-negative STEC isolates examined in this study display both whole genome phylogenetic diversity and variation in the Shiga toxin alleles they harbor. A phylogeny was inferred from the conserved genomic core (∼2.5 Mbp) of a diverse set of 39 *E. coli*/*Shigella* genome sequences including representatives of all the major pathotypes (**Figure 1**). The phylogeny demonstrates that the LEE-negative STEC do not form a tight phylogenetic

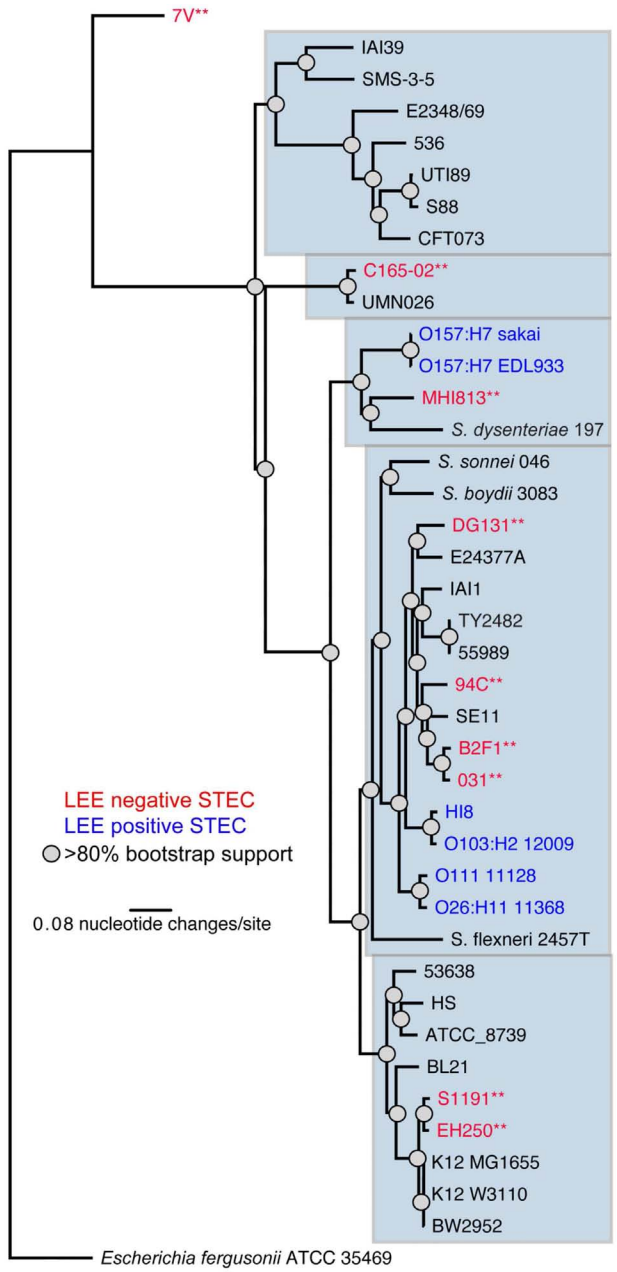

FIGURE 1 | A whole genome phylogeny of nine LEE-negative (red) and four LEE-positive (blue) STEC compared in this study. Whole genome sequences for the LEE-negative STEC sequenced in this study (indicated by asterisks) was combined with sequence data obtained from GenBank for *E. coli/Shigella* genomes representing the major pathotypes (**Table A1** in Appendix), and aligned based on concatenated regions of shared sequence as determined from analysis using Mugsy (Angiuoli and Salzberg, 2011). The phylogenetic tree was inferred with *E. fergusonii* isolate 35469 as the outgroup.

grouping suggesting that they have evolved multiple times and acquired the *stx* phage multiple times. Additionally, the phylogenetic analysis identified the early evolutionary divergence of the 7V isolate, which had been noted previously by MLST (Tarr et al., 2008; Newton et al., 2009; Walk et al., 2009; Steyert et al., 2011). The MHI813 isolate is more closely related to the EHEC 1 clonal group containing the O157:H7 isolates, while DG131 is the isolate most closely related to the EHEC 2 clonal group. The remaining isolates were distributed throughout the phylogeny. In general, the *stx* gene phylogeny (**Figure A1** in Appendix) does not parallel the result found for whole genome phylogenetic analysis. This is not unexpected since *stx* genes are carried on mobile genetic elements.

WHOLE GENOME SEQUENCE COMPARISON

Comparative genomics was utilized to determine whether there were any genes shared by all the LEE-negative STEC isolates that were not in the reference LEE-positive STEC genomes, and conversely, whether the LEE pathogenicity island was the only feature that distinguished LEE-positive from LEE-negative STEC. In addition to the nine LEE-negative STEC isolates, four representative LEE-positive STEC genomes were included in the comparative analysis including one from the EHEC 1 clonal group, O157:H7 str. EDL933, two from the EHEC 2 clonal group, O111:H-str. 11128, and O26:H11 str. 11368, and one that is a member of neither group, O103:H2 str. 12009. Whole genome comparative analysis was performed on this set of 13 genomes and identified a shared core alignment length of ∼3.66 Mb. This core sequence size is greater than the ∼2.5 Mb identified when including the 39 isolates used to construct the *E. coli* phylogeny in **Figure 1**. The whole genome comparison revealed no genomic regions (>500 bp) that are common to all nine LEE-negative STEC and absent in the four LEE-positive STEC genomes. Conversely, in addition to the LEE pathogenicity island, there were six genomic regions identified in all four LEE-positive STEC genomes that were not present in any of the LEE-negative STEC genomes. These include the five non-LEE encoded effectors *espK*, *espN*, *espX7*, *nleA*, and *nleG*, along with two other phage-encoded genes; one gene encodes the transcriptional regulator PerC1 (also termed PchABC in STEC), a homolog of PerC in EPEC, while the other encodes a hypothetical protein (locus tag ECO103_2361 in isolate 12009 and further referred to as *hyp*).

To determine whether the 7V isolate, having diverged earlier from other *E. coli* genomes, was lacking genes that were present in the other 12 genomes. The whole genome comparison revealed that the 7V isolate lacked an 8.9 kb cluster containing seven genes; these genes were identified as Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated proteins (Barrangou et al., 2007). The reverse analysis (i.e., unique in 7V when compared to the other genomes) identified that 7V contains 120 blocks of sequence >300 bases each, totaling ∼298 kb, that are unique. This quantity of unique sequence was greater than any of the other LEE-negative STEC isolates included in this analysis (**Table 3**). The number of unique sequence regions >300 bp and total length of unique sequence, along with selected possible virulence factors identified by BLASTX contained in the sequence blocks, are listed in **Table 3**. Although some putative virulence factors were identified, the majority of the sequence regions contain hypothetical proteins. Other unique regions with predicted functions include phage structural genes and some metabolic-related genes. For example a gene cluster coding for proteins involved in propanediol utilization was discovered in the C165-02

Table 3 | Properties of unique sequence regions and selected factors identified.

Isolate	#seq*	Total(kb)	Selected factors identified in unique regions
7V	120	298	2 Autotransporters, adhesion/hemaggluntin, type VI secretion Vgr family cluster, DNA transfer protein, 2 major facilitator superfamily transporters, fimbrial protein cluster, F4 fimbriae homolog, fimbrial protein homologs HtrE, PapC, and LpfD, outer membrane protein YopM homolog, reverse transcriptase, serine/threonine phosphatase, RatA-like protein, SWIM zinc finger family protein, tellurite resistance protein TciA, zeta toxin, insecticidal toxin SepC
94C	35	69	2 Adhesin/hemagglutinin, protease regulator PrtR homolog, conjugal transfer proteins including PilT homolog
B2F1	20	35	2 Adhesin/hemagglutinin
C165-02	98	216	Adhesin/invasion TibA homolog, autotransporter adhesion, adhesion/hemagglutinin, AidA-I family autotransporter, type I fimbriae, PapC homolog, type VI secretion family protein, transcriptional regulator YdeO homolog, HtrE homolog, MarR family protein, ArsA, and ArsD, iron uptake IroE and IroN homologs, Clp protease, reverse transcriptase, colicin B, and colicin B immunity proteins
DG131	59	113	3 Hemagglutinin family proteins, type IV secretion pilin homologs PilP, and PilT, FhuA homolog, siderophore receptor IreA homolog, toxin/antitoxin proteins YfjF/YfjZ, reverse transcriptase, colicin E5 immunity protein
EH250	41	91	AfaD homolog, AFA-III adhesion operon regulator, YadA family protein, autoagglutinating adhesion, K88 fimbriae homolog, immunoglobulin binding protein, DprA homolog, capsule polysaccharide biosynthesis family proteins, HipA kinase family protein, SEC-C family protein, MarR homolog
MHI813	86	248	3 Adhesin/hemagglutinins including HecA homolog, AidA-I homolog, 2 fimbrial clusters, type VI secretion system cluster, immunoglobulin A1 protease, AfaC homolog, transcriptional regulator HilD, M23 peptidase family protein, S-type colicin, YkfI/YafW toxin-antitoxin system, RadC, catalase/peroxidase
031	56	163	Adhesion/hemagglutinin, conjugal transfer proteins TraJ and TraX homologs, pilus regulatory protein PapB homolog, fimbrial protein PixA, and PixB homologs, transcriptional regulator YfjR homolog, protein kinase domain protein, ShiA homolog, tellurite resistance protein TehB, reverse transcriptase, programmed cell death toxin MazF
S1191	80	202	Autotransporter EatA homolog, 2 AidA-I autotransporter homologs, hemolysin, type IV secretion conjugal transfer proteins, Kappa-fimbriae cluster, AadA streptomycin resistance, microcin H47

**Number of unique sequence regions >300 bp as determined by Mugsy (Angiuoli and Salzberg, 2011) analysis.*

isolate. Overall, the LEE-negative STEC isolates are phylogenetically diverse and each isolate contains features that may contribute to virulence; however further functional analysis will be required to determine the role in virulence, if any.

VIRULENCE PROFILES OF THE LEE-NEGATIVE STEC ISOLATES

Since some of the LEE-negative isolates included in this study cluster more closely with pathotypes other than LEE-positive STEC (**Figure 1**), we queried the nine LEE-negative STEC genomes using the BSR for virulence factors that are typically associated with pathotypes other than LEE-positive STEC [BfpA (EPEC), AggR (EAEC), PapA (UPEC), STa, STb, LT-A, and LT-B(ETEC); Kaper et al., 2004]. The results revealed the presence of enterotoxin genes typically associated with enterotoxigenic *E. coli* in four of the LEE-negative STEC genomes; the 7V genome encodes a homolog of the heat stable enterotoxin STa, while the S1191 and C165-02 genomes encode STb. The genome of the C165-02 isolate also contains a gene similar to that encoding the B subunit of the heat-labile enterotoxin LT-IIa, while the MHI813 isolate carries a homolog of the gene encoding the A subunit of LT-IIb. These observed intersections of pathotype virulence factors highlight the diversity of *E. coli* as a species as well as the LEE-negative STEC.

There are few putative virulence factors have been definitively associated exclusively with LEE-negative STEC. Using a BSR analysis we examined the presence and level of sequence similarity of LEE-positive and LEE-negative STEC virulence factors in the 13 genomes (**Figure 2**). The analysis can be broadly divided into groups of virulence genes: toxins, adhesins, fimbriae, ATs, and plasmid associated genes from pO157 (marker of O157:H7) and pO113 (marker of some LEE-negative STEC; **Figure 2**). As predicted, all isolates encode one or more of the Shiga toxins (**Table 1**, **Figure 2**). The LEE encoded adhesion, intimin, is restricted to the LEE-positive STEC isolates and lacking in the LEE-negative STEC isolates, whereas common fimbriae and ATs are distributed in all types of STEC. The plasmid features appear to be more restricted, but not exclusive, with the LEE-positive STEC isolates containing more pO157 features and the pO113 features being more common among the LEE-negative STEC (**Figure 2**). Features previously predicted to be restricted to LEE-negative STEC include the adhesin protein Saa, encoded on pO113 (Paton et al., 2001). We confirm that this feature is restricted to the LEE-negative STEC, but is not found widely among LEE-negative STEC isolates. In addition to *saa*, the genes *sab*, *epeA*, and *subAB*, have been reportedly observed only in LEE-negative STEC isolates (Paton and Paton, 2005; Cergole-Novella et al., 2007; Herold et al., 2009; Newton et al., 2009; Bugarel et al., 2010; Irino et al., 2010; Wu et al., 2010). As with *saa*, these genes are present in several of the LEE-negative STEC, but not all. As above, these findings further

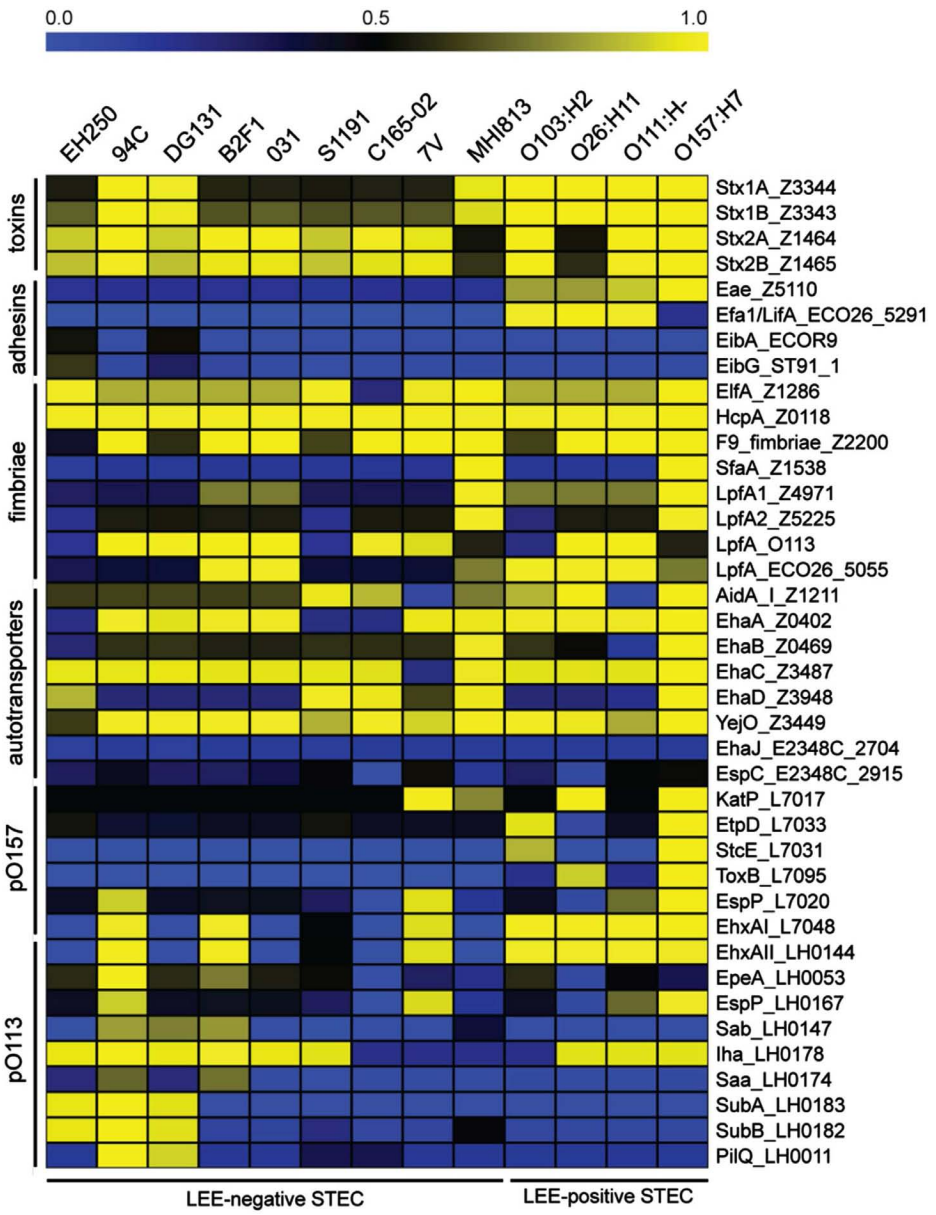

FIGURE 2 | A virulence gene profile based on BLAST score ratio (BSR) analysis. BSR analysis was performed on the genomes to determine the presence and level of protein sequence identity of selected virulence factors. Unless an *E. coli* isolate is otherwise indicated in the gene label, reference protein sequences were taken from the LEE-positive O157:H7 EDL933 isolate with the exception of the proteins encoded on pO113, which were taken from STEC O113:H21 isolate EH41. Yellow indicates a higher level of similarity, blue indicates a lower level of similarity, and black indicates ∼50% identity over the length of the sequence queried.

support the diversity of the LEE-negative STEC isolates within *E. coli*.

DISTRIBUTION OF GENES OF INTEREST IN *E. COLI* COLLECTIONS

Since a limited number of genomes were used in the whole genome analysis, we determined the frequency of the *saa*, *perC1*, and *hyp* genes in a larger collection of *E. coli* genomes. Polymerase chain reaction assays were developed for each of these features, and the prevalence was determined in both the *E. coli* ECOR (environmental) and DECA (diarrheagenic) collections[7]. Among the environmental isolates, only the ECOR37 isolate encodes the LEE pathogenicity island, *perC1* and *hyp*. Seven other ECOR isolates (7/72, 9.7%) also carry *hyp*, but no other isolate contained *perC1*. In the DECA collection, 100% of the EHEC (*LEE+/stx+*) genomes (18 of 78 total isolates) harbor both *perC1* and *hyp*, whereas none of the EPEC1 clonal group carries either of the

[7]http://www.shigatox.net/

genes. However, the *perC1* gene was found in 100% (24/24), and *hyp* in 58% (14/24) of the remaining LEE-positive *stx*-negative isolates whereas these two genes were present in only 1 of 25 LEE-negative *stx*-negative isolates. The reported absence of the *saa* gene in LEE-positive STEC genomes prompted us to include *saa* in our PCR analysis, which demonstrated the absence of *saa* in all isolates in both the ECOR and DECA collections. These analyses support the previous assertion that Saa is LEE-negative STEC restricted, and that LEE-positive STEC genomes contain *perC1* and *hyp*, but that these genes are not highly conserved among *E. coli* in general.

INSERTION SEQUENCE SITES AND INTEGRASE GENE PHYLOGENY

Several common *stx* phage insertion sites such as *wrbA*, *yecE*, *yehV*, *argW*, *ssrA*, and *prfC* have been reported in LEE-positive STEC genomes (Ogura et al., 2007). Those sites, however, were determined to be unoccupied in many LEE-negative STEC isolates and thus the insertion sites of the *stx* phages in these isolates were essentially unknown (Garcia-Aljaro et al., 2006, 2009; Prager et al., 2011). The *stx* phage insertion sites, as well as the genomic locations of other identifiable phages, were determined in the LEE-negative isolates by examining the integrase genes. Unless a particular insertion site is already occupied, insertion sequences can integrate at preferred locations having a DNA sequence specificity associated with the encoded integrase (Groth and Calos, 2004; Serra-Moreno et al., 2007). The results demonstrate that the *stx* phages are located at a variety of sites in the LEE-negative genomes in this study, many of which appear to be novel insertion sites for *stx* phages (**Figure 3**). However, this was not because the more widely known insertion sites were already occupied, but rather because of the variety of integrase proteins carried on the phages. The phage integrase sequences were examined, and as expected, integrase phylogeny reveals clusters of genes that utilize the same insertion site (**Figure A2** in Appendix). As displayed in **Figure A2** in Appendix, there are integrases that are more commonly associated with *stx*-encoding phages; however the integrase sequences are phylogenetically diverse, and no association between a particular *stx* variant and integrase was observed. There are 59 phage insertion sites that have been identified in the 13 genomes examined, but some appear to be more frequently occupied than others (**Figure 3**). There also does not appear to be an association between phage occupation and phylogeny, as no correlation is seen when the phylogenetic analysis in **Figure 1** is combined with the phage insertions sites in **Figure 3**. This confirms that the phage insertions are governed by the phage integrases and not the core genome, other than containing the insertion site.

Stx-CONTAINING PHAGE SEQUENCE DIVERSITY

Lambda phages are known to often undergo a significant amount of genetic exchange (Johansen et al., 2001; Brussow et al., 2004; Casjens, 2005). Comparison of the 20 *stx* phage sequences contained in the 13 genomes allowed examination of the potential diversity of the *stx* phages. Complete phage sequences were obtained for the majority of the phages; however, in some draft genomes phage sequences were not contiguous and phages were reconstructed from multiple contigs (**Figure A3** in Appendix). In **Figure A3** in Appendix, the colored blocks indicate regions of homology and the *stx* genes are indicated by the asterisk. The analysis clearly demonstrates the mosaic nature of the *stx* phages. Furthermore, phages sharing either insertion site or *stx* gene variant often contain extensive non-homologous regions.

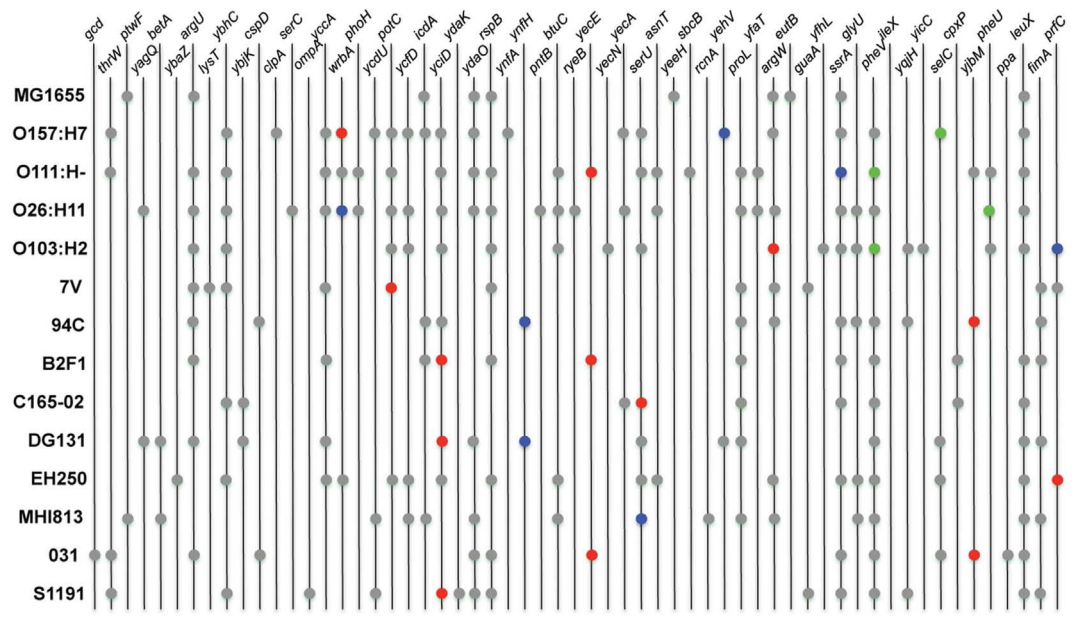

FIGURE 3 | Chromosomal location of phage integration. Locations of phage were determined by identifying integrase genes in the genomes of the LEE-negative STEC isolates. Insertion sites were obtained from GenBank for the four reference LEE-positive STEC isolates and *E. coli* MG1655 K12. Prophages encoding stx_1 and stx_2 are represented in blue and red, respectively. The LEE pathogenicity island is indicated by green, and locations of all other insertion elements are represented in gray.

For example, the B2F1 stx_{2d2}, DG131 stx_{2b}, and S1191 stx_{2e}-encoding phages share the *yciD* insertion site, but display very little sequence homology within the phage. These comparisons suggest a significant degree of diversity among *stx*-containing phages.

SHIGA TOXIN TRANSCRIPTION

Potential Shiga toxin induction and production are important as severe complications such as HUS result from the Shiga toxin produced by the bacteria during infection (Karch et al., 1999; Kaper et al., 2004). To determine if the phages in the LEE-negative STEC could be induced to express greater levels of *stx* transcript, mid-log phase cultures were incubated for 2 h either in the presence or absence of mitomycin C, and *stx* gene expression was determined by qRT-PCR. Primers were designed to be specific to either stx_1 or stx_2 alleles; the expression of stx_1 and stx_2 were measured separately in isolates carrying both Shiga toxin types. Two isolates, B2F1 and 031, each harbor 2 distinct stx_2 alleles; however, due to sequence similarity the signal from each stx_2 gene allele could not be determined for these isolates. Levels of *stx* transcripts in induced cultures were normalized to *stx* mRNA levels from untreated cultures for each isolate (**Figure 4A**). The most highly induced *stx* gene was 94C stx_{2a}, where the level of induction was over 10 times greater than that observed for EDL933 stx_2. Not only is stx_2 more highly induced in the 94C isolate compared to EDL933, but stx_1 is as well. The results demonstrate that the induction level of the *stx* genes in isolates B2F1 and 031 is also greater than for EDL933 stx_2, but it is not clear if this is due to one of the *stx* genes or both. Elevated levels of *stx* mRNA were not observed under inducing conditions for five isolates. Overall, there does not appear to be a consistent *stx* induction pattern based on STEC genome phylogeny or phage insertion site. Our results also reveal a wide variation in basal level expression of the *stx* genes in the isolates studied. Calculations of the basal and induced expression levels of the stx_1 and stx_2 alleles carried in the LEE-negative STEC isolates relative to those carried by EDL933 are reported in **Table A2** in Appendix. From these results it becomes evident that the stx_2 genes are expressed at similar levels in the 94C and EDL933 isolates when induced.

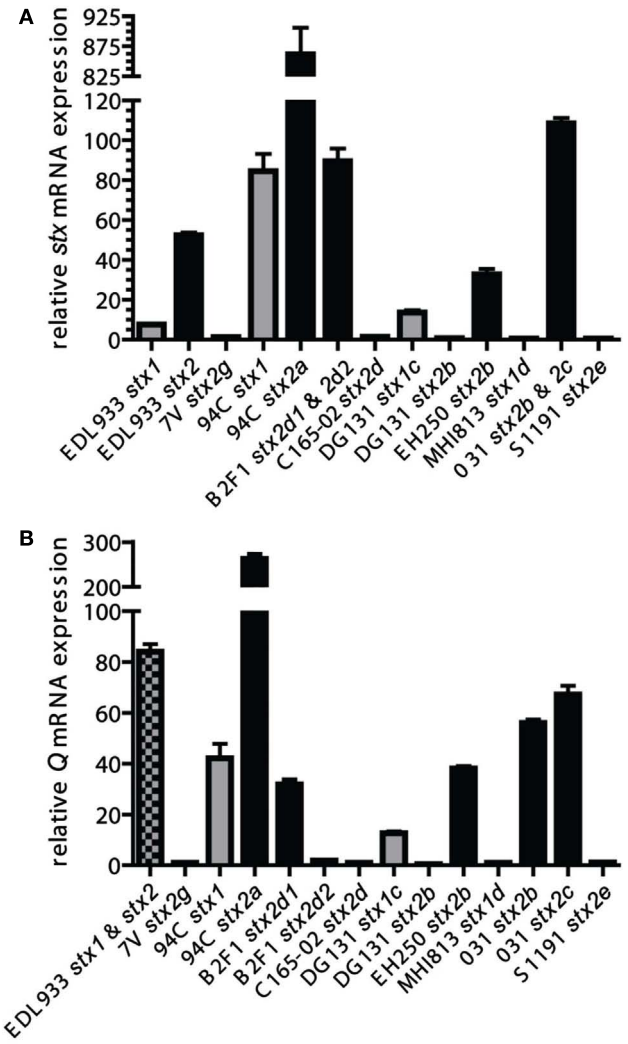

FIGURE 4 | A comparison of induced *stx* and *Q* gene expression. Mid-log phase cultures were incubated for 2 h either in the presence or absence of mitomycin C and relative mRNA levels were determined with qRT-PCR. *stx* **(A)** and *Q* **(B)** mRNA expression comparisons were made of mitomycin C-treated cultures relative to un-induced cultures (value of 1 signifies no induction for that particular *stx* in the isolate). Values and standard errors are presented and are based on results from three independent biological replicates each measured with technical triplicates. Results are displayed in gray for stx_1-encoding phages, black for stx_2-encoding phages, and checkered where the expression from the stx_1 and stx_2 phages could not be distinguished. The *Q* genes associated with the stx_{2b} and stx_{2g} phages in isolates EH250 and 7V, respectively, were each found to be associated with another phage in the isolate, thus the measured *Q* expression might have a contribution from that *Q* gene as well.

Q ANTITERMINATOR PHYLOGENY AND TRANSCRIPTION

Expression of *stx* genes within lambdoid phages is believed to be largely under the control of the Q antiterminator protein (Brussow et al., 2004). In lambdoid phages the *Q* gene transcription is increased under inducing conditions allowing for increased transcription of the *stx* genes that are downstream of the Q binding site (Brussow et al., 2004). The variety of genetic structures within the Shiga toxin cassettes in the phages can be observed when examining the genes upstream of the *Q* gene through the endolysin gene for each *stx*-encoding phage (**Figures 5A,B** for stx_1 and stx_2-encoding phages, respectively). Interestingly, the phage gene organization in the vicinity of the Shiga toxin genes 94C stx_{2a}, B2F1 stx_{2d1}, and 031 stx_{2c} is quite similar and these three phages display the greatest *stx* expression induction. However the genetic architecture does not appear to be the only factor affecting *stx* expression. To further examine the involvement of the Q protein in the regulation of *stx*, the *Q* gene sequences associated with each *stx*-encoding phage were aligned and an inferred phylogeny based on the alignment confirms the broad phylogenetic diversity observed with the whole genome phylogeny (**Figure 5C**). Interestingly, the three isolates exhibiting the highest level of *stx* induction share similar Q proteins (94C stx_{2a}, 031 stx_{2c}, and B2F1 stx_{2d1}). This suggests that the primary sequence of Q may play a role in the regulation of Shiga toxin, however further experimental evidence is required.

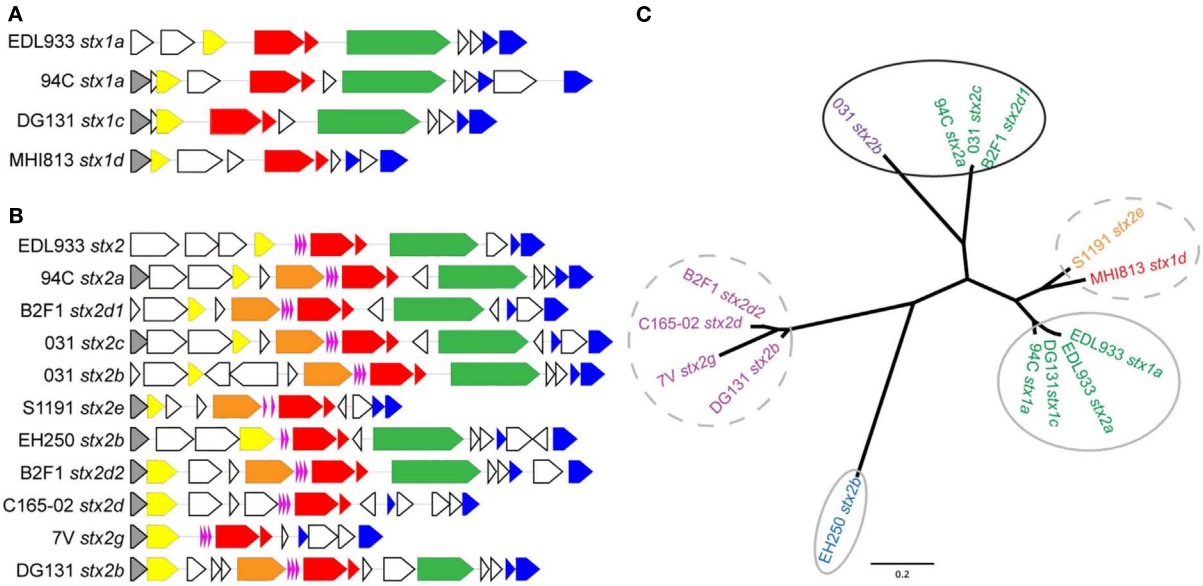

FIGURE 5 | Gene organization flanking the *stx* genes and *Q* gene phylogeny for the *stx* phages in the LEE-negative STEC isolates and LEE-positive O157:H7 EDL933. Gene organization comparisons are shown for **(A)** stx_1-encoding phages and **(B)** stx_2-encoding phages. The colors correspond to the following gene designations: gray, *rusA*; yellow, *Q*; orange, DNA methylase; pink, tRNA genes; red, *stxAB*; green, *yjhS*; blue, lysis S, and endolysin genes; white, all other genes, predominantly encoding hypothetical proteins. A cluster diagram based on the *Q* gene sequences was determined **(C)** and primers (**Table 2**) were designed to be specific for each cluster according to the colors: Q1 green, Q2 purple, Q3 turquoise, Q4 blue, Q5 magenta, Q6a orange, and Q6b red. Clusters circled by a solid black line denote a high level of *stx* induction, gray circles denote intermediate level induction, and broken lines denote lack of induction.

To determine if the induction of the *Q* genes with mitomycin C correlates with the *stx* gene expression, specific primers were designed for each cluster of *Q* gene sequences in an attempt to maximize qRT-PCR efficiency and minimize potential signal from *Q* genes associated with phages in the genome other than the specific *stx*-encoding phage. In the isolates EDL933, EH250, and 7V, the contribution to the *Q* gene qRT-PCR signal from two phages (both *stx*-encoding in EDL933) cannot be distinguished, but independent determination of *Q* expression in the *stx*-encoding phages was possible for all other isolates. The induction pattern for *Q* gene expression parallels the *stx* gene expression, but there is not a perfect quantitative correlation (**Figure 4B**), suggesting other factors may be involved. These studies confirm that the stx_{2d1} gene expression is inducible in isolate B2F1, but not stx_{2d2} gene expression (Teel et al., 2002). Our results also indicate that basal level *stx* and *Q* gene expression are not correlated (data not shown), thus expression of *stx* is at least partially dependent on some factor other than levels of *Q* transcripts produced under non-inducing conditions.

DISCUSSION

Recently, there has been an increased interest in characterizing LEE-negative STEC isolates because certain isolates have been associated with diarrheal symptoms and HUS, as results from infection with certain LEE-positive STEC isolates (Johnson et al., 2006; Mellmann et al., 2008; Newton et al., 2009; Kappeli et al., 2011). Detailed characterization of LEE-negative STEC has indicated that the association to HUS is especially significant for the activatable stx_{2d} subtype (Bielaszewska et al., 2006) and that other toxin subtypes are primarily associated with a milder course of disease (Friedrich et al., 2002; Persson et al., 2007). A limited number of reports have partially characterized these *stx*-encoding phages and detailed PCR screens for virulence factors associated with LEE-negative STEC isolates (Muniesa et al., 2000; Reckten-wald and Schmidt, 2002; Teel et al., 2002; Cergole-Novella et al., 2007; Beutin et al., 2008; Newton et al., 2009; Wu et al., 2010; Prager et al., 2011), but there remains a paucity of whole genome studies. To fill this knowledge gap a comparative genomics study of nine phylogenetically diverse LEE-negative STEC isolates and four reference LEE-positive STEC isolates was undertaken. Utilizing a gene-independent whole genome alignment method we determined that as a subset of STEC, the LEE-negative STEC isolates, do not share any genes in common that are lacking in all the LEE-positive STEC genomes examined. The phylogenetic diversity of the LEE-negative STEC may preclude the identification of a molecular marker that can differentiate the LEE-negative STEC isolates as a group from all other *E. coli* (**Figure 1**). Traditionally, LEE-positive STEC isolates are defined as STEC that carry the LEE pathogenicity island in their genome. Our results suggest that genes encoded outside the LEE such as the non-LEE encoded effectors *espK*, *espN*, *espX7*, *nleA*, and *nleG*, as well as the *perC1* gene (also termed *pchABC*) and a hypothetical gene marker, *hyp*, may be suitable biomarkers for LEE-positive STEC. Indeed, the presence of *perC1* and *hyp* in an additional 18 LEE-positive STEC genomes examined, and the lack of these genes in a selection of LEE-negative genomes, suggest that these may be reliable LEE-positive STEC

biomarkers. Nonetheless, the set of LEE-negative isolates queried will need to be expanded for a more conclusive result.

The definition of a pathotype of *E. coli* based on a single feature, especially one encoded on a mobile element such as the phage-borne Shiga toxin genes, is likely to reveal highly diverse host isolate backgrounds when examined on a genomic scale. The whole genome phylogeny based on conserved core sequence, utilizing approximately half the genome, determined that the majority of the LEE-negative isolates are more similar to other *E. coli* pathotypes than to LEE-positive STEC (**Figure 1**). The 7V isolate also appears to be on a deep rooting branch of this phylogeny, previously described as a "cryptic lineage" (Walk et al., 2009). Although the 7V isolate is not phylogenetically related to any prototype ETEC isolates, we determined that it does harbor the heat stable enterotoxin gene STa (ST-IA). These results confirm a recent report that identified the genes encoding STa and KatP carried on the 7V plasmid (Prager et al., 2011). The S1191 and C165-02 isolates also appear to have a STEC/ETEC intermediate pathotype based on virulence factors, as their genomes encode both Stx and heat stable enterotoxin b, STb. Additionally, the C165-02 genome encodes the gene for the B subunit of LT-IIa, whereas the gene coding for the A subunit of LT-IIb was found in the MHI813 genome. As these features are usually plasmid-borne, it is possible that these isolates contain a novel virulence plasmid that is different than pO157, pO113, or the 7V plasmid, but since these are draft genomes it also does not preclude chromosomal insertion of these virulence factors. Without more detailed information from sequencing the isolated plasmids, the comparative genomic analyses suggest that there is a variety of substantially different virulence plasmids harbored by LEE-negative STEC isolates that, in some cases, encode enterotoxin genes.

Without the LEE pathogenicity island, LEE-negative STEC must adhere to the intestinal epithelium by means other than the tight binding brought about by the Intimin/Tir complex (Mellies et al., 2007). The focused analysis on the presence/absence of multiple fimbriae and ATs, some of which may function as adhesions, in the 13 genomes examined, identified further variability (**Figure 2**). While some of the traditional adhesins were identified in the core of the LEE-negative STEC, additional isolate – specific adhesins and fimbrial genes were identified (**Table 3**). In fact, additional adherence factors were identified in each of the LEE-negative STEC genomes (**Table 3**). The combined results of the whole genome sequence comparison, virulence factor profiling analysis and the identification of factors encoded in the isolate-specific sequence regions indicate that there is no common adherence factor in all LEE-negative STEC isolates, but rather that each isolate encodes a particular assortment of adherence factors that allows pathogenic success.

In general, analysis of LEE-positive STEC genomes has revealed the presence of a great number of prophages in each genome, some of which contain virulence-associated genes (Schmidt and Hensel, 2004; Asadulghani et al., 2009). The genomic location of insertion elements and phages in the LEE-negative STEC genomes were cataloged (**Figure 3**). By inspecting the various insertion site occupancies in the genomes, it is clear that while some genomic sites are occupied by phage more frequently, there appears to be no discernable pattern of phage insertion that correlates to the phylogenetic relationship. Most sites occupied by prophages in the nine LEE-negative STEC genomes are also utilized in at least one of the four LEE-positive STEC genomes, but a few novel insertion sites are identified. We determined that insertion elements are predominately inserted at specific genomic locations that can be correlated to the integrase gene carried on the mobile genetic element (**Figure A2** in Appendix). Of note the absence of the LEE pathogenicity island in the LEE-negative STEC genomes is not due to lack of availability of the usual insertion sites adjacent to *selC*, *pheV*, or *pheU* (**Figure 3**). Interestingly, the *pheV* site is occupied in all LEE-negative STEC isolates, except 7V. The *pheU* site is unoccupied in all nine LEE-negative genomes and the *selC* site is occupied in only the DG131, EH250, and 031 genomes (**Figure 3**). Thus the LEE pathogenicity island could potentially insert in any of these genomes, but has not.

A comparison of *stx* phage sequences demonstrates the modular structure and sequence heterogeneity present even between phages encoding the same *stx* allele variant (**Figures 5** and **A3** in Appendix). This heterogeneity, especially in the integrase genes has led to the insertion of *stx*-encoding phages at a variety of genomic locations in the LEE-negative STEC isolates, such that an *stx* allele variant cannot necessarily be correlated with a particular genomic location. As a further example of this fact, we determined the integration site of the stx_{2e}-encoding phage carried in the S1191 isolate to be *yciD* (**Figure 3**), whereas, *yecE* is the integration site of the stx_{2e}-encoding phage in the 2771/97 isolate (Recktenwald and Schmidt, 2002). We also determined that the Q protein sequences are divergent in these two stx_{2e}-encoding phages, and that the phage gene organization is not shared (Recktenwald and Schmidt, 2002; Beutin et al., 2008; **Figure 5B**). Q proteins with low sequence identity have been noted previously between LEE-positive O157:H7 stx_{2c}-encoding phages (Eppinger et al., 2011a) and this work demonstrates the same phenomenon in the LEE-negative STEC isolates (**Figure 5C**). The extent to which dissimilar Q proteins and/or genetic organization upstream of the *stx* genes affects *stx* expression is not known (Brussow et al., 2004). It is of significance that in a detailed analysis of the *Q* gene sequences, the four Q proteins associated with phages that were not induced by mitomycin C, namely, B2F1 stx_{2d2}, C165-02 stx_{2d}, 7V stx_{2g}, and DG131 stx_{2b}, are more similar (**Figure 5C**). Likewise, the *Q* sequences corresponding to the most highly induced *stx* transcript cluster together. In fact, there is a general trend between the *Q* gene induction and the associated *stx* gene induction (**Figures 5A,B**); however further work would be required to elucidate the reason for the lack of increase in *Q* expression under inducing conditions noted for some of the phages included in this work.

Conflicting reports exist as to whether the Shiga toxin genotype or the level of Shiga toxin production can be used as an indicator for severity of clinical symptoms and progression to HUS associated with STEC infection (Friedrich et al., 2002; Bielaszewska et al., 2006; Orth et al., 2007; De Sablet et al., 2008; Neupane et al., 2011). Not all of the LEE-negative STEC isolates included in this work were isolated from humans, thus a complete comparison aimed at associating Shiga toxin characteristics with virulence in humans cannot be made. All of the Shiga toxins carried in the LEE-negative STEC isolates in this work are prophage-encoded and possibly inducible. Cultures of the LEE-negative STEC and LEE-positive

EDL933 were incubated either with or without mitomycin C followed by qRT-PCR utilizing primers having either $stxA_1$ or $stxA_2$ as a target (**Table 2**). Heterogeneity in *stx* expression between isolates has been previously reported (Ritchie et al., 2003; Beutin et al., 2008; De Sablet et al., 2008; Zhang et al., 2010). Variation in basal *stx* expression and level of *stx* induction was observed among the LEE-negative isolates in this work, and a number of the *stx* genes did not appear to be inducible under conditions tested (**Table A2** in Appendix and **Figure 4A**). The isolates demonstrating the greatest induction of stx_2 are EDL933, 94C, B2F1, and 031. This induction may be related to the severe clinical outcome associated with each isolate (**Table 1**) and the potential to exacerbate the disease with the administration of antibiotics. Overall the *Q* gene induction matched the trend of the associated *stx* gene, suggesting that there was a phage-based regulation of the toxin.

In conclusion, this study highlights the broad phylogenetic diversity of LEE-negative STEC isolates as well as the *stx*-encoding prophages harbored in their genomes. Our genome-wide comparative results indicate that LEE-negative STEC isolates as a group vary significantly in the assortment of adhesins and other virulence factors they encode. Sequence comparisons of the *stx*-encoding prophages demonstrate the extensive variation in terms of overall mosaic structures, *stx* allele variants, integrase sequence, Q antiterminator homologs and even the gene organization flanking the *stxAB* genes. These results suggests that extensive genetic exchange has taken place between phages and the possibility may arise from continued genetic exchange. Various genomic insertion sites of the *stx*-encoding phages in the LEE-negative STEC isolates were identified, revealing five sites not previously reported to be utilized by *stx*-encoding phages. The qRT-PCR results of the *stx* and *Q* genes determined that *stx* expression levels are increased in isolates in which *Q* expression levels are also increased under inducing conditions. Finally, this study demonstrates that the overall genome content, phage location and combination of potential virulence factors are variable in the LEE-negative STEC, requiring a larger set of isolates and further functional analyses before conclusions about this group can be made.

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APPENDIX

Table A1 | *E. coli*/*Shigella* genomes used in whole genome analysis.

Name	Accession
IAI39	NC_011750.1
SMS-3-5	NC_010498.1
E2348/69	NC_011601.1
536	NC_008253.1
UTI89	NC_007946.1
S88	NC_011742.1
CFT073	NC_004431.1
UMN026	NC_011751.1
Sakai	NC_002695.1
EDL933	NC_002655.2
S. dysenteriae 197	NC_007606.1
S. sonnei 046	NC_007384.1
S. boydii 3083	NC_010658.1
E24377A	NC_009801.1
IAI1	NC_011741.1
TY-2482	AFOG00000000
55989	NC_011748.1
SE11	NC_011415.1|
H.I.8.	AFDY00000000
2009	NC_013353.1
11128	NC_013364.1
1368	NC_013361.1
S. flexneri 2457T	NC_004741.1
53638	NZ_AAKB00000000
HS	NC_009800.1
ATCC 8739	NC_010468.1
BL21	NC_012947.1
K12 MG1655	NC_000913.2
K12 W3110	AC_000091.1
BW2952	NC_012759.1

Table A2 | *stx* mRNA expression relative to *stx* expression in EHEC O157:H7 EDL933.

Isolate	Basal expression*	Induced expression*
stx1		
EDL933	1.000 ± 0.052	1.000 ± 0.052
94C	0.427 ± 0.014	4.81 ± 0.51
DG131	0.265 ± 0.012	0.475 ± 0.043
MHI813	0.708 ± 0.140	0.0384 ± 0.0047
stx2		
EDL933	1.000 ± 0.025	1.000 ± 0.025
7V	$8.67 \pm 0.35 \times 10^{-4}$	$2.58 \pm 0.14 \times 10^{-5}$
94C	0.0728 ± 0.0034	1.12 ± 0.05
B2F1	0.0404 ± 0.0031	0.0679 ± 0.0028
C165-02	$7.11 \pm 0.30 \times 10^{-3}$	$2.21 \pm 0.09 \times 10^{-4}$
DG131	$5.73 \pm 0.42 \times 10^{-3}$	$8.21 \pm 0.37 \times 10^{-5}$
EH250	0.0182 ± 0.0006	0.0121 ± 0.0011
031	0.182 ± 0.005	0.381 ± 0.018
S1191	$8.33 \pm 0.11 \times 10^{-4}$	$1.07 \pm 0.05 \times 10^{-5}$

**Values and standard errors are based on results from three independent biological replicates each measured by qRT-PCR in technical triplicates.*

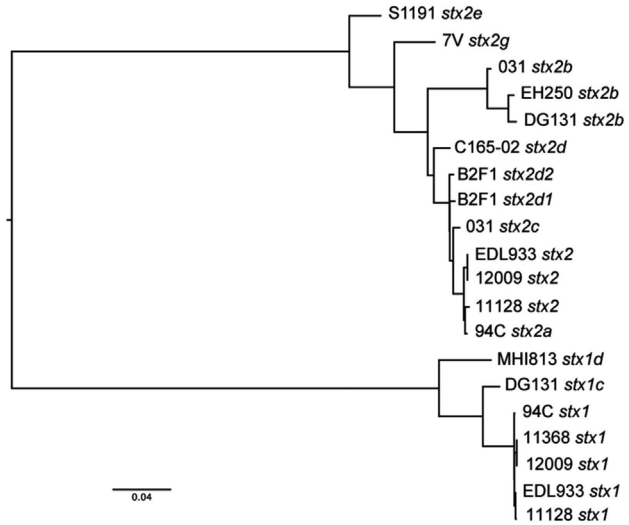

FIGURE A1 | Shiga toxin gene phylogeny. A phylogenetic tree was constructed from an alignment of concatenated *stxA* and *stxB* gene subunits for each of the Shiga toxins encoded in the 13 isolates compared in this study.

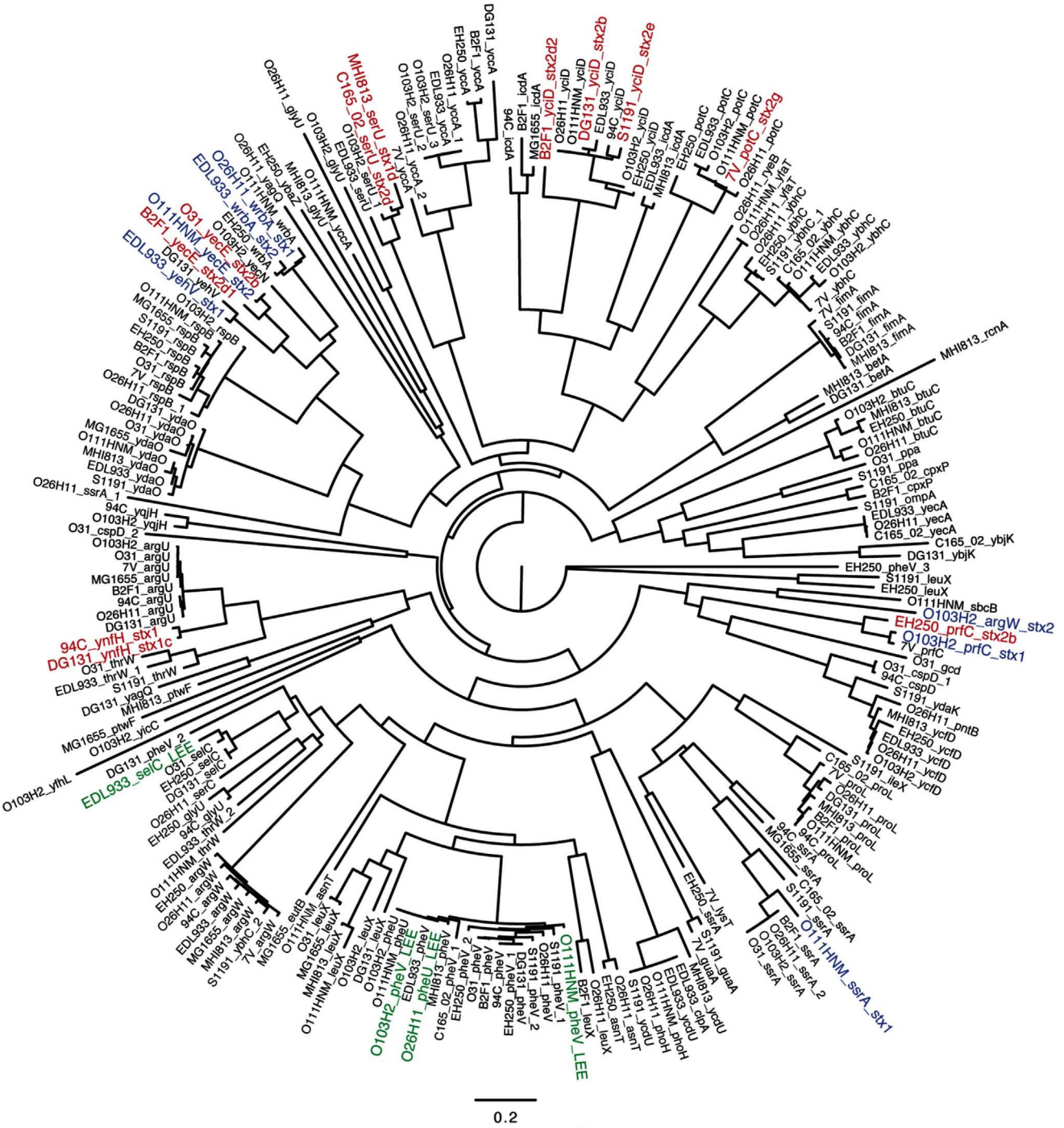

FIGURE A2 | Relationship between integrase gene phylogeny and chromosomal location of insertion elements. Integrase gene sequences were extracted from the LEE-negative STEC genomes and the gene adjacent to the integrase gene was designated as the insertion site. Integrase gene sequences were obtained from GenBank for the *E. coli* K12 MG1655 genome along with the four reference LEE-positive STEC genomes. A phylogenetic tree was inferred from an alignment of the integrase genes, and displays the predominant correlation between integrase gene sequence and chromosomal location of the insertion element. Integrase genes extracted from *stx*-encoding phages in the LEE-negative STEC genomes are depicted in red, while those from the reference LEE-positive STEC genomes are depicted in blue and the integrase genes associated with the LEE pathogenicity island are denoted in green. An integrase gene could not be identified in the STEC 94C stx_{2a} and STEC O31 stx_{2c} prophages, thus those phages are not included in this analysis.

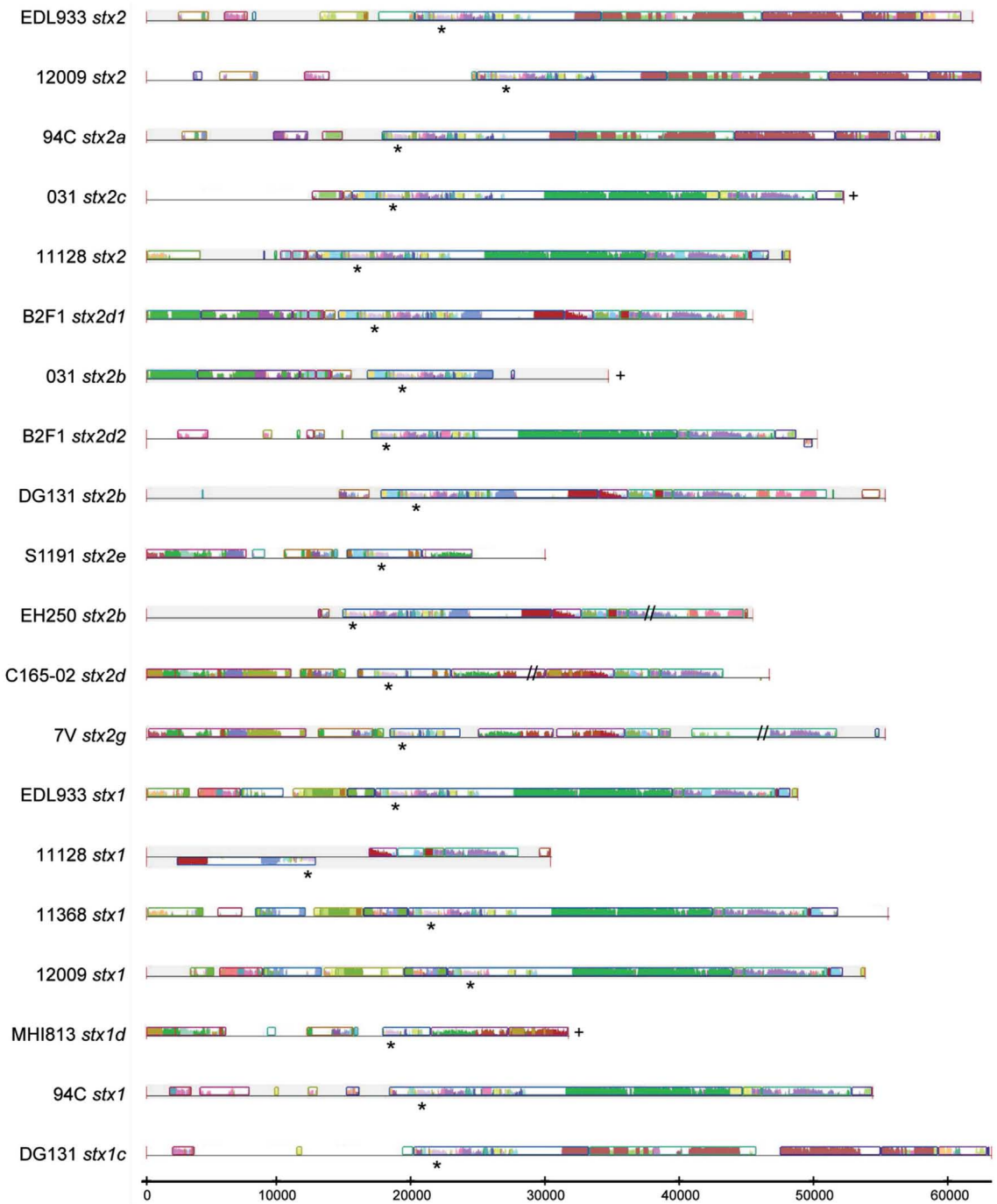

FIGURE A3 | Sequence comparison of the *stx*-encoding prophages. Phage sequences extracted from the genomes of the nine LEE-negative STEC isolates and obtained from GenBank for the four reference LEE-positive STEC genomes were subjected to sequence analysis using Mauve (Darling et al., 2010). Similar color denotes regions of shared sequence and the height of the bars denotes level of similarity of the shared sequence regions. Regions where there is a line, but no colored bars, indicate a lack of homology with any of the other phages in the comparison. The location of the *stx* genes is identified with an asterisk (*), the plus (+) signifies that the 3′ end of phage could not be determined unambiguously from the sequence data, and the double hash (//) denotes a gap in known sequence data.

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Escherichia coli O157:H7—Clinical aspects and novel treatment approaches

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Escherichia coli O157:H7 is a notorious pathogen often contracted by intake of contaminated water or food. Infection with this agent is associated with a broad spectrum of illness ranging from mild diarrhea and hemorrhagic colitis to the potentially fatal hemolytic uremic syndrome (HUS). Treating *E. coli* O157:H7 infection with antimicrobial agents is associated with an increased risk of severe sequelae such as HUS. The difficulty in treating this bacterium using conventional modalities of antimicrobial agent administration has sparked an interest in investigating new therapeutic approaches to this bacterium. These approaches have included the use of probiotic agents and natural products with variable success rates. In addition, novel modalities and regimen of antimicrobial agent administration have been assessed in an attempt at decreasing their association with aggravating infection outcomes.

Keywords: *Escherichia coli* O157:H7, hemolytic uremic syndrome, hemorrhagic colitis, shiga toxins, antimicrobial chemotherapy

GENERAL CHARACTERISTICS OF *E. coli* O157:H7

The identification of *E. coli* O157:H7 as the etiologic agent of an outbreak of gastroenteritis that occurred in 1982 (Riley et al., 1983) has led to the recognition of a novel class of *E. coli*, the Enterohemorrhagic *E. coli* (EHEC). This group of pathogenic *E. coli* includes those that cause a clinical disease similar to that caused by *E. coli* O157:H7 and that possess few other characteristics of this organism, namely producing one or more phage-encoded Shiga toxins, possessing a hemolysin-encoding 60 MDa plasmid and that cause attaching and effacing (A/E)lesions (Levine, 1987; Nataro and Kaper, 1998).*E. coli* O157:H7 produces either or both of two toxins, one neutralized by antisera to shiga toxin produced by *Shigella dysenteriae* type 1 and referred to as Shiga toxin 1 (Stx1) while the other, Shiga toxin 2 (Stx2), is not neutralized by these antisera (Strockbine et al., 1986). Although *E. coli* O157:H7, like other *E. coli* ferments lactose, it does not ferment sorbitol within 48 h, unlike 80–95% of *E. coli* isolated from human stools (March and Ratnam, 1986). On the other hand, it does not grow well at 44–45.5°C, which is the default incubation temperature for detection of *E. coli* in food and water sources (Raghubeer and Matches, 1990).

Disease caused by *E. coli* O157:H7 has been reported from more than 30 countries on six continents (Doyle et al., 2001). In a 20-year surveillance period in the USA, 350 outbreaks were reported (Rangel et al., 2005). The Center for Disease Control and Prevention (CDC) estimates that *E. coli* O157: H7 causes 73,480 illnesses, 2168 hospitalizations and 61 deaths per year in the USA alone (Mead et al., 1999). *E. coli* O157:H7 has been found in cattle of several countries including the USA, Canada, Germany, Spain, England, and Scotland (Armstrong et al., 1996). Outbreaks have also occurred in these countries, as well as in Japan (Michino et al., 1999).

Cattle are considered to be the chief animal reservoir for *E. coli* O157:H7, which is a temporary member of their normal gut micro flora (Caprioli et al., 2005). *E. coli* O157:H7 has been isolated from many healthy cattle and has not been shown to be a pathogen in these animals. Cattle seem to lack vascular receptors for shiga-like toxins (Pruimboom-Brees et al., 2000). *E. coli* O157:H7 has also been isolated from other animals including deer (Diaz et al., 2011), sheep (Urdahl et al., 2003), horses (Lengacher et al., 2010), goats (Mersha et al., 2010), and dogs (Kataoka et al., 2010).

The first outbreak of *E. coli* O157:H7 occurred in 1982 and was traced to contaminated hamburger meat (Riley et al., 1983). Most outbreaks, particularly those that occurred during the 1980s were food borne with the main culprits being beef products particularly undercooked hamburgers in addition to unpasteurized milk (Griffin and Tauxe, 1991). During the past decade, however, marked changes in the epidemiology of human infections have taken place and outbreaks traced to vegetable and fruit sources, in addition to other food sources are on the rise. Infections traced to white radish sprouts (Michino et al., 1999), fresh spinach (Brandl, 2008), and lettuce (Hilborn et al., 1999). Consumption of tomatoes and apple juice has been frequently involved in outbreaks as well (McDowell and Sheridan, 2001). In addition, waterborne outbreaks have occurred (Swerdlow et al.,

1992; Olsen et al., 2002; Bopp et al., 2003). *E. coli* O157:H7 appears to be capable of survival for prolonged times in water particularly at lower temperatures (Wang and Doyle, 1998). This microorganism was demonstrated to survive for more than eight months in a farm water gutter, and the surviving organisms were able to colonize cattle (Kudva et al., 1998). Swimming in contaminated water has also resulted in outbreaks (Keene et al., 1994; Friedman et al., 1999; Paunio et al., 1999). Person-to-person transmission has also been reported in day care centers and nursing homes as well (Panaro et al., 1990; Reida et al., 1994).

The rather easy spreading of *E. coli* O157:H7 from one person to another indicates that the infectious dose is rather low. Moreover, transmission by water, which would tend to dilute the organisms, substantiates this suggestion. The estimated infectious dose from outbreak data is 10–100 CFU (Griffin et al., 1994).

VIRULENCE FACTORS OF *E. coli* O157:H7

The ability to produce one or more shiga toxins is a hallmark *E. coli* O157:H7 infection. However, toxin production is not sufficient to cause disease. Two other factors are indicted in contributing to the virulence of *E. coli* O157:H7. The first of these two factors is harboring a 60 MDa virulence plasmid (pO157), which encodes a hemolysin (Schmidt et al., 1996; Mead and Griffin, 1998). The other factor is the locus of enterocyte effacement (LEE) (Kresse et al., 1998; Ogierman et al., 2000).

THE LOCUS OF ENTEROCYTE EFFACEMENT (LEE)

The LEE contains all the genes necessary for inducing the A/E lesions typical of *E. coli* O157: H7 infection (Louie et al., 1993; Vallance and Finlay, 2000). As *E. coli* O157:H7 attaches to the gut mucosa and interacts with it, histopathological changes are produced in the epithelium. These changes are collectively known as A/E lesions (Kresse et al., 2000). These lesions are characterized by effacement of the epithelial brush border microvilli and the formation of actin-rich pedestals within the host cell underneath the attached bacterial cells. The presumed functions of these pedestals are prevention of dislodgement of the bacterium during the host diarrheal response and inhibition of bacterial phagocytosis (DeVinney et al., 1999).

PO157

All isolates of *E. coli* O157:H7 harbor the 60 MDa pO157 plasmid. This plasmid contains the *hly* operon encoding an enterohemolysin (Schmidt et al., 1996). This hemolysin, with the aid of specialized transport systems, may allow the bacterium to utilize the blood released into the intestine as a source of iron (Mead and Griffin, 1998).

SHIGA TOXINS

The Shiga toxin family comprises three members. Shiga toxin, produced by *Shigella dysenteriae* type 1, is the prototype Shiga toxin. On the other hand, Stx1 and Stx2 are produced by the EHEC. Several variants of Stx2 have been identified as well and these include Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g. These share 84–99% of the amino-acid sequence of Stx2 but differ in some of its biological characteristics (Ito et al., 1990; Melton-Celsa and O'Brien, 1998; Schmidt et al., 2000; Melton-Celsa et al., 2002; Zheng et al., 2008). Three functional properties characterize the Shiga toxin family. These toxins are cytotoxic to HeLa and Vero cells. They lead to fluid accumulation in ligated rabbit illeal loops; therefore, they are "enterotoxic" and they are capable of inducing paralysis of the hind-legs and death in rabbit and mouse models (Jackson, 1990).

The binding moiety of these toxins, which aids them in binding to human and animal cells, consists of five B subunits. These subunits are non-covalently associated with an A subunit, which in turn consists of an A_1 and an A_2 subunit (Sandvig and Van Deurs, 1996). Shiga toxin and Stx1 differ only by a single amino acid in the B subunit (Calderwood et al., 1987; Hofmann, 1993). Thus, they are essentially identical; moreover, Stx1 is neutralized by antiserum to Shiga toxin (O'Brien and Holmes, 1987; Qadri and Kayali, 1998). Stx2 is antigenically distinct and unrelated. It is approximately 55% homologous to Shiga toxin/Stx1 (Jackson, 1990) and is not neutralized by antiserum to Shiga toxin (Qadri and Kayali, 1998).

The cellular receptors for the Shiga toxins are the neutral glycolipids globotriosylceramide (Gb3) and globotetraosylceramide (Gb4) (Betz et al., 2011). Various cell types are sensitive to Shiga toxins. These include enterocytes, renal, aortic, and brain endothelial cells, mesangial cells, renal tubular and lung epithelial cells, cells of the monocytic lineage, polymorphonuclear cells, in addition to platelets and erythrocytes among other cell types (Meyers and Kaplan, 2000).

After the toxin binds its receptor on the cell membrane, a short incubation leads to aggregation of toxin-receptor complexes in clathrin-coated pits. Next, the A fragment is endocytosed. The toxin is transported through endosomes to the Trans Golgi network (TGN). In the TGN, the toxin is cleaved by the enzyme furin into the A_1 and A_2 subunits. From the TGN, the toxin is transported to the endoplasmic reticulum where translocation into the cytosol takes place. If toxin was not cleaved by furin, then the cytosolic enzyme caplain may cleave the molecule (Hofmann, 1993; Sandvig and Van Deurs, 1996). The A_1 subunit is a 28S rRNA N-glycosidase (Jackson, 1990). The toxin cleaves an adenine residue from a specific nucleotide of the 28S rRNA component of the 60S ribosomal subunit. This blocks tRNA binding to the 60S ribosomal subunit thus preventing peptide elongation and disrupting protein synthesis. This leads to cell death (Hofmann, 1993).

Shiga toxins induce an increase in chemokine synthesis from intestinal epithelial cells. This augments host mucosal inflammatory responses with release of interleukins, such as IL-8 and IL-1, in addition to Tumor Necrosis Factor (TNF). Activation of human endothelium by TNF or IL-1 leads to an increase in toxin receptor synthesis and hence increased sensitivity of the cell leading to increased cell death after exposure to the toxins (Meyers and Kaplan, 2000).

E. coli O157:H7 strains may produce either Stx1, Stx2, or both; however, most strains produce Stx2 (Mead and Griffin, 1998). Stx1 remains mostly cell-associated and stored in the periplasmic space while Stx2 is released from bacterial cells. Therefore, Stx1 is typically predominantly detected in cell lysates, while Stx2 is

found in higher titers in culture supernatants (Strockbine et al., 1986; Yoh et al., 1997; Sato et al., 2003; Shimizu et al., 2009).

OTHER VIRULENCE FACTORS

While the LEE, pO157 and Shiga toxin production are defining virulence factors of *E. coli* O157:H7, other factors contribute to its pathogenicity. Some strains harbors EspP, which belongs to the family of serine protease autotransporters of Enterobacteriaceae (SPATE). This protease cleaves pepsin A and human coagulation factor V, which probably contributes to increased hemorrhage into the intestinal tract (Brunder et al., 1997). Moreover, EspP cleaves multiple complement system components hence protecting the bacterium from immune system-mediated elimination (Orth et al., 2010). On the other hand, in addition to LEE members such as intimin and Tir, bacterial attachment to host intestinal cells is also mediated by a type IV pilus referred to as the hemorrhagic coli pilus (HCP) (Xicohtencatl-Cortes et al., 2007). Multiple fimbriae and fimbrial gene clusters have also been implicated in contributing to adherence of this organism to host cells (Low et al., 2006).

CLINICAL ILLNESSES ASSOCIATED WITH *E. coli* O157:H7 INFECTIONS

Infection with *E. coli* O157:H7 can be asymptomatic or may manifest as non-bloody diarrhea, hemorrhagic colitis, the hemolytic uremic syndrome (HUS), thrombocytopenia purpura and death (Griffin et al., 1988).

HEMORRHAGIC COLITIS

Unless infection with *E. coli* O157:H7 is asymptomatic, following an incubation period of 3–4 days (Nauschuetz, 1998), the illness starts with severe abdominal cramps accompanied by a non-bloody diarrhea. In most patients the watery diarrhea becomes grossly bloody after two or three days (Boyce et al., 1995). Fever may be totally absent or may be of the low-grade type and its presence is more common in patients with severe illness (Griffin et al., 1988; Slutsker et al., 1997).

The duration of *E. coli* O157:H7 shedding seems to be age-dependent. Children under five years of age carry the organism after the resolution of symptoms longer than older children and adults (Pai et al., 1988). Intermittent shedding has also been reported (Belongia et al., 1993).

THE HEMOLYTIC UREMIC SYNDROME

Gastrointestinal symptoms due to infection with *E. coli* O157:H7 usually resolve within a week. Patients then mostly recover with no major sequelae. Nevertheless, 5–10% of patients under the age of 10 years develop the HUS approximately one week after onset of hemorrhagic colitis. The release of Shiga toxins is believed to play a central role in the development of HUS (Karmali et al., 1983). HUS most commonly occurs in children between 1 and 5 years of age but it can also occur in other groups particularly hospitalized patients over the age of 60 years. HUS displays a classical triad of microangiopathic hemolytic anemia (with fragmented RBCs on blood film), thrombocytopenia and renal failure (Gasser et al., 1955; Gianantonio et al., 1964). The patient's hematocrit may decline by 10%. Oligouria and hypertension (with elevated serum potassium, blood urea nitrogen, and uric acid levels) may occur as well. A condition known as thrombotic thrombocytopenia purpura (TTP) strikes mostly the adult population and is rarer than HUS. In TTP less marked renal damage is noted and fewer cases have a diarrheal prodrome. Both HUS and TTP can present with neurological abnormalities including seizures, coma and hemiparesis. These two conditions need not always be differentiated and may be referred to as HUS/TTP (Nauschuetz, 1998).

HUS and TTP are non-consumptive coagulopathies i.e., characterized by the consumption of platelets but not of clotting factors. They are regarded as variants of a single syndrome (Van Gorp et al., 1999). While fever and central nervous system (CNS) involvement are more frequent in TTP, renal dysfunction is less, and mortality and recurrences are greater. Although TTP can be initiated by *E. coli* O157:H7 infection, a diarrheal prodrome is uncommon (Siegler, 1995).

Classical postdiarrheal HUS always involves the colon and the kidney; however, other organ systems may be affected. The brain is most commonly affected with an evidence of CNS dysfunction in nearly one-third of HUS cases. Generalized seizures are common and occur in <20% of children affected. CNS injury symptoms range from disorders of posture, movement, and muscle tone to coma. Transient hepatocellular damage occurs in 40% of cases. The pancreas may be involved leading to diabetes mellitus, pancreatitis, and rarely, exocrine dysfunction. Other organs such as the heart, the lung and the skin are involved in rare cases (Siegler, 1995).

After the onset of the acute phase of HUS, characterized by the already mentioned triad of hemolytic anemia, thrombocytopenia, and acute renal injury, the patient's clinical disease may follow one of several patterns. More than 95% of cases recover from the acute phase of the disease. Thus, the mortality rate is 5% (McLigeyo, 1999). Grave sequelae, such as end-stage renal disease or permanent neurologic damage, occur in about 5% of subjects who survive the acute phase of HUS (Boyce et al., 1995).

Although an *E. coli* O157:H7 infection may result in HUS and TTP, numerous other causes may result in these diseases.

PATHOGENESIS OF *E. coli* O157:H7 INFECTIONS

The infectious process of *E. coli* O157:H7 (**Figure 1**) is initiated by the ingestion a relatively small inoculum of 10–100 CFUs. Only a few organisms are needed to allow enteric colonization (Mead and Griffin, 1998). The process by which these bacteria become attached to the mucosa of the distal ileum and the colon is complex and likely starts by bacterial fimbrial attachment followed by translocation of the bacterial Tir protein to the host cell membrane. Tir serves as the receptor for intimin, which is a bacterial outer membrane protein that plays a major role in attachment and production of A/E lesions characterized by effacement of microvilli (DeVinney et al., 1999).

Owing to this bacterium's tremendous ability to produce the potent cytotoxic Shiga toxins, invasion of the host cells is not necessary for the progression to hemorrhagic colitis. Although the toxins are probably also not necessary for triggering the diarrhea, they most likely cause the intestinal lesions, characterized by

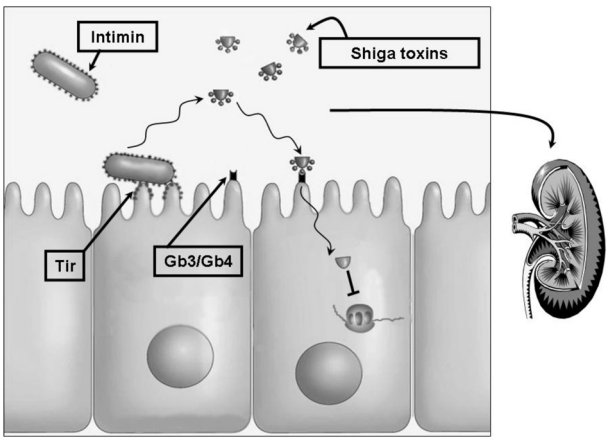

FIGURE 1 | The pathobiologic process of *E. coli* O157:H7. The complex process by which *E. coli* O157:H7 attaches to the intestinal mucosa starts by bacterial fimbrial attachment followed by translocation of the bacterial Tir protein into the host cell membrane. Tir serves as the receptor for the bacterial outer membrane attachment protein intimin. One or more types of Shiga toxins are released which then bind to their cellular receptors, the neutral glycolipids Gb3 and Gb4. Internalization and cellular activation of these toxins blocks ribosomal peptide elongation hence disrupting protein synthesis leading to cell death. Intestinal damage permits Shiga toxins and other bacterial factors to gain entrance to the circulation. These may reach multiple host tissues including the kidneys where damage to the microvasculature results in the potentially lethal hemolytic uremic syndrome. Treatment of this disease remains largely supportive with no widely accepted antibacterial or toxin-targeted regimen. Antibacterial agents are believed to result in bacterial lysis and release of stored toxins. One potential treatment method may rely on inhibition of toxin expression prior to administration of a bactericidal agent.

hemorrhage and ulcerations, via damaging the microvasculature of the intestinal wall (Tesh and O'Brien, 1992).

Once the gut-blood barrier has been compromised by intestinal damage, Shiga toxins, and other bacterial products like lipopolysaccharide (LPS) gain entrance to the circulation. LPS can by itself, independent of Shiga toxins, damage endothelial cells, increase TNF levels, activate platelets and induce the blood coagulation cascade. It can also increase levels of interleukins such as IL-8, which is a potent activator of white blood cells (WBCs). WBCs participate in the pathogenic process by elaborating tissue-damaging enzymes such as elastase. Shiga toxins induce an increase in chemokine synthesis from intestinal epithelial cells probably augmenting host mucosal inflammatory responses with release of IL-8, TNF, and IL-1. As mentioned above, activation of human endothelium by TNF or IL-1 leads to an increase in expression of the Shiga toxin cellular receptors. This leads to an increased cell death after exposure to the toxins. Since the toxin receptors are widely distributed on various types of cells, thus many host tissues are affected (Meyers and Kaplan, 2000).

CURRENT AND NOVEL TREATMENT APPROACHES

Treatment of infection with EHEC strains, including *E. coli* O157:H7, is mainly based on supportive therapy, particularly rehydration. The use of antimotility agents, which inhibit peristalsis and delay clearance of the organism, poses a risk factor for progression to HUS (Cimolai et al., 1994; Bell et al., 1997). The use of antimicrobial agents in the treatment of *E. coli* O157:H7 infection is not recommended but remains a debatable issue (Safdar et al., 2002). This is based on studies that have shown it to be a risk factor for the development of HUS (Wong et al., 2000; Smith et al., 2012). Additionally, the use of trimethoprim, the quinolones, or furazolidone enhances the production of Shiga toxins from *E. coli* O157:*H7 in vitro* presumably due to lysis of bacterial cells and the release of stored toxins (Kimmitt et al., 2000). This enhanced release of toxins may alternatively be due to induction of Stx-producing prophages harbored by the bacterium. These prophages would be activated by the SOS response, a damage response triggered in these bacteria mostly due to genomic insult which may be exerted by antimicrobial treatment (Kimmitt et al., 2000).

In light of the difficulties in treating this agent, alternate treatment approaches were investigated by multiple groups. Antibodies to Stx2 were shown to enhance the survival of infected gnotobiotic piglets (Donohue-Rolfe et al., 1999). These antibodies were also demonstrated to be well tolerated in humans and thus may be useful for preventing HUS in pediatric subjects (Lopez et al., 2010). On the other hand, carbosilane dendrimers were shown to specifically bind to Shiga toxins with high affinity and to inhibit cellular entry of the toxin. Intravenous administration of these carbosilane dendrimers decreased the brain damage and prevented the lethal effect of the toxins in infected mice (Nishikawa et al., 2002).

The use of natural products for the treatment and prevention of *E. coli* O157:H7 has been assessed by multiple groups. Studies in infant rabbits show that the administration of *Lactobacillus casei*, commonly known for its probiotic efficiency, had a protective effect against the toxins of *E. coli* O157:H7 (Ogawa et al., 2001). Multiple other probiotic agents have been shown to be effective in curbing the growth or the pathogenic effect of this organism (Shu and Gill, 2001, 2002; Asahara et al., 2004; Takahashi et al., 2004; Gagnon et al., 2006; Kim et al., 2009; Etienne-Mesmin et al., 2011; Tahamtan et al., 2011). Certain herbs such as Chinese cinnamon, Spanish oregano and other essential oils have been shown to have mechanisms of action against the cell membrane and cell wall of *E. coli* O157:H7 (Oussalah et al., 2006). Green tea components (Lee et al., 2009) in addition to cranberry constituents (Lacombe et al., 2010) have also been shown to have an effect against this bacterium. In an attempt at implementing antimicrobial agents in the treatment of an *E. coli* O157:H7-infected animal model, azithromycin was shown to enhance the survival of infected piglets (Zhang et al., 2009). The majority of the studies mentioned herein have limited their testing to *in vitro* assays or employed animals that were gnotobiotic or treated with antimicrobial agents to limit the growth of their normal flora prior to infection with *E. coli* O157:H7. Consequently, the response to the tested agents in a host with a normal range of flora may be different.

Our group examined whether employing an agent that would inhibit toxin expression prior to treatment with a bactericidal antibiotic may be effective in treating such an infection. We assessed the effects of rifampicin, an RNA polymerase inhibitor, and gentamicin, a ribosome inhibitor, on the expression of the

Stx1 and Stx2 encoding genes, *stx1* and *stx2* (Kanbar et al., 2003; Matar and Rahal, 2003; Rahal et al., 2011a,b). After incubation with antimicrobial agents, levels of *stx1* gene transcripts notably decreased by more than 99% in a sample treated with the minimum inhibitory concentration (MIC) of rifampicin, in that treated with the MIC of rifampicin followed by the minimum bactericidal concentration (MBC) of rifampicin and in the sample treated with the MIC of rifampicin followed by the MBC of gentamicin. The sample treated with the MBC of gentamicin alone showed a 51.37% decrease, which was the least noted toxin gene expression inhibition. A 77% decrease in *stx2* transcript detection was seen in the sample treated with the MBC of gentamcin alone. Samples treated with the MIC of rifmapicin, or with the MIC of rifampicin followed by the MBC of rifampicin, or with the MIC of rifampicin followed by the MBC of gentamicin showed complete inhibition of *stx2* transcript detection. Detection of toxin release from these bacteria using reverse passive latex agglutination (RPLA) yielded results that were mostly concurrent with the decrease in RNA synthesis except for samples treated with the MBC of gentamicin alone. Gentamicin seemed to trigger an enhanced release of toxins from treated cultures.

To assess the utility of an antimicrobial regimen for the treatment of an animal model of infection, multiple groups of BALB/c mice received 3× LD50 of an *E. coli* O157:H7 strain via intraperitoneal injection. These were then treated with various rifampicin/gentamicin regimen and were monitored for survival. None of the mice infected and left untreated and none of the mice infected but treated with the *in vivo* MBC equivalent dose of gentamicin survived. On the other hand, the highest survival rate was obtained with the group treated with the *in vivo* MIC equivalent dose of rifampicin followed by the *in vivo* MBC equivalent dose of gentamicin (50% survival rate). In comparison, 25% of the mice infected and treated with the *in vivo* MIC equivalent dose of rifampicin survived while mice treated post-infection with the *in vivo* MIC equivalent dose of rifampicin followed by the *in vivo* MBC equivalent dose of rifampicin showed a 12.5% survival rate.

Therefore, preliminary data support that antimicrobial agents may be used for the treatment of an *E. coli* O157:H7 infection. One promising treatment modality, as evidenced by our *in vivo* data, may be to implement an expression inhibitory dose of an agent that would limit toxin production prior to using a bactericidal dose of an antimicrobial. Such a treatment modality may also be of use in treating other Shiga toxin producing organisms including emerging agents such as *Escherichia coli* O104:H4; however, potential implementation of such a treatment remains to be assessed.

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Enterohemorrhagic *E. coli* (EHEC) pathogenesis

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Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is a human pathogen responsible for outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS) worldwide. Conventional antimicrobials trigger an SOS response in EHEC that promotes the release of the potent Shiga toxin that is responsible for much of the morbidity and mortality associated with EHEC infection. Cattle are a natural reservoir of EHEC, and approximately 75% of EHEC outbreaks are linked to the consumption of contaminated bovine-derived products. This review will discuss how EHEC causes disease in humans but is asymptomatic in adult ruminants. It will also analyze factors utilized by EHEC as it travels through the bovine gastrointestinal (GI) tract that allow for its survival through the acidic environment of the distal stomachs, and for its ultimate colonization in the recto-anal junction (RAJ). Understanding the factors crucial for EHEC survival and colonization in cattle will aid in the development of alternative strategies to prevent EHEC shedding into the environment and consequent human infection.

Keywords: EHEC, LEE, acid resistance, cattle, colonization

INTRODUCTION

Verocytoxin-producing *Escherichia coli* (VTEC), also known as Shiga-toxin producing *E. coli* (STEC), is a food-borne zoonotic agent associated with outbreaks worldwide that poses a serious public health concern. Over 380 different VTEC serotypes have been isolated from humans and animals, but only a small number of serotypes are linked to human disease. Serotype O157:H7 is the major source of *E. coli* food poisoning outbreaks in the United States (US) (Karmali et al., 2010). Characteristics of *E. coli* serotype O157:H7 (EHEC) infection includes abdominal cramps and bloody diarrhea, as well as the life-threatening complication hemolytic uremic syndrome (HUS) (Karmali et al., 1983; Karmali, 1989; Griffin and Tauxe, 1991). Karmali and colleagues first identified VTEC as the infectious agent responsible for HUS after correlating *E. coli* infection in patients with diarrhea and HUS with the presence of a toxin that produced significant irreversible cytotoxic effects in Vero cells (Konowalchuk et al., 1977; Karmali et al., 1985). O'Brien and LaVeck later purified the toxin from an enteropathogenic strain of *E. coli* and determined that the toxin was structurally and antigenically similar to the Shiga toxin produced by *Shigella dysenteriae* type 1 (O'Brien and LaVeck, 1983).

Shiga toxin is composed of two major subunits, designated A and B (O'Brien et al., 1992; Paton and Paton, 1998). The B subunit forms a pentamer that binds to globotriaosylceramide-3 (Gb3) (Lingwood et al., 1987), and this specificity determines where Shiga toxin mediates its pathophysiology. The A subunit exhibits an RNA N-glycosidase activity against the 28S rRNA (Endo et al., 1988) that inhibits host protein synthesis and induces apotosis (Sandvig, 2001; Karmali et al., 2010). In humans, EHEC colonizes the large intestine (Phillips et al., 2000). Shiga toxin released by EHEC binds to endothelial cells expressing Gb3, allowing absorption into the bloodstream and dissemination of the toxin to other organs (Sandvig, 2001). The tissues and cell types expressing Gb3 varies among hosts, and the distribution of Gb3 targets the pathology of toxin-mediated disease to cells expressing Gb3 (Pruimboom-Brees et al., 2000). For example, renal glomerular endothelium expresses high levels of Gb3 in humans, and Shiga toxin production results in acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, all typical characteristic of HUS (Karmali et al., 1983).

Currently no treatment is available for EHEC infections (Goldwater and Bettelheim, 2012). The use of conventional antibiotics exacerbates Shiga toxin-mediated cytotoxicity. In an epidemiology study conducted by the Centers for Disease Control and Prevention, patients treated with antibiotics for EHEC enteritis had a higher risk of developing HUS (Slutsker et al., 1998). Additional studies support the contraindication of antibiotics in EHEC infection; children on antibiotic therapy for hemorrhagic colitis associated with EHEC had an increased chance of developing HUS (Wong et al., 2000; Zimmerhackl, 2000; Safdar et al., 2002; Tarr et al., 2005). Antibiotics promote Shiga toxin production by enhancing the replication and expression of *stx* genes that are encoded within a chromosomally integrated lambdoid prophage genome. *Stx* induction also promotes phage-mediated lysis of the EHEC cell envelope, allowing for the release and dissemination of Shiga toxin into the environment (Karch et al., 1999; Matsushiro et al., 1999; Wagner et al., 2002).

Cattle are a major reservoir of EHEC, but unlike in humans, EHEC colonization in adult ruminants is asymptomatic (Cray and Moon, 1995; Brown et al., 1997; Dean-Nystrom et al., 1997; Woodward et al., 1999; Wray et al., 2000). While humans express Gb3 on their vascular endothelium that promotes much of the

pathophysiology associated with Shiga toxin, cattle lack vascular expression of Gb3 (Pruimboom-Brees et al., 2000). Although Gb3 receptors are detected in the kidney and brain of cattle, Shiga toxin was unable to bind to the blood vessels in the cattle gastrointestinal (GI) tract (Pruimboom-Brees et al., 2000). As a result, Shiga toxin cannot be endocytosed and transported to other organs to induce vascular damage in cattle. In contrast to humans where EHEC colonizes in the colon and causes electrolyte imbalances, EHEC colonizes the recto-anal junction (RAJ) of cattle where it is impervious to the effects of Shiga toxin (Naylor et al., 2003). The insensitivity to Shiga toxin and differential preference in colonization sites make cattle a more tolerant host for EHEC and may contribute to persistence and transmission of this human pathogen.

Cattle transmit EHEC to humans by shedding the pathogen in their feces. Fecal shedding may be brief or more extended (Rice et al., 2003). A proportion of positive animals called "super shedders" excrete more EHEC than others. Although the "super shedders" comprise a small ratio of cattle, it has been estimated that they may be responsible for over 95% of all EHEC bacteria shed (Omisakin et al., 2003; Chase-Topping et al., 2007). Evidence supports that high concentrations of EHEC in feces or prolonged shedding may result from intimate colonization at the RAJ (Cobbold et al., 2007). Once shed into the environment, humans acquire EHEC by consuming contaminated bovine-derived products such as meat, milk, and dairy products (Armstrong et al., 1996) or contaminated water, unpasteurized apple drinks, and vegetables (Cody et al., 1999; Hilborn et al., 1999; Olsen et al., 2002). Direct contact with ruminants at petting zoos or through interactions with infected people within families, daycare centers, and healthcare institutes represent another source of EHEC transmission (Spika et al., 1986; Carter et al., 1987; Rowe et al., 1993; Rangel et al., 2005). Bovine manure can harbor viable EHEC for more than seven weeks (Wang et al., 1996), and the long-term environmental persistence of EHEC poses an increased risk for transmission of EHEC through the fecal-oral route through wash-off to nearby farms or in contaminated grass consumed by other cattle. By gaining a better understanding of how EHEC colonizes the cow, methods can be devised to limit fecal shedding of EHEC into the environment and limit sources of contamination and consequent human infection.

FACTORS IMPORTANT FOR EHEC SURVIVAL AND COLONIZATION IN CATTLE

ACID RESISTANCE SYSTEMS

EHEC adapts an oral-fecal lifestyle in cattle and other ruminants. After being ingested, EHEC enters the rumen of cattle. In order to reach the RAJ for colonization, EHEC must first breach the acidic barrier of the stomachs. EHEC has an intricate acid resistance (AR) system that enables it to survive through the acidic environment of the stomach, as exemplified by its low infectious dose of 10–100 colony-forming units (Tuttle et al., 1999). Three important AR systems have been identified in *E. coli*: the AR system 1 (glucose-repressed or oxidative), AR system 2 (glutamate-dependent), and AR system 3 (arginine-dependent). The relative importance of each AR system *in vivo* is still being delineated; however, induction and function of these systems *in vitro* varies depending on the type of culture medium used and growth conditions (Lin et al., 1995, 1996; Hersh et al., 1996).

Among the three AR systems, the mechanism of glucose-repressed AR system is the least understood. The glucose-repressed AR system is activated in stationary phase in Luria-Bertani broth (LB) and repressed by addition of glucose to culture media. Activation of the glucose-repressed AR system depends on two global regulators: the cAMP receptor protein (CRP), and the stress response alternative sigma factor RpoS. (Castanie-Cornet et al., 1999). Calves inoculated with equal numbers of wild type EHEC and an *rpoS* mutant strain shed the *rpoS* mutant significantly less than the wild type, indicating an important role for RpoS and the glucose-repressed AR system in passage through the GI tract of cattle (Price et al., 2000). Since RpoS is a global stress regulator, eliminating this transcription factor may have other pleiotropic effects that can alter the ability of EHEC to colonize the host.

The glutamate-and arginine-dependent AR systems have similar modes of action (**Figure 1**). The glutamate decarboxylases

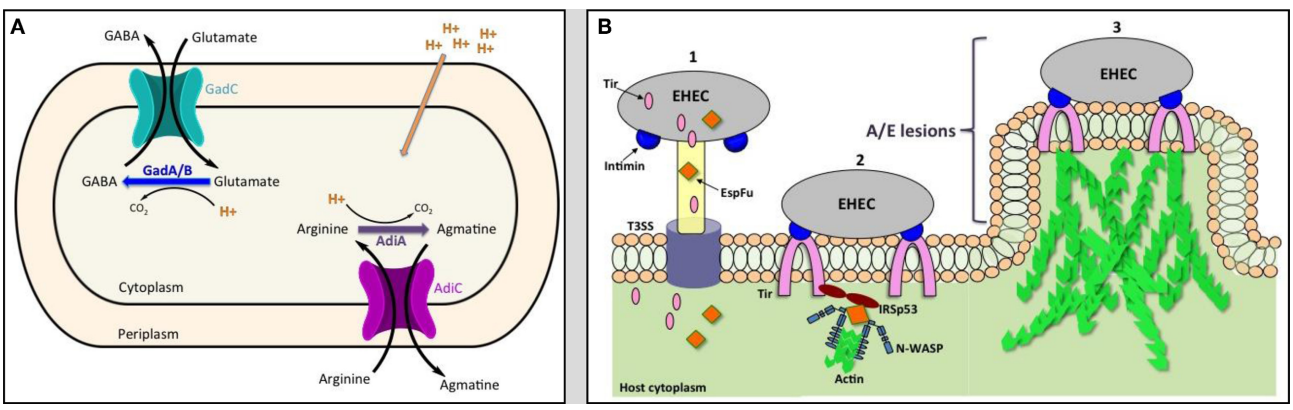

FIGURE 1 | The model of acid resistance system 2 and 3 (A) and the schematic diagram of the formation of attaching and effacing (A/E) lesions (B). EHEC injects effector proteins such as Tir and EspFu into the host cytoplasm through the T3SS (1). Tir localizes to the host membrane and binds to intimin to intimately attach the bacteria to the cell. Tir and EspFu recruit host factors (2) to subvert host cytoskeleton and actin polymerization (3).

GadA and GadB and the arginine decarboxylase AdiA convert glutamate or arginine to γ-amino butyric acid (GABA) or agmatine, respectively, by displacing the α-carboxyl group of the amino acids with a proton that is transported from the environment into the cytoplasm. GABA and agmatine are exchanged for new amino acids through their cognate antiporters GadC and AdiC, respectively (Hersh et al., 1996; Castanie-Cornet et al., 1999). The uptake of the protons increases the internal pH and helps maintain pH homeostasis.

Regulation of the glutamate-dependent AR system is complex and varies with different environmental conditions (detailed review in Foster, 2004). Of the three AR systems, the glutamate-dependent AR system provides the highest level of acid protection (Lin et al., 1996; Castanie-Cornet et al., 1999). Additionally, Price et al. demonstrated that among the three AR systems, glutamate-dependent AR system is necessary for passage through the acidic stomachs and colonization in cattle. Interestingly, the glutamate-dependent AR system was not required for EHEC survival in acidic foods such as apple cider. Instead EHEC utilizes the glucose-repressed AR system to withstand the acid challenge when stored in foods containing low pH (Price et al., 2004). This versatility allows EHEC to utilize different AR systems to persist in diverse environments. Further investigation into the mechanisms EHEC uses to activate the AR systems in cattle will be useful for developing new techniques to reduce EHEC survival through the acidic stomachs and its subsequent colonization at the RAJ.

FORMATION OF ATTACHING AND EFFACING LESIONS ON EPITHELIAL CELLS

After passage through the acidic barrier, EHEC forms attaching and effacing (A/E) lesions on the mucosal epithelium at the RAJ, allowing for its colonization at the RAJ. A/E lesions are characterized by destruction of microvilli, intimate attachment of the bacteria to the cell, and accumulation of polymerized actin beneath the site of bacterial attachment to form a pedestal-like structure cupping individual bacteria (**Figure 1**) (Nataro and Kaper, 1998). The genes required for formation of A/E lesions are encoded within the chromosomal pathogenicity island known as the locus for enterocyte effacement (LEE) (McDaniel et al., 1995; Elliott et al., 1998). The LEE consists of approximately 41 genes, divided into five major operons (*LEE1-5*), that encode for a type 3 secretion system (T3SS), regulators, chaperones, and effector proteins. The LEE-encoded regulator (Ler), the first gene encoded in *LEE1*, acts as the master transcription factor of the pathogenicity island, regulating expression of the entire LEE (Elliott et al., 1998; Muller et al., 2009).

The structure of the T3SS resembles a "molecular syringe" where EHEC can inject effector proteins through the T3SS needle directly into the cytoplasm of the target cells. One important secreted protein that is injected into the host is the translocated intimin receptor (Tir). Once released into the host cytoplasm, Tir is directed to the host cytoplasmic membrane and is inserted as a hairpin-like structure, with its N- and C-terminus in the cytoplasm and central domain exposed to the surface. The central domain of Tir interacts with the LEE-encoded surface protein intimin to form a tight attachment of the bacteria to the eukaryotic cell (Kenny et al., 1997; Deibel et al., 1998). Another non-LEE encoded effector protein, *E. coli* secreted protein F-like protein from prophage U (EspFu), is secreted into the cell and works co-operatively with Tir to recruit host proteins to subvert host cytoskeleton and actin polymerization. EspFu recruits actin nucleation-promoting factor Wiskott-Aldrich syndrome protein (N-WASP) and insulin receptor tyrosine kinase substrate p53 (IRSp53), an important regulator for actin cytoskeleton reorganization. This results in accumulation of actin beneath attached bacteria, forming the characteristic pedestal-like structure (**Figure 1**) (Campellone et al., 2004; Weiss et al., 2009).

In vitro studies demonstrate the crucial role A/E lesion formation plays in EHEC attachment to cultured cells. Various groups have investigated whether the formation of A/E lesions is also required for EHEC to attach to bovine intestinal epithelial cells to promote colonization in cattle. Immunofluorescence staining of tissues reveals that EHEC tightly adheres predominately to the epithelial cells in the RAJ of cattle (Naylor et al., 2003). Dziva et al. used signature-tagged transposon mutagenesis (STM) to identify EHEC genes required for colonization and survival in cattle. Transposon insertions in the genes encoding for the T3SS machinery resulted in reduced fecal shedding of EHEC (Dziva et al., 2004). Similarly, deletion of the *LEE4* operon, which encodes for essential structural components of the T3SS, resulted in reduced EHEC ability to colonize cattle (Naylor et al., 2005). These data suggest that the secretion apparatus is important for colonization in cattle. Tir and intimin have also been shown to play an important role in intestinal colonization in neonatal calves and piglets (Donnenberg et al., 1993; McKee et al., 1995; Tzipori et al., 1995; Dean-Nystrom et al., 1998) and in adult cattle and sheep (Cornick et al., 2002). Together the data indicate that LEE-mediated adherence of EHEC to intestinal epithelia is important for promoting colonization in cattle.

In recent years, several non-LEE encoded effectors—EspJ, NleB, NleE, NleF, and NleH—also have been shown to influence EHEC survival and colonization. Although EspJ is not required for A/E lesion formation in HEp-2 cells or human intestinal explants, *in vivo* studies in mice show that EspJ aids in the passage of EHEC through the host's intestinal tract, suggesting a role for EspJ in host survival and pathogen transmission (Dahan et al., 2005). The mouse pathogen *Citrobacter rodentium*, which shares homology of many virulence factors with EHEC, had reduced colonization of *nleB*, *nleH, nleF* mutants in mice compared to the wild-type strain (Kelly et al., 2006; Echtenkamp et al., 2008; Garcia-Angulo et al., 2008). Wild-type EHEC also outcompeted the *nleF* mutant in gnotobiotic piglets for colonization of the piglet colon and RAJ (Echtenkamp et al., 2008). Co-infection of lambs with wild-type EHEC and an *nleH* mutant demonstrated a competitive advantage of the wild-type strain over the mutant (Hemrajani et al., 2008). In contrast, Hemrajani et al. found that the *nleH* mutant colonized the bovine gut more efficiently than wild-type EHEC. While studies in mice and other animal models provide insight into the roles of EHEC virulence genes, further studies are required to evaluate the role that these EHEC effectors perform in cattle.

REGULATION OF ACID FITNESS AND LEE GENES BY QUORUM-SENSING

Acid resistance and formation of A/E lesions are crucial for EHEC to establish a persistent oral-fecal lifestyle in cattle. Elucidating the mechanisms by which EHEC regulates these two systems in its natural reservoir provides insight for developing better preventative strategies to reduce EHEC carriage. Progress toward understanding how EHEC regulates both acid resistance and the LEE genes was made recently with the discovery that the transcriptional regulator SdiA regulates both transcription of the LEE genes for A/E lesion formation (Kanamaru et al., 2000; Hughes et al., 2010) and the *gad* genes for acid resistance in cattle (Kanamaru et al., 2000; Hughes et al., 2010). A member of the LuxR family of transcription factors, SdiA senses acyl-homoserine lactones (AHLs) produced by other bacteria.

Bacteria coordinate their behavior through the production and sensing of chemical signals, a mechanism termed quorum-sensing (Fuqua et al., 2001). The LuxR/I system in *Vibrio fischeri* represents the prototypical quorum-sensing system (Nealson et al., 1970; Nealson and Hastings, 1979). Briefly, through LuxI, *V. fischeri* synthesizes AHLs, which diffuse freely across the bacterial membrane into the environment. When the bacterial population reaches a sufficient density, AHLs diffuse back into the bacterial cytoplasm where they bind to the transcriptional regulator LuxR. LuxR senses AHLs through an AHL-binding region at the amino terminus, enabling LuxR to bind DNA through a helix-turn-helix at the carboxyl terminus to modulate expression of target genes (Nealson et al., 1970; Fuqua et al., 2001; Lupp et al., 2003).

Unlike *V. fischeri*, EHEC lacks a LuxI-like synthase; therefore, SdiA function depends on AHLs produced by other bacteria in the environment (Ahmer et al., 1998). Hughes et al. found that AHLs present in the rumen of cattle activate the *gad* genes that are vital to acid resistance for the passage through the acidic stomachs, and repress the LEE genes to prevent colonization within the rumen. EHEC lacking the SdiA sensor results in significantly reduced acid survival compared to wild type both *in vitro* and in cattle rumen (Hughes et al., 2010). Additionally, wild-type EHEC outcompetes the *sdiA* mutant for colonization at the RAJ (Hughes et al., 2010). Based on these studies, a model has been proposed in which SdiA senses AHLs in rumen to activate acid fitness genes that allow EHEC passage through the acidic stomachs but also downregulate LEE genes to ensure colonization does not occur in hostile environments. EHEC does not encounter AHLs beyond the rumen, alleviating the SdiA-mediated repression of the LEE and allowing EHEC to colonize the RAJ (**Figure 2**). Intervention in quorum-sensing provides an alternative strategy to reduce carriage in cattle and subsequently, shedding and contamination of EHEC in the environment.

Developing strategies to reduce EHEC survival and colonization in cattle have been an ongoing challenge. Strategies to increase cattle resistance to EHEC colonization include supplementation with probiotics, administration of antibiotics, and vaccination against T3SS machinery (detailed review in Callaway et al., 2009; Jacob et al., 2009). Conflicting results from these studies has thwarted efforts to control EHEC populations within cattle (Potter et al., 2004; Van Donkersgoed et al., 2005; Peterson et al., 2007; Sargeant et al., 2007) and

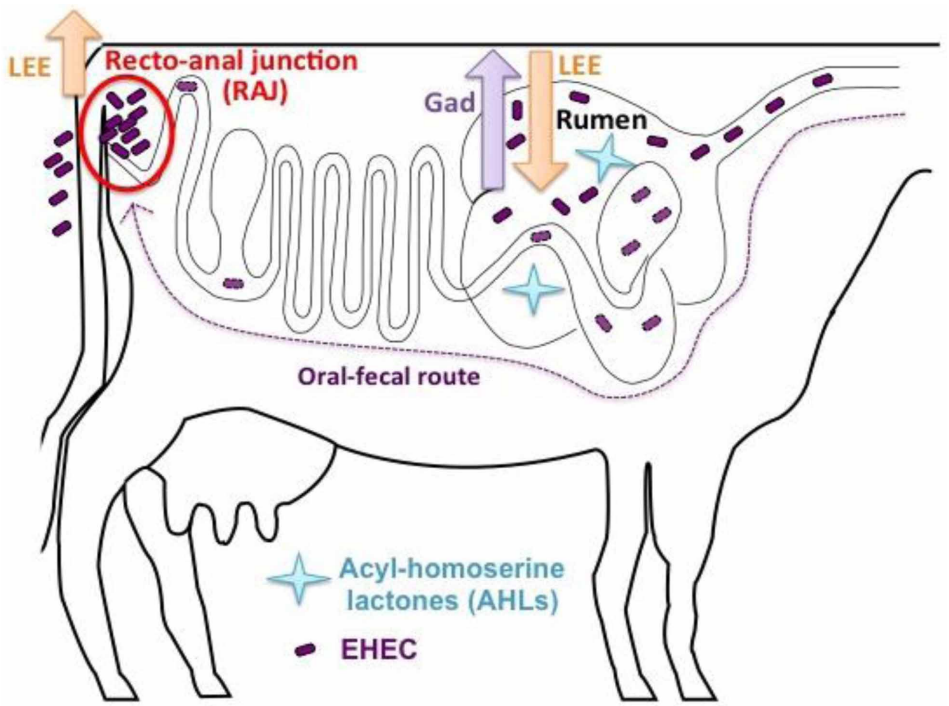

FIGURE 2 | Proposed EHEC SdiA-AHL signaling in cattle.

emphasizes the necessity for additional research to be performed. The dearth of knowledge on the mechanisms regulating intestinal colonization of ruminants by EHEC has hindered these strategies. With cattle being the major reservoir of EHEC and bovine-derived products as the prominent source of EHEC outbreaks, understanding the biology of EHEC colonization in cattle is vital to the development of new preventative strategies.

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Characterization of Shiga toxin-producing *Escherichia coli* O130:H11 and O178:H19 isolated from dairy cows

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Shiga toxin-producing *E. coli* (STEC) are isolated from human patients with bloody diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS). In the last years, the infections with non-O157 serotypes are increasing their frequency of association with human disease. STEC produce Shiga toxin (Stx) and other virulence factors that could contribute to human pathogenesis. Cattle are the main reservoir and the transmission to humans is through the consumption of undercooked meat, non-pasteurized dairy products, and vegetables or water contaminated with feces. We have previously determined that O130:H11 and O178:H19 serotypes were the most prevalent in dairy cows from Argentina. In the present study, 37 and 25 STEC isolates from dairy cows belonging to O130:H11 and O178:H19 serotypes, respectively, were characterized regarding to their cytotoxicity on Vero cells, *stx* subtypes, presence of *sab* and typing by multiple-locus variable-number tandem repeat analysis (MLVA). All strains demonstrated a cytotoxic effect, and in O130:H11 isolates, $stx2_{EDL933}$ was the predominant subtype. In O178:H19 isolates the main *stx2* subtype was $stx2_{vha}$. The *sab* gene was detected in 65 and 24% of the isolates belonging to O130:H11 and O178:H19, respectively. Only one MLVA profile was identified among the O130:H11 isolates meanwhile 10 MLVA profiles were detected among the O178:H19 isolates which were grouped in two main clusters. In conclusion, our data show that O130:H11 and O178:H19 STEC isolates encode virulence factors associated with severe human disease and both serotypes should be considered for routinely testing. Our subtyping experiments showed that isolates could be distinguished based on the stx_2 subtype and the presence/absence of *sab* gene, and for isolates belonging to O178:H19, also when the MLVA type was considered. However, MLVA subtyping of O130:H11 isolates will require the development of more specific markers.

Keywords: STEC, dairy cattle, MLVA, Shiga toxin

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) cause bloody diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans (Pearce et al., 2004; Giugno et al., 2007). Most outbreaks have been attributed to O157:H7 serotype (Mora et al., 2004) but infections with non-O157 serotypes are also being frequently associated with HC and HUS (Bettelheim, 2007). In several countries STEC O157:H7 have been frequently isolated from cattle but several studies in Argentina have detected mainly non-O157:H7 serotypes (Meichtri et al., 2004; Padola et al., 2004; Fernández et al., 2010). Cattle are the main reservoir of STEC and the transmission to humans occurs through the consumption of undercooked meat, non-pasteurized dairy products, and vegetables or water contaminated with feces (Hussein and Sakuma, 2005). Direct contact with cattle and dairy farm environment has been reported also as a possible source for STEC human transmission (Oliver et al., 2005).

The main virulence factor of STEC is the production of Shiga toxins (Stx1 and Stx2) (Paton and Paton, 1998; Gyles, 2007). Stx1 group includes few subtypes, while the Stx2 is a more heterogeneous group and comprises an expanding number of subtypes (such as Stx2EDL933, Stx2vha, Stx2vhb, Stx2O118, Stx2dact, Stx2e, Stx2f, and Stx2g). Stx subtypes differ in their degree of association with HC and HUS cases, being Stx2O118 (formerly identified as Stx2d-Ount), Stx2e, Stx2f, and Stx2g not frequently associated with severe human disease (Friedrich et al., 2002; Karch et al., 2005; Prager et al., 2009, 2011). Other virulence factors that could contribute to the pathogenesis are intimin, encoded by the *eae* gene and responsible for the intimate attachment of STEC to intestinal epithelial cells, an enterohaemolysin (EhxA), an autoagglutinating adhesin (Saa) and a novel STEC autotransporter (Sab) described for first time in a *saa*-positive O113:H21 strain, which participates in adhesion and biofilm formation (Herold et al., 2009). The *ehxA*, *saa,* and *sab* genes are

located in a megaplasmid (Paton and Paton, 1998; Paton et al., 2001; Herold et al., 2009).

In Argentina, O130:H11 and O178:H19 were the most prevalent serotypes isolated from dairy cows (Fernández et al., 2010) and were also identified by Masana et al. (2011) in beef abattoirs and by López et al. (2012) in feedlot cattle. Both serotypes have been isolated from HC and HUS cases in several countries and have been found among human STEC isolates received between 2000–2010 by the CDC National *E. coli* Reference Laboratory (Blanco et al., 2004; Fremaux et al., 2006; Giugno et al., 2007).

In the present study, we further characterized O130:H11 and O178:H19 STEC isolated by Fernández et al. (2010) from dairy farms regarding their cytotoxicity on Vero cells, *stx* subtypes, presence of *sab* gene and typing by multiple-locus variable-number tandem repeat analysis (MLVA), in order to evaluate the genetic diversity of isolates belonging to these serotypes which are prevalent in dairy cattle.

MATERIALS AND METHODS

BACTERIAL STRAINS

The bacterial strains used in this study were 37 STEC O130:H11 and 25 STEC O178:H19 isolated from dairy cows in five farms (named A, B, C, D, and E) from Argentina (Fernández et al., 2010).

CYTOTOXIC ACTIVITY ON VERO CELLS

The cytotoxicity of the isolates was evaluated by Vero cells assay. Briefly, each strain was cultured overnight into 25 ml of Microbiological broth (No. 3, Merck) and was centrifuged 120× g (10 min at 4°C) and the supernatant was centrifuged again 17,228× g (10 min at 4°C) and identified as S1. The cell pellet was washed with PBS, resuspended in 3 ml of polymyxin sulfate (0.1 mg/ml) and incubated 30 min. Polymyxin B-treated cultures were centrifuged at 120× g (10 min at 4°C). The supernatant was centrifuged at 17,228× g, 10 min at 4°C, and was identified as S2. Fifty and 25 µl of each one S1 and S2 were inoculated in each one of the 96-well-plates containing 4×10^4 freshly trypsinized Vero cells and were incubated 48 h at 37°C in a 5% CO_2 atmosphere. The cell monolayers were fixed with 10% (v/v) formaldehyde and then stained with 0.2% (w/v) crystal violet in phosphate-buffered saline solution. *E. coli* EDL933 strain was used as positive control and a strain *stx* positive without cytotoxic effect as negative control (*E. coli* serotype O15:H21). Wells having 50% or greater cytotoxicity, compared to a standard control well were considered positive.

stx SUBTYPING

The strategy to detect stx_2 subtypes was similar to that previously described by Krüger et al. (2011). Briefly, all stx_2-positive STEC were subjected to PCR with the primer pair VT2-c/VT2-d, and amplification products were independently digested with restriction endonucleases *Hae*III, *Rsa*I, and *Nci*I to detect $stx_{2EDL933}$, stx_{2vha}, stx_{2vhb}, stx_{2g}, and stx_{2NV206} (Tyler et al., 1991; Bertin et al., 2001; Krüger et al., 2007). All isolates were also evaluated with the VT2-cm/VT2-f primer set (Pierard et al., 1998) specific for stx_{2O118} (first termed stx_{2d} by Piérard and renamed stx_{2O118} as proposed by Scheutz and Strockbine, 2005). The strains used as positive controls for each subtype and the references corresponding to the primers are detailed in Krüger et al. (2011).

sab GENE

The detection of the *sab* gene was performed by PCR using the primers described by Herold et al. (2009) and the following amplification conditions: initial cycle of 94°C for 120 s, 30 cycles with denaturation step (94°C, 30 s), annealing step (54°C, 30 s) and extension step (68°C, 30 s), and a 60 s cycle at 72°C. STEC O20:H19 was used as positive control and *Salmonella spp, Staphylococcus aureus,* and *Pseudomonas aeruginosa* as negative controls.

MLVA ASSAY

We performed an MLVA assay that previously showed a high level of discrimination among STEC isolates belonging to different non-O157:H7 serotypes (Schimmer et al., 2008; Bustamante et al., 2010; Franci et al., 2011). The seven VNTR loci studied in this assay were analyzed as described by Bustamante et al. (2010). Representative alleles were sequenced with an ABI PRISM 3730XL genetic analyzer (Macrogen, Korea). The dendrogram was constructed using the UPGMA clustering method implemented by START Vs. 1.0.5 software (Joley et al., 2001). The alleles were indicated in a string order CVN001-CVN002-CVN003-CVN004-CVN007-CVN014-CVN015, named according to the number of tandem repeat sequences. If no amplification product was detected, the allele was designated with an arbitrary number (30).

In all PCR assays, Inbio-Highway (Argentina) DNA polymerase was used.

RESULTS AND DISCUSSION

Using Vero cell assay, the S1 and S2 supernatants of all isolates from both serotypes demonstrated cytotoxic effect after 48 h post-inoculation on Vero cells.

Among 36 stx_2-positive O130:H11 isolates, $stx_{2EDL933}$ was the predominant subtype (81%), and the other subtype present was stx_{2vhb} (**Table 1**). Only three isolates harbored both subtypes.

The most frequent stx_2 subtype among O178:H19 isolates was stx_{2vha} (72%), while $stx_{2EDL933}$ and stx_{2vhb} subtypes were found less frequently (20 and 8%, respectively) and no isolates harboring more than one stx_2 subtype were found.

It is interesting to note that the $stx_{2EDL933}$-positive strains, belonging to either O130:H11 or O178:H19 serotypes, (**Tables 1** and **2**) corresponded mainly to isolates harboring the profile stx_1-stx_2-*ehxA*-*saa*.

The subtypes found in this work have been reported as the predominant sxt_2-subtypes in bovine STEC strains in Argentina and other countries (Bertin et al., 2001; Brett et al., 2003; Meichtri et al., 2004; Galli et al., 2010; Krüger et al., 2011) and have been associated with the development of HC and HUS (Friedrich et al., 2002; Persson et al., 2007). In a study performed by Masana et al. (2011) O130:H11 and O178:H19 were also among the most prevalent serotypes found in carcasses and bovine feces sampled

Table 1 | Origin and virulence genotypes of O130:H11 isolates.

Strain number	Farm	Virulence genotype*	*sab*	*stx*$_2$ subtype
1	A	*stx*$_1$-*ehxA-saa*	–	–
2	A	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2vhb}$
3	A	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2vhb}$
4	A	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2vhb}$
5	A	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$
6	A	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$
7	A	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$
8	B	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$ *stx*$_{2vhb}$
9	B	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$ *stx*$_{2vhb}$
10	B	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
11	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
12	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
13	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
14	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
15	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
16	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
17	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
18	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
19	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
20	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
21	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
22	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$ *stx*$_{2vhb}$
23	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$
24	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
25	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
26	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2vhb}$
27	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2vhb}$
28	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
29	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
30	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
31	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2vhb}$
32	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
33	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
34	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
35	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	–	*stx*$_{2EDL933}$
36	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$
37	E	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$

*Previously determined (Fernández et al., 2010).

Table 2 | Origin and characterization of O178:H19 isolates.

Strain number	Farm	Virulence genotype*	*sab*	*stx*$_2$ subtype	MLVA profile
1	A	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
2	A	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
3	A	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
4	A	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
5	A	*stx*$_2$	–	*stx*$_{2vha}$	I$_2$
6	C	*stx*$_2$	–	*stx*$_{2vha}$	I$_3$
7	C	*stx*$_2$	–	*stx*$_{2vha}$	I$_5$
8	D	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
9	D	*stx*$_2$	+	*stx*$_{2vhb}$	II$_2$
10	E	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
11	E	*stx*$_2$	+	*stx*$_{2EDL933}$	II$_4$
12	E	*stx*$_2$	–	*stx*$_{2vhb}$	I$_4$
13	E	*stx*$_2$	–	*stx*$_{2vha}$	I$_2$
14	E	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
15	B	*stx*$_2$	–	*stx*$_{2vha}$	I$_3$
16	A	*stx*$_2$	–	*stx*$_{2vha}$	I$_2$
17	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$	II$_3$
18	C	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$	II$_5$
19	D	*stx*$_2$	–	*stx*$_{2vha}$	I$_2$
20	D	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$	II$_2$
21	D	*stx*$_2$-*ehxA-saa*	–	*stx*$_{2vha}$	I$_2$
22	D	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
23	E	*stx*$_1$-*stx*$_2$-*ehxA-saa*	+	*stx*$_{2EDL933}$	II$_1$
24	C	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$
25	C	*stx*$_2$	–	*stx*$_{2vha}$	I$_1$

*Previously determined (Fernández et al., 2010).

at abattoirs in Argentina. In that study, O130:H11 isolates presented the same virulence genotypes (in regard to the presence of *stx*$_1$, *stx*$_2$ subtypes, *ehxA* and *saa*) as the ones detected in the present report. Regarding to O178:H19, some virulence genotypes (*stx*$_{2vha}$; *stx*$_1$-*stx*$_{2EDL933}$-*ehxA-saa*; *stx*$_{2vhb}$) found by Masana et al. (2011) were detected also in the present study, but there were other profiles (*stx*$_{2NT}$; *stx*$_{2EDL933}$-*stx*$_{2vha}$) not shared between these studies.

The gene encoding Sab, a protein which mediates biofilm formation and promotes intestinal adherence, was detected in 65% of the isolates belonging to O130:H11. This study is the first, to our knowledge, to describe O130:H11 as a serotype carrying *sab*. In O178:H19 isolates *sab* was detected in 24% of the isolates (**Table 2**). Buvens et al. (2010) did not detect *sab* in a STEC O178:H19 strain isolated from HUS. All *sab*-positive STEC strains identified to date were also positive for *ehx* as well as *saa*, all genes located in a megaplasmid, noteworthy, in the present study some of the O178:H19 isolates were *sab*-positive but negative for *ehx*A and *saa*.

Most of the MLVA loci could be amplified, although there were differences between serotypes. To our knowledge this is the first time that STEC O130:H11 is typed by MLVA and notably, only one MLVA profile (5-2-30-9-8-30-6) was detected among these isolates. We have used this MLVA assay to subtype several isolates belonging to different non-O157:H7 serotypes and we found a high level of discrimination (Bustamante et al., 2010; Franci et al., 2011). Other authors have also applied this protocol to successfully resolve outbreaks due to a non-O157 strain (Schimmer et al., 2008). In our experience, this is the first time that all isolates from a same serotype and different origin present a unique MLVA profile. The lack of diversity found in this serotype would indicate that the chosen VNTR loci are not variable enough for typing O130:H11 strains since they did show variability in relation with the presence/absence of *sab* and also with the *stx2* subtype present.

Therefore, there is a need to identify VNTR loci that are variable among STEC strains belonging to this serotype.

On the other hand, among the 25 O178:H19 isolates, 10 MLVA profiles were detected, which were grouped in two main clusters (**Figure 1**). A relationship could not be found with regard to MLVA profiles and farm origin (**Table 2**). Cluster I included isolates from all the farms, and cluster II, isolates from dairy farms C, D, and E. A high variability was found among isolates from farms C and E, detecting in each farm 5 MLVA profiles among 6 isolates (**Table 2**). All isolates belonging to clade I, were *sab*-negative and, with the exception of isolate 12, they presented the subtype stx_{2vha} (**Table 2**). Clade II was the most variable, presenting five different profiles among six isolates. Moreover, isolates 9 and 20 shared the MLVA profile but not their virulence profile. Within this clade, all the isolates were *sab*-positive and carried $stx_{2EDL933}$, with the exception of isolate 9 (positive for *sab* but negative for that stx_2 subtype) (**Table 2**). Although a relationship between the MLVA profile and the stx_2 subtype is not expected, with the exception of isolates from a same clone, all stx_{2vha}-positive isolates belonged to cluster I and all stx_{EDL933}-positive isolates, to cluster II. Regarding isolates carrying stx_{2vhb}, one belonged to cluster I and the other to cluster II. Noteworthy, all the MLVA profiles present in these isolates were quite different from the ones detected previously in STEC O178:H19 isolated from minced meat of the same geographic region (Franci et al., 2011). Taking into account all these results, a high genetic variability was evidenced among isolates belonging to this serotype. Our results showed different STEC O178:H19 clonal lineages and determined that some clones may be present in more than one farm.

CONCLUSION

The data suggest differences in the genetic variability for the two serotypes. It could be assessed when the stx_2 subtype and the presence/absence of *sab* gene were taken into account, and for isolates belonging to O178:H19, also when the MLVA type was considered. The MLVA typing assay chosen seems not suitable for detecting genetic differences among O130:H11 STEC isolates, and further loci need to be analyzed.

STEC non-O157 serotypes are nowadays frequently associated with outbreaks and sporadic cases of HUS and particularly, O130:H11 and O178:H19 STEC have been isolated from human patients. In our study isolates from dairy cows belonging to these serotypes possess virulence characteristics associated with the development of severe disease in humans and

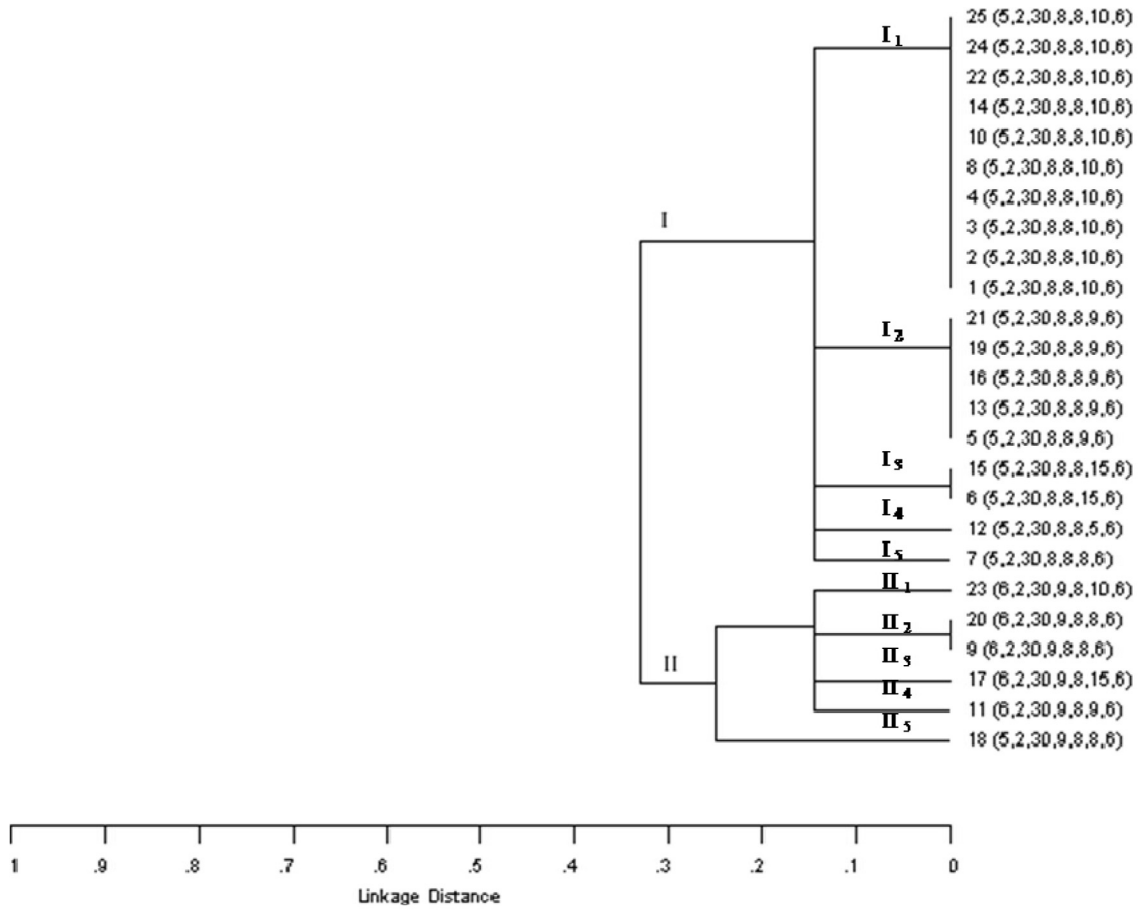

FIGURE 1 | Dendrogram based on MLVA profiles of STEC O178:H19 isolated from dairy cows in Argentina. Order of the allele string: CVN001-CVN002-CVN003-CVN004-CVN007-CVN014-CVN015.

it would be desirable to consider them in the group of serotypes routinely investigated.

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Synanthropic rodents as possible reservoirs of shigatoxigenic *Escherichia coli* strains

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Shigatoxigenic *Escherichia coli* (STEC) strains are worldwide zoonotic pathogen responsible for different cases of human disease including hemolytic uremic syndrome (HUS). Transmission of STEC to humans occurs through the consumption of food and water contaminated by faeces of carriers and by person-to-person contact. The objective of this study was two-fold: (1) to investigate whether synanthropic rodents are possible reservoirs of STEC in the urban area and (2) whether a particular genus out of synanthropic rodent is the principal carrier of STEC. One hundred and forty-five rodents were captured in Buenos Aires City. Screening for *stx*1/*stx*2 and *rfb*O157 was done by PCR from the confluence zone. STEC isolates were further characterized with biochemical tests by standard methods. Additional virulence factors (*eae*, *ehx*A, and *saa*) were also determined by PCR. Forty-one of the rodents were necropsied and sample of kidney and small and large intestine were taken for histopathological diagnosis. The samples sections were stained with hematoxylin-eosin, and observed by light microscopy to evaluate the systemic involvement of these species in natural infections. STEC was isolated from seven out of 27 suspect animals at screening. The following genotypes were found in the STEC strains: *stx*1/*stx*2/*ehx*A (1), *stx*2 (4), *stx*2/*ehx*A (1), *stx*2/*ehx*A/*eae* (1). Neither gross nor microscopic lesions compatible with those produced by Shiga toxin were observed in the studied organs of necropsied rodents. The bivariate analysis including the 145 rodent's data showed that the isolation of STEC is associated positively to *Rattus* genus. This synanthropic species may play a role in the transmissibility of the agent thus being a risk to the susceptible population. Their control should be included specifically in actions to dismiss the contamination of food and water by STEC in the urban area, as additional strategies for epidemiological control.

Keywords: STEC, hemolytic uremic syndrome, *Rattus*, reservoir, synanthropic

INTRODUCTION

Shigatoxigenic *Escherichia coli* (STEC) strains are a worldwide zoonotic pathogen responsible for different cases of human disease including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Karmali, 1989).

Unlike other commensal *E. coli* strains, STEC strains have several virulence genes which permit the evaluation of its pathogenic nature in the laboratory (*stx*1, *stx*2, *eae*, *ehx*A, *saa*) (Paton and Paton, 1998a, 2002). These strains may be screened by PCR of *stx* genes in cultured bacteriological samples and subsequent isolation of colonies stx^+.

STEC was reported as inhabitant in the intestine of various animal species: cattle, sheep, pigs, goats (Beutin et al., 1993). There are few references of STEC in synanthropic animals (Hancock et al., 1998; Čižek et al., 1999; Nielsen et al., 2004).

The fecal-oral route is recognized as the major route of transmission, associated with raw or processed food, probably contaminated at some point of the production chain (Meichtri et al., 2004). Over years cattle has been involved as the principal reservoir of STEC, however, isolated strains are partially similar to those which have impact on public health (Boerlin et al., 1999; Rosser et al., 2008).

In the present work we investigated STEC in synanthropic rodents of Buenos Aires City, Argentina. The aim of this study was twofold: (1) to investigate whether synanthropic rodents are possible reservoirs of STEC in the urban area and (2) whether a particular genus of synanthropic rodent is a carrier of STEC.

MATERIALS AND METHODS

STUDY DESIGN

A non-probabilistic sampling by convenience was carried out in Buenos Aires City. One hundred and forty-five rodents were captured using live traps (Sherman and cage traps). The traps were placed in parks at intervals of five meters, varying in number

according to the surface and the characteristics of each site of capture. The traps remained active for four consecutive nights and they were checked daily in the morning.

The inclusion criteria for the sample from the rodent population of Buenos Aires City were "animals from parks which were captured alive." We studied the population groups that were validated by relevant epidemiological characteristics. The animals were classified according to genus and species. Each rodent was anesthetized to be weighed, measured, sexed, and their reproductive status was recorded. They were also sampled by two rectal swabs.

The study was approved by the Institutional Animal Care and Use of Experimental Animals, CICUAL, Faculty of Veterinary Science, University of Buenos Aires.

DETECTION OF *stx*$^+$ AND ISOLATION OF STEC STRAINS

The samples were examined according to standard procedures for isolation of O157:H7 STEC and non-O157 STEC previously described (Bentancor et al., 2007). Briefly, for O157 STEC detection the samples were inoculated in 5 ml of cefixime-tellurite (CT) tripteine soy broth (TBS) for enrichment and incubated 18 h at 37°C. The cultures were streaked onto Sorbitol MacConkey Agar (CT-SMAC) and incubated overnight. Detection of non-O157 was performed inoculating the samples in 5 ml of TSB and incubated overnight at 37°C. The cultures were streaked onto MacConkey Agar (MAC) and incubated overnight.

Screening for *stx*1/*stx*2 and *rfb*O157 from the confluence area (CT-SMAC and MAC) was done by Multiplex PCR (Leotta et al., 2005) using the primers of Pollard et al. (1990) and of Paton and Paton (1998b) (**Table 1**). To obtain the templates for PCR a sample from the confluence area was taken and suspended in 200 µl of sterile distilled water. The suspension was boiled 10 min in a water bath and centrifuged at 1300 rpm for 5 min. The supernatant was used as the DNA extract. The PCR mixture for each sample had a final volume of 50 µl, and contained 1.5 mM of the two *stx*1 specific primers, 0.3 mM of the two *stx*2 and *rfb*O157 specific primers, 200 mM of each deoxyribonucleoside triphosphate and 1 U of Go Taq (Promega) DNA polymerase in 1 × buffer according to the manufacturer's instructions, and finally 6 µl of the template from sample. PCR was performed in a Mastercycler Gradient Eppendorf under the following conditions: 5 min at 94°C, followed by 30 cycles of: 45 s at 94°C, 30 s at 56°C, 30 s at 72°C and a final step of 7 min at 72°C, maintaining it at 4°C until analysis. 10 µl of each PCR product obtained were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Positive samples at screening from the confluence zone were considered suspect. Each suspect sample was evaluated for the presence of *stx*$^+$ strains up to 50 CFU.

According to the results obtained by PCR, animals were classified into positive (positive screening with isolation of a *stx*$^+$ strain), suspect (positive screening without isolation) and negative (pathogen was not detected at screening).

BIOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF VIRULENCE FACTORS

STEC isolates were further characterized as *E. coli* after gram staining, oxidase, catalase, glucose oxidation-fermentation, indole, methyl red (MR), Voges Proskauer (VP), Simmons citrate, fermentation of sugars (lactose, sucrose, cellobiose, sorbitol, raffinose, dulcitol, rhamnose) H_2S production, yellow pigment, β-glucuronidase activity, lysine decarboxylase, ornithine decarboxylase, and mobility assessment (Mac Faddin, 2003). RapidID ONE System 20 test (Remel) was also done, and additional virulence factors (*saa*, *eae*, *ehx*A) were determined by PCR (Paton and Paton, 1998a, 2002).

Table 1 | Primers used for PCR analysis.

Primer	Sequence	Amplicon	Reference
stx 1 *a**	GAAGAGTCCGTGGGATTACG	130	Pollard et al., 1990
stx 1 *b**	AGCGATGCAGCTATTAATAA		
stx 2 *a**	TTAACCACACCCCACCGGGCAGT	346	Ziebell et al., 2002
stx 2 *b**	GCTCTGGATGCATCTCTGGT		
*rfbO157 f**	CGGACATCCATGTGATATGG	259	Paton and Paton, 1998a
*rfbO157 r**	TTGCCTATGTACAGCTAATCC		
ehxAf†	GCATCATCAAGCGTACGTTCC	534	Paton and Paton, 1998a
ehxAr†	AATGAGCCAAGCTGGTTAAGCT		
eaeAf†	GACCCGGCACAAGCATAAGC	384	Paton and Paton, 1998a
eaeAr†	CCACCTGCAGCAACAAGAGG		
stx2f†	GGCACTGTCTGAAACTGCTCC	255	Paton and Paton, 1998a
stx2r†	TCGCCAGTTATCTGACATTCTG		
stx1f†	ATAAATCGCCATTCGTTGACTAC	180	Paton and Paton, 1998a
stx1r†	AGAACGCCCACTGAGATCATC		
saa f†	CGTGATGAACAGGCTATTGC	119	Paton and Paton, 2002
saa r†	ATGGACATGCCTGTGGCAAC		

**Primers used at PCR screening.*

†*Primers used in the study of virulence factors.*

HISTOPATHOLOGY

Necropsy of captured animals was performed to determine if STEC carriers had any injury. We looked for gross changes. Kidney, small, and large intestine were removed for histopathological diagnosis. The samples were fixed for 48 h in 10% buffered formalin and processed routinely. Sections of paraffined-embedded tissue were stained with hematoxylin-eosin and evaluated by light microscopy.

STATISTICAL ANALYSIS

The data obtained from each rodent were analyzed with EpiInfo 3.2 (CDC-WHO). We performed bivariate analysis (Chi-squared) and estimated OR and 95% confidence interval for significant variables.

RESULTS

Rodents captured belonged to the following species: *Deltamys kempi* (*n*:4), *Mus musculus* (*n*:66), *Oligoryzomys flavescens* (*n*:16), *Rattus norvegicus* (*n*:31), *Rattus rattus* (*n*:28).

PCR analysis was performed for all the rodents captured (145 animals). Twenty-seven out of 145 animals were suspected at PCR screening from the confluence area: *Deltamys kempi* (0/4); *Mus musculus* (10/66); *Oligoryzomys flavescens* (1/16); *Rattus norvegicus* (6/31); *Rattus rattus* (10/28) (**Table 2**).

Isolation of *stx*$^+$ strains was obtained in seven cases out of 27 suspect samples: *Mus musculus* (1/66), *Rattus norvergicus* (2/31), and *Rattus rattus* (4/28). Each isolated strain was identified as *Escherichia coli* by conventional bacteriological test and by RapidID ONE System 20 test. All the isolates were detected as non-O157 strains by PCR.

The following genotypes were found in the STEC strains: *stx*1/*stx*2/*ehx*A (1), *stx*2 (4), *stx*2/*ehx*A (1), *stx*2/*ehx*A/*eae* (1).

Forty-one out of 145 animals evaluated (five positive, four suspect and 32 negative) were necropsied. Neither gross nor microscopic lesions compatible with those produced by Shiga toxin were not observed in the studied organs.

The bivariate analysis including the 145 rodent data showed that the isolation of STEC is associated positively to *Rattus* genus (*p*: 0.01). The possibility of isolating STEC from rodents is higher in *Rattus* than in others genus (OR: 10,62, IC 95%: 1,10-218,07).

DISCUSSION

Very little is known about the occurrence of STEC in synanthropic rodents. This study differs from others studies which captured rodents and searched for O157 STEC in sampling methods and procedures for isolation of strains (Hancock et al., 1998; Čižek et al., 1999), in our case the presence of O157 was not detected in these samples.

We isolated STEC strains in seven out to 27 suspect animals. This low number of isolates could be related to the number of CFU evaluated in positive animals at PCR screening from the confluence zone. We analyzed 50 CFU from each suspect animal to avoid overestimated rodent species. It is possible that a low proportion of STEC in the suspect samples determinate a low recovery. On the other hand, sampling methods could be the cause of the low efficiency of isolation according to the low sample obtained.

Although rodents from different species were suspected at PCR screening, we observed a higher proportion in individuals of the *Rattus* genus. This fact is important because rodents from *Rattus* genus live in urban areas, overlaying their territory with men, feeding on waste and stowed food which can pollute. The growth of this population is related to their availability to food and water. When we discriminated these individuals by species, the study of carriage in *R. norvegicus* was lower than in *R. rattus*, opposed to the findings of different researchers who have not detected *R. rattus* as a carrier of STEC (Čižek et al., 1999; Nielsen et al., 2004). Differences between *R. rattus* and *R. norvegicus* could not be explained.

In Argentina the epidemiology of HUS included sporadic cases, opposite to large outbreaks of food borne diseases described in others countries. Synanthropic species as other sources deserve to be evaluated to mitigate the endemic presentation of the disease.

The capture of these animals was performed in the urban area, mainly in parks. Although the population of rats of Buenos Aires City is not determinate, larger sample with animals that come from other urban areas would be needed to establish whether there are differences between species in the urban cycle.

In the present study, one strain isolated genotype had *stx*1 and all strains carried *stx*2. The *stx*2 is strongly associated with increased risk of HUS (Rivas et al., 2006). However, none of

Table 2 | Rodents evaluated as carrier of STEC and virulence profile of isolated strains.

Rodent evaluated	*N*°	STEC suspicious carrier at screening	Isolation of *stx*$^+$ strain	*Escherichia coli* bacteriological identification	O157 serogroup	Genotype				
						*stx*1	*stx*2	*eae*	*ehxA*	*saa*
Rattus norvegicus	31	6	2	2	0	0	2	0	0	0
Rattus rattus	28	10	4	4	0	1	4	1	3	0
Mus musculus	66	10	1	1	0	0	1	0	0	0
Deltamis kempi	4	0	NA	NA	NA	NA	NA	NA	NA	NA
Oligoryzom ys flavescens	16	1	0	NA	NA	NA	NA	NA	NA	NA

NA, not applicable.

the isolated strains was from the O157 serogroup, which is most frequent as HUS producer.

The animals captured had no signs of disease; neither gross changes nor microscopic lesions compatible with those produced by Shiga toxin were observed in the studied organs. At the present time, lesions in rodents have been described in animal models which were inoculated orally or gastrointestinally with STEC, or by injection with Stx alone or with lipopolysaccharide component on the outer membrane of this bacterium (Obata, 2010). The absence of lesions may be due to Stx production, amount of Stx receptor (globotriaosylceramide, Gb3) in host cells and their sensitivity to Stx or complex biochemical aspects of the Gb3 membrane microenvironment (Obrig, 2010). Anyway, healthy synanthropic rodents could serve as a reservoir of STEC as occurs in other animal species (Beutin et al., 1993). On the other hand the possibility that the animals consumed contaminated food and could be colonized transiently by STEC could not be excluded. It is difficult to estimate the time of excretion of STEC in wild animals that do not survive in captivity to determine if their carrier status is due to a transient colonization or whether we are in presence of a new reservoir of STEC. In order to obtain a finally conclusion about their role as reservoirs, larger sample would be need.

The results of the present study represent the first finding of STEC in *R. rattus* and show STEC no-O157 is circulating in different synanthropic rodent species of the urban area.

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Shiga toxin-producing *Escherichia coli* in beef retail markets from Argentina

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Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens that cause mild or serious diseases and can lead to people death. This study reports the prevalence and characteristics of STEC O157 and non-O157 in commercial ground beef and environmental samples, including meat table, knife, meat mincing machine, and manipulator hands ($n = 450$) obtained from 90 retail markets over a nine-month period. The STEC isolates were serotyped and virulence genes as *stx* (Shiga toxin), rfb_{O157}] (O157 lipopolysaccharide), $fliC_{H7}$ (H7 flagellin), *eae* (intimin), *ehx*A (enterohemolysin) and *saa* (STEC autoagglutinating adhesin), were determined. STEC O157 were identified in 23 (25.5%) beef samples and 16 (4.4%) environmental samples, while STEC non-O157 were present in 47 (52.2%) and 182 (50.5%), respectively. Among 54 strains isolated, 17 were STEC O157:H7 and 37 were STEC non-O157. The prevalent genotype for O157 was $stx_2/eae/ehxA/fliC_{H7}$ (83.4%), and for STEC non-O157 the most frequent ones were $stx_1/stx_2/saa/ehxA$ (29.7%); stx_2 (29.7%); and $stx_2/saa/ehxA$ (27%). None of the STEC non-O157 strains were *eae*-positive. Besides O157:H7, other 20 different serotypes were identified, being O8:H19, O178:H19, and O174:H28 the prevalent. Strains belonging to the same serotype could be isolated from different sources of the same retail market. Also, the same serotype could be detected in different stores. In conclusion, screening techniques are increasingly sensitive, but the isolation of STEC non-O157 is still a challenge. Moreover, with the results obtained from the present work, although more studies are needed, cross-contamination between meat and the environment could be suspected.

Keywords: STEC, raw ground beef, retail markets, environmental samples, Argentina

INTRODUCTION

Foodborne illnesses are defined as diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food. Shiga toxin-producing *Escherichia coli* (STEC) are a group of food and water-borne pathogens associated with a wide spectrum of human diseases, ranging from mild diarrhea to hemorrhagic colitis (HC), thrombocytopenia, hemolytic uremic syndrome (HUS), and can also lead to people death (Karmali et al., 2010). Argentina has a high incidence of HUS: 17 cases per 100,000 children younger than 5 years, one of the highest in the world (Rivas et al., 2010). This rate is 10-fold higher than in other industrialized countries (Mead and Griffin, 1998). In Argentina, STEC is the primary etiological agent of HUS (Leotta et al., 2008), and *E. coli* O157:H7 is the predominant serotype isolated. However, other serotypes were associated with HUS (Guth et al., 2011).

Ruminants have been identified as the major reservoir of STEC (Karmali et al., 2010) and a variety of foods have been identified as vehicles of illnesses. However, approximately 52% of outbreaks have been associated with bovine products. Contamination of carcasses with STEC can occur when gut contents or fecal matter contact the meat surfaces, also cross-contamination between carcasses may occur during processing (Edwards and Fung, 2006). Studies of STEC prevalence in feces and carcasses of bovines from selected beef exporting abattoirs of Argentina showed that STEC non-O157 was present in 22.3% and in 9.0% of the feces and bovine carcasses (Masana et al., 2011), and the prevalence of STEC O157 was 4.1% and 2.6%, respectively (Masana et al., 2010). In other study 12.34% and 18.64% of STEC in carcasses, were detected at the slaughter and sanitary control cabin, respectively. These percentages increased at butcheries (24.52%) from the same city. The 25% of retail beef cuts were STEC-positive with significant differences among the different cuts of meat (chuck: 12.12%, rump roast: 12.12% and minced beef: 40.74%) (Etcheverría et al., 2010). Comparatively, the prevalence of STEC non-O157 in the USA beef cattle feces has been shown to range from 19 to 30%

(Barkocy-Gallagher et al., 2003; Renter et al., 2005) and the prevalence in hides was 56.3% (Barkocy-Gallagher et al., 2003).

Some studies of STEC prevalence in minced beef samples were made in Argentina, in one of them, 3.8% of O157:H7 was detected (Chinen et al., 2001) while Parma et al. (2000) found serotypes mainly non-O157. Studies in France, Australia, and the USA determined a STEC prevalence of approximately 16% in minced beef samples (Bohaychuk et al., 2006), while in Spain (Mora et al., 2007) the prevalence was 12%.

Until present, there have not been systematic studies on local ground beef retail markets to assess the microbiological quality of meat, but also including verification of good hygiene practices, handlers' habits and traceability of the raw material. In Argentina, the microbiological quality of the meat sold at retail markets is based on the parameters specified at the Argentine Food Code. However, for STEC strains only O157:H7 serotype is mandatory, and the searching for potentially pathogenic bacteria for consumers on surfaces that contact with the meat is not established.

The aims of this study were the detection, isolation, and characterization of STEC strains in ground beef and environmental sponge samples, including meat table, knife, meat mincing machine, and manipulator hands, in ground beef retail markets, and to determine the pathogenic potential of the circulating strains. It also attempts to establish potential cross-contamination throw surfaces.

MATERIALS AND METHODS

SAMPLE COLLECTION

From October 2010 to July 2011, 450 samples (90 raw ground beef and 360 environmental samples) were weekly collected from 90 retail stores in Berisso city, Buenos Aires province, Argentina. Sampling collection was randomly performed and covered all the geographic areas of the city. Environmental samples were obtained from meat contact surfaces such as meat tables, knives, meat mincing machines, and manipulator hands, following the protocol described below. From meat tables, three 400 cm^2 areas were sampled with a sterile sponge soaked in buffered peptone water (Biokar, Zac de Ther, France). The entire surface of the knife blade and the intersection between the blade and the blade handle were sponged. The meat mincing machine was disassembled and the sample was taken from the meat container, the worm meat grinder, and the screw ring. Both hands (front, back, and nails) of the manipulator were sampled. All samples were ice refrigerated and sent to the laboratory to be analyzed immediately.

CULTURE ENRICHMENT, PCR ASSAY, AND ISOLATION

The samples were analyzed by duplicate. One replicate was processed according to US Department of Agriculture, Food Safety and Inspection Service (FSIS) methodology MLG 5.05 for *E. coli* O157:H7 (USDA-FSIS, 2010), and the other for STEC screening. Briefly, a 65 g portion of raw ground beef was placed aseptically in a plastic bag with 585 mL of modified tryptic soy broth (mTSB) with 20 mg/L of novobiocin and casaminoacids (Acumedia Manufacturers, USA). To the sponges 100 ml of mTSB with 20 mg/L novobiocin plus casaminoacids were added. After homogenizing in a stomacher (Interscience, Saint Nom, France), each sample was incubated for 15–22 h at 42°C. All the samples were processed by immunomagnetic separation (IMS) (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions, and plated onto CT-SMAC (Oxoid, Basingstoke, Inglaterra) and SD39 (Acumedia). Suspect colonies were screened for stx_1, stx_2, and rfb_{O157} genes by multiplex PCR (Leotta et al., 2005). For STEC screening 25 g of raw ground beef and environmental samples were incubated at 41.5°C for 15–22 h in 225 mL modified *E. coli* broth (mEC) (Acumedia) and 90 mL of mEC (Acumedia), respectively. One milliliter of the broth culture was taken for DNA extraction, using triton 1% in TE 1×, to be analyzed by an intralaboratory validated SYBR-Green real time PCR analysis (Brusa et al., 2011). The PCR was performed in a 20 µl reaction mixture containing 4 µl of DNA template, 10 µl of PerfeCTa SYBR Green SuperMix, low ROX (Quanta, Biosciences), and 0.2 µl of 100 µM of each primer. Primer sequences were: stx1-F GCAGATAAATCGCCATTCG, stx1-R TGTTGTACGAAATCCCCTCTG, stx2-F CATGACAAC GGACAGCAGTTA, and stx2-R TGTGGATGCATCTCTGGTCA (Operon, Huntsville, AL, USA). Thermal cycle condition was as follows: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 10 s, and 56°C for 30 s, followed by a cycle at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. *E. coli* O157:H7 EDL933 and *E. coli* K-12 were used as positive and negative controls, respectively. One milliliter of PCR-positive samples for stx_1 and/or stx_2 genes were spin down and the pellet was plated onto MacConkey agar (MAC) (Becton Dickinson Co., Sparks, MD, USA) and subsequently, in three consecutive eosin-methylene blue–Levine (Biokard, Zac de Ther, France) agar plates and incubated at 37°C for 18 h. The confluent growth zone onto MAC agar was screened for stx_1 and stx_2 genes by multiplex PCR (Leotta et al., 2005). At least 30 presumptive *E. coli* colonies were selected from any *stx*-positive plate for PCR confirmation. STEC strains were isolated in Trypticase soy agar (TSA) (BD Co.), confirmed by multiplex PCR (Leotta et al., 2005), and stored in Trypticase soy broth (TSB) with 30% and 40% glycerol at −20°C and −70°C, respectively, for further phenotypic and genotypic characterization.

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF ISOLATES

Confirmation of isolates as *E. coli* was performed through biochemical tests according to Ewing and Edwards (1986). In all STEC O157 isolates, stx_1, stx_2, and rfb_{O157} genes were detected by multiplex PCR as described above, while the *eae* (intimin), *ehx*A (enterohemolysin), and $fliC_{H7}$ (H7 flagellin) genes were investigated as described by Karch et al. (1993), Schmidt et al. (1995), and Gannon et al. (1997), respectively.

Virulence factors for STEC non-O157 isolates were detected as previously described for O157, and also *saa* (autoagglutinating adhesin) gene was investigated in intimin-negative strains (Toma et al., 2004). The O and H antigens were determined by a microagglutination technique in plates and by tube agglutination technique, respectively, with an antisera kit (O1-O186) and 56 H antisera produced by Laboratorio de Referencia de *E. coli* (LREC) (Lugo, Spain), as described by Blanco et al. (1997).

RESULTS

PREVALENCE OF O157:H7 AND NON-O157 STEC STRAINS

Among the 90 raw ground beef samples and 360 environmental samples, 25.5% beef and 4.4% environmental samples, were positive for STEC O157:H7 while 52.2% beef and 50.5% environmental samples were positive for non-O157 STEC strains, by the PCR screening (**Table 1**). From the 90 meat tables, 90 knives, 90 mincing machines, and 90 manipulator hands analyzed, 2.2, 3.3, 6.6, and 5.5%, respectively, were positive for STEC O157:H7; while, 55.5, 46.6, 61.1, and 38.8%, respectively, were positive for STEC non-O157 (**Table 1**). Overall, STEC O157:H7 were isolated from 11/23 (47.8%) raw ground beef samples and 6/16 (37.5%) environmental samples ($n = 2$ meat table, $n = 1$ knife, $n = 1$ mincing machine, $n = 2$ manipulator hands) (**Table 1**). STEC non-O157 were isolated from 13/47 (27.6%) raw ground beef samples and 24/182 (13.2%) environmental samples ($n = 9$ meat table, $n = 7$ knife, $n = 6$ mincing machine, $n = 2$ manipulator hands) (**Table 1**).

CHARACTERIZATION OF O157 AND NON-O157 STEC ISOLATES

Fifty-four STEC isolates ($n = 17$ O157, $n = 37$ non-O157) were characterized by phenotypic and genotypic techniques, and then serotyped. STEC O157 characterization proved that all isolates were sorbitol and cellobiose negatives. β-glucuronidase negative and Biotype C (rhamnose + / rafinose + / dulcitol +) was predominant, with only two strains belonging to biotype D (rhamnose + / rafinose + / dulcitol −). All STEC O157 isolates harbored *eae*, *ehx*A, and *fli*C_{H7} genes, while 83.4% were *stx*$_2$, and 16.6% were *stx*$_1$/*stx*$_2$-positive. Although all O157 were *fli*C_{H7}-positive, 6% were non-motile.

All STEC non-O157 strains were sorbitol and ortho-Nitrophenyl-β-galactoside (ONPG) positives. Most of the non-O157 strains were β-glucuronidase positive (73%), and motile (97.3%). Among 37 STEC non-O157 isolates, 20 different serotypes were identified, comprising 12 O serogroups (O8, O41, O44, O49, O79, O113, O116, O130, O171, O174, O178, and O181) and 8 H antigens, being H19 ($n = 16$) and H21 ($n = 6$) the prevalent ones (**Table 2**). Five strains were O-non-typeable and two strains H-non-typeable. The most prevalent serotypes were O8:H19 and O178:H19 (13.5%); O174:H28 (10.8%); O41:H14, O79:H19, O113:H21, O174:H21, and O181:H49 (5.4%). Other serotypes were isolated just one time (**Table 2**).

Different *stx* genotypes occurred among non-O157 strains (**Table 2**). The frequency of *stx* genotypes was *stx*$_2$ (23/37, 62%), *stx*$_1$/*stx*$_2$ (13/37, 35%), and *stx*1 (1/37, 3%). Distinct virulence profiles were also found. The most frequent ones were *stx*$_1$/*stx*$_2$*saa*/*ehxA* (11 strains), *stx*$_2$ (11 strains), and *stx*$_2$/*saa*/*ehx*A (10 strains).

It is interesting to notice that in one beef retail market three different serotypes were isolated from the same raw ground beef (O8:H19, O79:H19, and ONT:H19), also the virulence profile among ONT:H19 isolates were different between them. From another retail market multiple strains could be isolated belonging to three different serotypes. One strain O41:H14 serotype isolated from the ground beef, one strain O8:H19 serotype isolated from the manipulator's hands and two strains O178:H19 isolated from meat table and knife. Also, the same serotype could be isolated from different sources in the same retail market. For example O157:H7 was isolated from ground beef, mincing machine, table meat, and manipulator's hands at the same retail market, O113:H2 was isolated from ground beef and mincing machine at another retail market, O174:H28 was isolated from knife and meat table at another retail market, and O181:H49 was isolated from ground beef and meat table at another retail market.

DISCUSSION

The isolation of STEC from foods is problematic because the bacterium is likely to be present in low numbers, may be sublethally injured and is usually accompanied by large population of competent microflora, including other *E. coli*. The IMS improved the isolation sensitivity of O157 strains at least 100-fold (Wright et al., 1994), but the isolation of STEC non-O157 is still a challenge, since non-O157 STEC strains show great genetic and biochemical diversity, and there is no unique phenotypic marker that can differentiate them from other *E. coli*. At the time the study was conducted, there were no standard methods to detect all the serotypes of STEC non-O157 in meat product, so an enrichment protocol originally used for detecting O157:H7 in meat products by USDA-FSIS was adapted to detect STEC in ground beef and environmental samples (USDA-FSIS, 2010). In order to support the growth of all STEC, novobiocin was not added (Vimont et al., 2007; Kanki et al., 2011) and instead, bile salt No. 3 was added to inhibit gram-positive bacteria (Hussein and Bollinger, 2008). Contamination of STEC in ground beef has been studied by researchers in Botswana, Republic of Ireland, Egypt, the Netherlands, Spain, and Canada samples (Heuvelink et al., 1999; Chapman et al., 2001; Cagney et al., 2004; Magwira et al., 2005; Bohaychuk et al., 2006; Mora et al., 2007; Rhoades et al., 2009) and it has been shown that the prevalence of STEC O157:H7 ranges from 0.4 to 3.7%, while STEC non-O157 ranges from 2.4

Table 1 | Number of STEC PCR-positives samples and strains isolated from different sources.

Sample category	No. of samples analyzed	No. (%) of STEC O157 PCR positive samples	No. (%) of STEC O157 strains isolated	No. (%) of STEC non-O157 PCR positive samples	No. of STEC non-O157 strains isolated
Raw ground beef	90	23 (25.5)	11 (47.8)	47 (52.2)	13 (27.6)
Meat tables	90	2 (2.2)	2 (100)	50 (55.5)	9 (18)
Knives	90	3 (3.3)	1 (33.3)	42 (46.6)	7 (16.6)
Mincing machine	90	6 (6.6)	1 (16.6)	55 (61.1)	6 (10.9)
Manipulator hands	90	5 (5.5)	2 (40)	35 (38.8)	2 (5.7)

Table 2 | Serotypes, source, Shiga toxin genotypes and other virulence markers of the strains studied.

Serotype	No.of strains	Sources					Virulence markers				
		GB	MT	K	MM	MH	*stx*$_1$	*stx*$_2$	*ehx*A	*saa*	*eae*
O157:H7	17	1	1		1		+	+	+	−	+
		10	1	1		2	−	+	+	−	+
O8:H19	5	1	2	1			+	+	+	+	−
		1					−	+	+	−	−
					1		−	+	+	+	−
O41:H14	2	2					−	+	−	−	−
O44:NT HNM	1		1				−	+	−	−	−
O49:H49	1		1				−	+	+	+	−
O79:H19	2	1	1				−	+	+	+	−
O113:H21	2	1			1		+	+	+	+	−
O116:H21	1			1			−	+	+	+	−
O130:H21	1			1			+	+	+	+	−
O171:H2	1	1					+	+	−	−	−
O174:H–	1					1	−	+	−	−	−
O174:H8	1	1					+	−	−	−	−
O174:H21	2	1	1				−	+	−	−	−
O174:H28	4		2	1	1		−	+	+	+	−
O178:H19	6	1	1	1	2		−	+	−	−	−
O181:H49	2	1	1				+	+	+	+	−
ONT:H18	1				1		+	+	+	+	−
ONT:H19	3	1					+	+	+	+	−
		1					+	+	+	−	−
		1					−	+	+	−	−
ONT:HNM	1			1			−	+	+	+	−

GB, ground beef; MT, meat table; K, knife; MM, mincing machine; MH, manipulator hands; ONT, O-untypeable; HNM, non-motile strains; NT, H-untypeable.

to 30% (Hussein, 2007). However, studies analyzing meat contact surfaces were not found. In the present study STEC O157 was isolated in 12.2% of ground beef and 2.2% of environmental samples, while STEC non-O157 was detected in 14.4% and 6.6% of the samples, respectively, although Etcheverría et al. (2010) found 40.74% in ground beef. Comparing these results with those reported by other countries as the USA showing that 0.3% of ground beef samples were positive for STEC O157 (Samadpour et al., 2006), or the United Kingdom that reported a 0.8% prevalence analyzing 6303 samples (Chapman et al., 2001), or Ireland with a 2.8% of positive samples from 1533 ground beefs analyzed (Cagney et al., 2004), the positive rates for STEC O157 reported in this study are much higher than the previously described above. A recent study in the USA showed that 7.3% ground beef samples were positive for STEC non-O157 (Bosilevac and Koohmaraie, 2011). In France, 11% of beef samples were contaminated (Mora et al., 2007), while in Australia the positive rate was 16% (Barlow et al., 2006), and was similar to the results obtained in the present study. However, it is very difficult to compare different studies as geographical locations, sampling procedures, isolation, and detection methods are different and can affect the prevalence data significantly.

Screening for the presence of *stx*$_1$ and *stx*$_2$ by PCR may not have been the best method for the initial determination of prevalence of STEC, since other species of bacteria can possess *stx* genes. The detection of *stx* in samples without performing the corresponding strain isolation is incomplete and is regarded as a presumptive diagnosis, but it is valid for the identification of reservoirs (Scheutz et al., 2001). The difference between O157 and non-O157 isolation rates is notably as observed from isolation results obtained in the present study, STEC O157 could be recovered from approximately the 50% of the beef and 37.5% of environmental samples analyzed, but STEC non-O157 could only be isolated from 27.6% of beef samples and 13.2% of environmental samples. The principal reason is the usage of IMS for O157 strains and the differential phenotypic characteristics that contribute to the identification of these strains. Although Wenting Ju et al. (2012) reported positive rates of 60% using a colony hybridization procedure targeting *stx* genes against 27.6% of isolates obtained in the present work, Bosilevac and Koohmaraie (2011) reported better values using PCR than using colony hybridization. Another difference between non-O157 isolation from ground beef samples and environmental samples lies in the fact that in inert surfaces the bacteria can survive but not multiply, so the number of microorganisms in environmental samples is lower than in the meat, making it harder to be recovered, as it is shown in the present work.

The STEC strains isolated from raw meat did not possess the Locus of Enterocyte Effacement pathogenicity island (LEE PAI) which is most commonly associated with STEC that

cause outbreaks and severe disease. Karmali et al. (2003) have classified STEC into seropathotypes according to their relative incidence, frequency of involvement in outbreaks and their association with severe disease. According to such a scheme, excluding O8:H19 and O113:H21, all the STEC non-O157 strains in this study are characterized as isolates that cause low human disease incidence, are rarely associated with outbreaks and do not cause severe human disease. Despite the high prevalence of STEC strains in ground beef, occurrence of human disease is low because most of the strains isolated from food lack adherence factors such as *eae* and *saa*, which contribute to intestinal colonization and therefore the pathogenicity of the strains. STEC O8:H19 and O113:H21 are important serotypes associated with HUS and HC worldwide, including Argentina (Rivas et al., 2011). They were also recovered from ground beef (Bosilevac and Koohmaraie, 2011) and are one of the most frequent serotypes in Argentine cattle (Masana et al., 2011).

Strains belonging to the same serotype could be isolated from different sources of the same retail market; also, the same serotype could be detected in different stores. However, molecular subtyping of the isolates, such as by pulsed field gel electrophoresis (PFGE), should be done to demonstrate possible clonal relatedness.

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Phage biocontrol of enteropathogenic and shiga toxin-producing *Escherichia coli* in meat products

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Ten bacteriophages were isolated from faeces and their lytic effects assayed on 103 pathogenic and non-pathogenic *Enterobacteriaceae*. Two phages (DT1 and DT6) were selected based on their host ranges, and their lytic effects on pathogenic *E. coli* strains inoculated on pieces of beef were determined. We evaluated the reductions of viable cells of *Escherichia coli* O157:H7 and non-O157 Shiga toxigenic *E. coli* strains on meat after exposure to DT6 at 5 and 24°C for 3, 6, and 24 h and the effect of both phages against an enteropathogenic *E. coli* strain. Significant viable cell reductions, compared to controls without phages, at both temperatures were observed, with the greatest decrease taking place within the first hours of the assays. Reductions were also influenced by phage concentration, being the highest concentrations, 1.7×10^{10} plaque forming units per milliliter (PFU/mL) for DT1 and 1.4×10^{10} PFU/mL for DT6, the most effective. When enteropathogenic *E. coli* and Shiga toxigenic *E. coli* (O157:H7) strains were tested, we obtained viable cell reductions of 0.67 log ($p = 0.01$) and 0.77 log ($p = 0.01$) after 3 h incubation and 0.80 log ($p = 0.01$) and 1.15 log ($p = 0.001$) after 6 h. In contrast, all nonpathogenic *E. coli* strains as well as other enterobacteria tested were resistant. In addition, phage cocktail was evaluated on two strains and further reductions were observed. However, *E. coli* bacteriophage insensitive mutants (BIMs) emerged in meat assays. BIMs isolated from meat along with those isolated by using the secondary culture method were tested to evaluate resistance phenotype stability and reversion. They presented low emergence frequencies (6.5×10^{-7}–1.8×10^{-6}) and variable stability and reversion. Results indicate that isolated phages were stable on storage, negative for all the virulence factors assayed, presented lytic activity for different *E. coli* virotypes and could be useful in reducing Shiga toxigenic *E. coli* and enteropathogenic *E. coli* viable cells in meat products.

Keywords: *Escherichia coli*, bacteriophage, phage biocontrol, bacteriophage insensitive mutant, phage cocktail

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are human pathogens that can cause diarrhea, as well as severe clinical manifestations including hemorrhagic enterocolitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (Su and Brandt, 1995; Griffin et al., 2002; Yoon and Hovde, 2008). STEC produce several virulence factors which contribute to their pathogenicity. Shiga toxins (Stx), AB type toxins that inhibit protein synthesis in target cells, are the most characterized virulence factors (Thorpe et al., 2002). Shiga toxins produced in the intestines by STEC are able to enter the systemic circulation causing severe damage to distal organs. The degree of damage is related to the amount of toxin produced during the infection (Ritchie et al., 2003). STEC synthesize two main types of Shiga toxins encoded by *stx1* and *stx2* genes. Moreover, the enterocyte attaching-and-effacing lesion gene (*eaeA*), which is also present in enteropathogenic strains (EPEC), can contribute to the virulence of STEC. The gene codes for the intimin protein, which allows bacteria to attach themselves to the intestinal epithelium (Frankel et al., 1998).

Foodborne disease-producing *Enterobacteriaceae*, such as *Shigella* spp., *Salmonella* spp., EPEC and STEC, are important etiologic agents of infantile gastroenteritis in Argentina (Binsztein et al., 1999; Rivas et al., 2008). In developing countries, EPEC are the cause of outbreaks of infantile diarrhea with high mortality in children under two years of age. In Argentina, HUS is endemic, with approximately 400 new cases being reported annually by National Health Surveillance System (Rivas et al., 2006), and more than 7000 cases being reported since 1965 (NCASP, 1995). In 2005, the annual incidence of HUS is 13.9 cases/100,000 children under five years of age (Rivas et al., 2006). Recent epidemiological studies showed that there is a sustained global increase in the isolation of non-O157 STEC strains from humans (Tozzi et al., 2003; Brooks et al., 2005; Bettelheim, 2007) and animals (Jenkins et al., 2003; Fernandez et al., 2009), particularly STEC of serogroups O26, O103, and O111 (Ogura et al., 2007).

The therapeutic potential of bacteriophages has been explored since they were discovered by Felix d'Herelle (Summers, 1999). Some of the attributes that make bacteriophages interesting as tools for biological control are: (i) their ability to infect and lyse

specific bacterial target cells and their inability to infect eukaryotic cells; (ii) phages generally do not cross bacterial species or genus barriers, and therefore do not affect desirable microorganisms commonly present in foods, the gastrointestinal tract or the normal bacterial microbiota (Carlton et al., 2005); (iii) phages need a bacterial host in which to multiply and therefore will persist only as long as the sensitive host is present (Clark and March, 2006). The potential of bacteriophages to control food pathogens is reflected in recent studies involving various pathogens including *Campylobacter jejuni* (Atterbury et al., 2003; Bigwood et al., 2008), *E. coli* O157:H7 (O'Flynn et al., 2004; Abuladze et al., 2008) and *Listeria monocytogenes* (Leverentz et al., 2003; Guenther et al., 2009; Holck and Berg, 2009). Several strategies are currently being applied to preserve perishable refrigerated foods and extend their shelf-life. However, physical processes and chemical compounds (preservatives) used for this purpose may alter meat organoleptic properties. Although bacteriophages represent a novel approach, there are no reports of their industrial use to improve safety, even if this "new, ecological, and specific" technology may be cheaper than "older" technologies, since phages can be isolated from the environment and are self-replicating entities. On the other hand, their inclusion into a meat product can be seen as a less aggressive approach.

The aim of this work was to isolate phages with specific lytic capacity for *E. coli* strains in order to determine phage host range and analyze their potential as biocontrol agents for STEC and EPEC strains in beef products.

MATERIALS AND METHODS

BACTERIOPHAGE ISOLATION AND PREPARATION OF STOCKS

E. coli DH5α was used to isolate bacteriophages from fifty stool samples of patients with diarrhea treated at the Centenary Hospital, Rosario. This strain was grown up to an optical absorbance of 1 ($A_{600} = 1$) in 10 mL of Hershey broth (8 g/L Bacto nutrient broth, 5 g/L Bacto peptone, 5 g/L NaCl, and 1 g/L glucose) (Difco, Detroit, MI, USA) supplemented with $MgSO_4$ (5 mM) (Cicarelli, San Lorenzo, Santa Fe, Argentina). A portion of faeces (5 g) was added and the culture was incubated for a further 12 h at 37°C. Next, chloroform (0.5 mL, Cicarelli) was added and the preparation was mixed and centrifuged at 15,000× g for 10 min. The supernatant was then filtered through a 0.45 μm pore size (Gamafil S.A., Buenos Aires, Argentina) (Kudva et al., 1999). Bacteriophage isolation and purification were performed by the double-layer plaque technique (Balagué et al., 2006). Briefly, aliquots of filtrates (10 and 100 μL) were mixed with 100 μL of recipient strain culture ($A_{600} = 1$), three mL of molten soft agar at 45°C (Hershey broth supplemented with 5 mM $MgSO_4$ and 0.7% agar) were added to each suspension and the mixture was poured onto pre-solidified Hershey agar plates and incubated overnight at 37°C. To isolate and purify phages, well-defined single plaques on the soft agar were picked and placed in 5 mL of Hershey medium supplemented with 5 mM $MgSO_4$. Tubes were kept at 4°C for 2 h and then inoculated with 100 μL of recipient strain culture ($A_{600} = 1$). Inoculated tubes were incubated at 37°C with intermittent shaking until complete lysis. Next, chloroform (0.1 mL) was added and cultures were centrifuged at 4000× g for 10 min. Phage stocks were stored at 4°C and enumerated by the double-layer plaque technique (Jamalludeen et al., 2007). These steps were repeated three times. Stability of phage stocks was evaluated after two months of storage at 4°C.

BACTERIOPHAGE AND BACTERIA CHARACTERIZATION

Phage electron micrographs were obtained by the procedure of Bolondi et al. (1995). Phage suspensions were concentrated by centrifugation (1 h, 70,000 × g, 5°C) and subsequently stained with phosphotungstic acid (2% w/v) (Biopack, Buenos Aires, Argentina). Electron micrographs were obtained using a JEOL 1200 EX II electron microscope (INTA Castelar, Buenos Aires, Argentina) operating at 85 kV. Phage morphologies and dimensions (head diameter, tail length, and diameter) were recorded.

Phages and strains of *E. coli* were tested for the presence of toxin-encoding genes (*stx1*, Shiga toxin 1; *stx2*, Shiga toxin 2; *eaeA*, attaching-and effacing; *LT1*, thermolabile toxin and *ST1*, thermostable toxin) of diarrheogenic *E. coli* by the polymerase chain reaction (PCR) using primers detailed in **Table 1** (Pass et al., 2000). PCR conditions were as follows: initial denaturing step at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, annealing at 63°C for 30 s and elongation at 72°C for 30 s, followed by a final step at 72°C for 5 min to achieve complete product elongation. *E. coli* ATCC43889 (*stx2* and *eaeA*), ATCC43890 (*stx1*), and ATCC43895 (*stx1*, *stx2*, and *eaeA*, and also harboring the *stx2* phage, 933W) were used as positive controls, while enterotoxigenic *E. coli* ATCC35401 was used for *LT1* and *ST1* genes. *E. coli* HB101 and ATCC98222 were utilized as negative controls. Amplified products were resolved by electrophoresis using 3% agarose gels in TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.0) (Promega, Madison, WI, USA) at 100 V for 3 h. Gels were stained with ethidium bromide (0.5 μg/mL) (Sigma, St. Louis, MO, USA) and PCR products were visualized under UV light.

BACTERIOPHAGE SPECIFICITY

The host range of each phage was determined by the double layer agar technique using 44 strains isolated from stool

Table 1 | Sequences of primers used in this study.

Gene	Primer[a]	Product size (bp) expected
stx1	fp: 5′-ACGTTACAGCGTGTTGCRGGGATC-3′	121
	bp: 5′-TTGCCACAGACTGCGTCAGTRAGG-3′	
stx2	fp: 5′-TGTGGCTGGGTTCGTTTATACGGC-3′	102
	bp: 5′-TCCGTTGTCATGGAAACCGTTGTC-3′	
eaeA	fp: 5′-TGAGCGGCTGGCATGATGCATAC-3′	241
	bp: 5′-TCGATCCCCATCGTCACCAGAGG-3′	
LT1	fp: 5′-TGGATTCATCATGCACCACAAGG-3′	360
	bp: 5′-CCATTTCTCTTTTGCCTGCCATC-3′	
ST1	fp: 5′-TTTCCCCTCTTTTAGTCAGTCAACTG-3′	160
	bp: 5′-GGCAGGACTACAACAAAGTTCACAG-3′	

[a] *fp, forward primer; bp, backward primer. stxl and stx2: Shiga toxin1 and 2 encoding genes; eaeA: intimin encoding gene; LTl and STl: thermolabile and thermostable toxins encoding genes.*

samples, and urine cultures (uropathogenic *E. coli*, UPEC). Stool and urine samples were streaked in Cystine Lactose Electrolyte Deficient (CLED) agar plates. Simmons citrate agar test was performed on growing lactose positive colonies. After incubation for 24 h at 35°C, only lactose positive and citrate negative colonies were further identified using API system (Biomerieux, Bs. As., Argentina). Sixteen *E. coli* strain from food (Balagué et al., 2006), one uropathogenic *E. coli* strain (*E. coli* T149) which expresses fimbriae P and α-hemolysin (Balagué et al., 2004) and five ATCC *E. coli* strains were also tested (ATCC 43890; 43889; 43895; 35401 and 98222). Previously characterized (API system) isolates from stool samples were also tested: *Shigella flexneri*, *S. sonnei*, *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Salmonella* Typhi and *Salmonella* Typhimurium. Strains tested against stock phages are listed in **Table 2**. Bacteriophage sensitivity was assayed by placing 10 μL of phage suspension on the solidified soft-agar layer inoculated with 100 μL of each bacterial culture, incubated for 24 h at 37°C, and the presence of lysis zones or plaques was examined (Goodridge et al., 2003).

MEAT ASSAYS

Beef from cow hindquarter purchased from retail was aseptically cut into pieces (1 cm^2 of surface and 0.4 cm thick), placed in petri dishes and pre-equilibrated to 5 or 24°C. The required pH was obtained by washing with sodium chloride-magnesium sulfate (SM) buffer (0.05 M TRIS, 0.1 M NaCl, 0.008 M $MgSO_4$, 0.01% w/v gelatin, pH = 7.5) prior to inoculation with bacteria and phage. Host strains employed in this study, namely non-O157 STEC (ARG4827; serogroup O18; harboring *stx*1 and *stx*2 genes) (Balagué et al., 2006), O157:H7 STEC (464; harboring *stx*1 and *eaeA* genes) and an EPEC (EPEC920; which harbors *eaeA* gene), were grown in Hershey medium supplemented with $MgSO_4$ (5 mM) for 12 h at 37°C. Bacterial strains and specific bacteriophages added to the meat samples are detailed in **Table 3**. Twenty μL of each diluted bacterial suspension (ranging from 5.9×10^5 to 3.9×10^7 CFU/mL) were pipetted onto the surface of each meat piece and allowed to attach for 10 min at room temperature. Another 20 μL of each bacteriophage were then pipetted on the meat, at low multiplicity of infection (MOI), 1.7×10^9 PFU/mL for DT1 and 1.4×10^9 PFU/mL for DT6, or high MOI, 1.7×10^{10} PFU/mL for DT1 and 1.4×10^{10} PFU/mL for DT6. Pieces of meat were also added with SM buffer (pH 7.5), instead of phage suspension, as controls. At 3, 6, and 24 h, meat pieces were transferred to a sterile bag, 5 mL SM buffer were added and samples processed for 2 min in a Stomacher (Seward, London, UK). A 1 mL portion of the stomacher fluid was transferred to a sterile tube and cells were pelleted by centrifugation at 3000× *g* for 10 min. The supernatant was removed and cells were resuspended in 1 mL SM buffer. Finally, a 0.1 mL sample was removed, serially diluted (10^2–10^4-fold) in SM buffer and 0.1 mL volumes of each dilution were plated on Hershey agar for viable cell enumeration (Bigwood et al., 2008). Phage cocktail (DT1 and DT6 in equal proportions) was assayed on *E. coli* DH5α (indicator strain used for phage isolation) and in O157:H7 STEC (464) using the methodology employed for each individual phage described above. Three replicates were performed for each treatment and one meat piece processed for replicate. Uninoculated controls were tested to determine the presence of naturally occurring bacteriophages. Plaques (PFU/mL) were evaluated by the double layer agar technique (Jamalludeen et al., 2007).

Table 2 | Strains tested against stock phages.

Source	Strains (amount)	Strains characteristics/ description
Food	*Escherichia coli* (10)	8 non-O157 STEC and 2 O157:H7 STEC
Stool sample	*Escherichia coli* (9)	4 O157:H7 STEC and 5 EPEC
	Escherichia coli (18)	Non-pathogenic
	Shigella spp. *Salmonella* spp. *Proteus mirabilis* *Citrobacter freundii* *Klebsiella pneumoniae* (17)	Other enterobacteria
Urine culture	*Escherichia coli* (17)	UPEC
ATCC	*Escherichia coli* (5)	35401; 43889; 43890; 43895 and 98222

EPEC, enteropathogenic E. coli; O157:H7 STEC, O157:H7 Shigatoxigenic E. coli; non-O157 STEC, Shigatoxigenic non-O157 E. coli; UPEC, urophatogenic E. coli; ATCC, american type culture collection.

BACTERIOPHAGE INSENSITIVE MUTANTS (BIMs) ISOLATION

Bacteriophage insensitive mutants (BIMs) were isolated by the secondary culture method described by Guglielmotti et al. (2007) with some modifications. *E. coli* sensitive strains (one EPEC, three O157:H7 STEC and one non-O157 STEC) ($A_{600} = 0.2 - 0.3$) were infected with a phage suspension at different infection ratios (multiplicity of infection, MOI of ≈ 10 and 1), incubated in Hershey broth at 37°C for 24 h and observed visually until complete lysis. An uninfected culture of each *E. coli* strain was used as a control. Cultures exhibiting complete and delayed lysis were the best candidates to isolate BIMs. After lysis, further incubation for 48 h at 37°C was required for secondary growth. Each tube with secondary growth was spread on Hershey agar plates for colony isolation.

BIMs were isolated from meat as described in meat assays methodology described above modified with an extended incubation time (48 h) at 37°C. For both of the aforementioned methodologies, after incubation of agar plates, eight different colonies were randomly isolated (on agar plates) and cultured overnight in Hershey broth at 37°C. These isolates were purified by three consecutive streakings on Hershey agar plates. The growing colonies were isolated as presumptive BIMs.

BIMs CONFIRMATION

Presumptive BIMs were confirmed by a liquid culture sensitivity test (Guglielmotti et al., 2007). Briefly, a log-phase culture

Table 3 | *E. coli* viable cell logarithmic reductions after phage treatment of contaminated meat products.

Phage stock/sensitive strain	Assay conditions		Log reduction in *E. coli* viable cells[a] after the incubation time (h)[b]		
	T (°C)	MOI	3	6	24
DT1/EPEC (920)	5	4.4×10^2	NS	**0.80 ± 0.14	NS
	24	4.8×10^2	**0.30 ± 0.05	NS	NS
	5	4.4×10^1	NS	**0.49 ± 0.09	NS
	24	4.8×10^1	NS	NS	**0.46 ± 0.08
DT6/EPEC (920)	5	5.2×10^2	**0.67 ± 0.12	**0.59 ± 0.11	*0.46 ± 0.15
	24	6.5×10^3	*0.32 ± 0.09	NS	NS
	5	5.2×10^1	NS	*0.30 ± 0.08	NS
	24	6.5×10^2	NS	NS	NS
DT6/non-O157 STEC (ARG4827)	5	2.4×10^4	*0.33 ± 0.09	*0.47 ± 0.12	*0.56 ± 0.17
	24	4.0×10^2	*0.43 ± 0.13	NS	NS
	5	2.4×10^3	NS	*0.37 ± 0.09	*0.50 ± 0.16
	24	4.0×10^1	*0.35 ± 0.11	NS	NS
DT6/O157:H7 STEC (464)	5	2.3×10^3	*0.59 ± 0.16	**0.86 ± 0.15	*0.38 ± 0.10
	24	5.8×10^3	**0.77 ± 0.14	***1.15 ± 0.12	NS
	5	2.3×10^2	*0.38 ± 0.09	*0.62 ± 0.18	NS
	24	5.8×10^2	NS	**0.74 ± 0.13	NS
Cocktail/DH5α	5	2.25×10^4	*0.91 ± 0.19	**2.16 ± 0.20	**2.23 ± 0.21
	24	1.75×10^4	*0.66 ± 0.15	NS	NS
Cocktail/O157:H7 STEC (464)	5	1.56×10^5	NS	NS	NS
	24	3.33×10^5	**1.43 ± 0.24	**2.58 ± 0.21	**2.20 ± 0.22

*MOI, multiplicity of infection (PFU/CFU); NS, not significant. Mean values of treated and control samples not significantly different using the scheffé method (*significant at p = 0.05; **significant at p = 0.01; ***significant at p = 0.001).*

[a] *Log reduction in E. coli viable cells with respect to phage-free control.*

[b] *Mean of three data points ± standard deviations.*

($A_{600} = 0.2 - 0.3$) of each presumptive BIM in Hershey broth was infected with the phage suspension at various MOI (≈ 10 and 1). Uninfected cultures of each *E. coli* strain were used as controls. BIMs cultures were incubated in Hershey broth at 37°C until growth of control strains was evident. Infected cultures that did not lyse at the first attempt were subcultured again. Each second subculture was prepared by transferring 2–3% of the final volume from the first culture to another test tube with 1 mL of fresh broth. When no bacterial lysis was evident, the resulting culture was stored at 4°C and subcultured under the same conditions. Presumptive BIMs that survived the third subculture were considered to be confirmed BIMs. Sensitivity of each parent strain (sensitive) was always determined in parallel to ensure lytic activity of phage suspensions.

DETERMINATION OF BACTERIOPHAGE-INSENSITIVE MUTANT FREQUENCY, REVERSION, AND STABILITY

The emergence frequency of BIMs was evaluated by mixing the appropriate volume of an overnight culture of each strain (EPEC920 and O157:H7 STEC 464) and phage suspension (DT1 and DT6) to obtain a MOI of 100. The bacterium–phage mixture was supplemented with $MgSO_4$ (5 mM), plated by the double-layer agar technique and incubated overnight at 37°C. BIM frequency was estimated as the ratio of the number of confirmed BIM to the initial bacterial number. All the experiments were performed in duplicate. Selected BIMs were propagated through 50 generations at 37°C and then checked by a plaque assay to evaluate reversion to phage sensitivity (O'Flynn et al., 2004).

Phage resistance stability was assayed by seven sequential subcultures of 2% portions of BIM cultures (Hershey broth) with independent addition of phage at each subculture (Guglielmotti et al., 2007). The loss of phage resistance was determined by comparing lysis of BIM culture with the control (mutant subculture without phage addition). The subculture where lysis first occurred was recorded.

STATISTICAL ANALYSIS

Means and standard deviations for data sets were calculated. Differences between means for control (untreated) and treated samples were compared by the Scheffé method and Origin 6.0 for graphics. Differences were considered statistically significant when p-values were <0.05.

RESULTS

ISOLATION, VIABILITY, AND CHARACTERISTICS OF BACTERIOPHAGE STOCKS

A total of 10 bacteriophage stocks were obtained from diarrheic stool samples, titred and stored at 4°C. **Figure 1** shows plaques produced by DT1 and DT6 on the EPEC920 and O157:H7 STEC strains. Phage concentration ranged between 1.2×10^{10} PFU/mL and 4.8×10^{10} PFU/mL. The viability of each bacteriophage stock stored at 4°C was evaluated after two months and similar titers were obtained, suggesting that the storage method used was adequate. Electronic microscopy allowed us to infer that bacteriophages DT1 and DT6, could taxonomically belong to T-even type of the *Myoviridae* family. Phages DT1 and DT6 had icosahedral heads and contractile tails. DT1 dimensions were of 89.3 nm (head diameter), 127.8 nm (tail length), 20.8 nm (tail thickness), and a total length of 217.1 nm; for DT6 measures were of 82.1 nm (head diameter), 125.7 nm (tail length), 17.7 nm (tail thickness), and 207.8 nm (total length) (**Figure 2**). PCR determinations of virulence factors (Stx1; Stx2; ST1; LT1 and Intimin) were negative for all bacteriophage stocks.

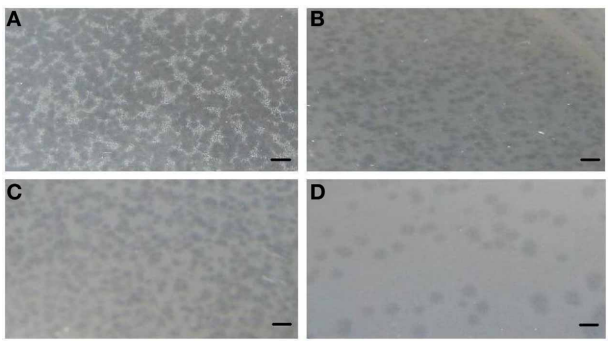

FIGURE 1 | Plaques produced by DT1 and DT6 on EPEC920 (A and B, respectively) and O157:H7 STEC strains (C and D, respectively). Bars represent 1 cm.

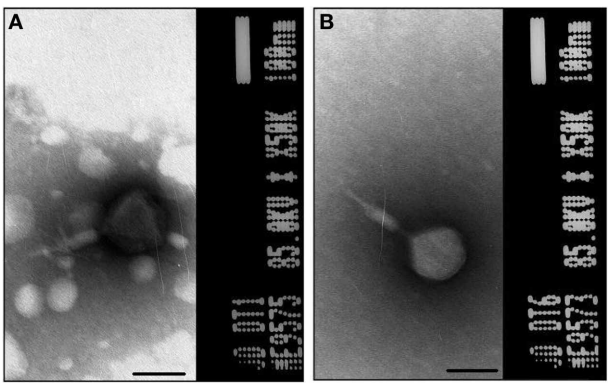

FIGURE 2 | Electron micrograph of phages DT1 (A) and DT6 (B) negatively stained with 2% phosphotungstic acid. Dimensions are given in the text. Bars represent 100 nm.

BACTERIOPHAGE SPECIFICITY

Non-*E. coli* and non-pathogenic *E. coli* strains were resistant to the lytic action of phages. Among the pathogenic *E. coli*, six (6) *E. coli* O157:H7 STEC, three (3) non-O157 STEC, five (5) EPEC, and two (2) UPEC strains were sensitive to phages evaluated (**Table 4**).

BIOCONTROL TESTS ON MEAT PRODUCTS

Two bacteriophages, which formed clearly defined plaques, with different host range were selected. The phage with the narrower range (DT1) was tested on an EPEC strain while the phage with the broader range (DT6) was tested on the same EPEC strain assayed previously and two STEC strains, one O157:H7 and one non-O157.

Significant decreases ($p = 0.05$) in viable cells (VC) for all tested strains were observed in comparison to the phage-free controls. **Table 3** shows log VC significant reductions ($p < 0.05$) at high MOI in EPEC920, non-O157 STEC and O157:H7 STEC strain/DT6 systems after 3-h incubation, and in all phage/strain systems after 6-h incubation at 5°C. At high MOI values, VC reduction were still significant ($p = 0.05$) after 24 h of DT6 phage exposure. When the test was carried out at 24°C, significant VC reduction ($p < 0.05$) were observed after 3 h for DT1/EPEC920, DT6/O157:H7 STEC, DT6/EPEC920, and DT6/non-O157 STEC. These reductions were not maintained after 6 or 24 h, with the exception of the 1.15 log reduction obtained for DT6/O157:H7 STEC after 6 h.

When the results obtained with different MOIs were compared, significant differences ($p = 0.05$) at both temperatures were observed for most phages/bacterium combinations. Reductions in a phage/bacterium system were higher at the highest temperature, up to 0.29 log (1.15 log – 0.86 log) CFU for

Table 4 | Host range of stock phages.

Phage stock	*E. coli* sensitive strains	Total sensitive strains
DT1	2 EPEC; 1 O157:H7 STEC; 1 non-O157 STEC	4
DT2	2 EPEC; 3 O157:H7 STEC	5
DT3	4 EPEC; 6 O157:H7 STEC; 3 non-O157 STEC	13
DT4	4 EPEC; 5 O157:H7 STEC; 3 non-O157 STEC	12
DT5	2 EPEC; 3 O157:H7 STEC	5
DT6	4 EPEC; 6 O157:H7 STEC; 3 non-O157 STEC	13
LM1	2 EPEC; 4 O157:H7 STEC; 1 non-O157 STEC	7
LM2	1 EPEC; 1 O157:H7 STEC; 2 non-O157 STEC	4
LM3	2 EPEC; 3 O157:H7 STEC; 2 non-O157 STEC; 1 UPEC	8
LM4	1 EPEC; 2 O157:H7 STEC; 2 non-O157 STEC; 1 UPEC	6

EPEC, enteropathogenic E. coli; O157:H7 STEC, O157:H7 Shigatoxigenic E. coli; non-O157 STEC, Shigatoxigenic non-O157 E. coli; UPEC, uropathogenic E. coli.

DT6/O157:H7 STEC at 6 h, with exception in a single case for the DT6/EPEC920 system, after 3-h incubation.

Phage stocks displayed different host range (**Table 4**), some having limited and others having a broader range. The lytic ability of phages at different MOI values and temperatures is shown in **Figure 3**. The biocontrol effectiveness against EPEC920 shown by DT1 (**Figures 3A,B**) and DT6 (**Figures 3C,D**) phages was different only at the lower temperature. At 5°C, DT1 produced a greater reduction in the number of VC after 6-h incubation than DT6, but DT6 biocontrol over EPEC920 was faster and prolonged. At 24°C there was no difference in the lytic effects of phages with the exception at the longer incubation time and lower MOI (4.8×10^1 PFU/CFU) where DT1 was more effective. DT6 was also active against O157:H7 strains; being more effective at 24°C (**Figure 3F**) though with a prolonged lytic action at 5°C (**Figure 3E**).

The phage cocktail successfully reduced DH5α VC only at 5°C, while for O157:H7 STEC reductions took place only at 24°C (**Figure 4**). DH5α was significantly reduced at 3, 6, and 24 h, being 2.23 log the major reduction value obtained at 24 h. For O157:H7 STEC, VC reductions up to 2.58 log at 6 h were observed, in addition, phage cocktail was able to achieve an effective and prolonged biocontrol effect (2.20 log at 24 h) (**Table 3**).

ISOLATION AND REVERSION OF BIMs

After incubating meat inoculated with phage/bacteria systems at 24°C for 24 h, significant bacterial growth was observed. To assess whether bacterial re-growth on meat experiments may be due to resistance, eight (8) potential BIMs were isolated from meat incubated for 48 h at 37°C and only 2 (25%) were confirmed as BIMs by the liquid culture sensitivity test method. Another eight (8) presumptive BIMs were isolated by the secondary culture method and 6 (75%) were confirmed. BIM frequencies observed for all the evaluated strains were low, ranging from 6.5×10^{-7} to 1.8×10^{-6} (**Table 5**).

One BIM, which could form a lawn, was found to be susceptible to phage DT6. BIMs evaluated for phage DT1 were resistant and showed no reversion to phage sensitivity. Mutants isolated from EPEC920 maintained their phenotype up to the fifth subculture. Mutants isolated from O157:H7 STEC maintained resistance up to the fourth subculture and only one was resistant at the seventh subculture.

DISCUSSION

Biocontrol by bacteriophages in meat products was developed in the last years and their recent "generally recognized as safe" (GRAS) designation and approval as food additives (FDA, 2006) has opened the discussion about "edible virus." Phages are naturally present in significant numbers in water and food (Sulakvelidze, 2011). In fresh meats and meat products more than 10^8 viable bacteriophages per gram may be present (Kennedy and Bitton, 1987), implying that phages are commonly consumed in large numbers. In addition, bacteriophages are especially abundant in the gastrointestinal tract (Breitbart et al., 2003; Hitch et al., 2004). However, most phage-host systems are highly specific, which is a general limitation (Carlton et al., 2005). Phage must be lytic and non-transducing as minimum requirements to ensure safety (Greer, 2005). Nevertheless, application of bacteriophages is a non-destructive, natural, self-perpetuating biocontrol method and can be as efficient as chemical agents for controlling specific bacterial pathogens (Leverentz et al., 2001). On the other hand, the high specificity of phages allows direct application onto a product or the possibility to use bacteriophages as "food additives," mixing them with foods without affecting their quality (Jay, 1996), hygiene and other normal microbiota viability of the food (Kudva et al., 1999) or the consumers' (Chibani-Chennoufi et al., 2004).

Previous studies have demonstrated the feasibility of isolating phages that specifically lyse O157:H7 *E. coli* strains (Sulakvelidze and Barrow, 2005; Raya et al., 2006). O'Flynn et al. (2004) developed a three-phage cocktail that was effective for reducing the numbers of *E. coli* O157:H7 on meat products. On the other hand, in a similar experiment, Dykes and Moorehead (2002) found no effect of bacteriophage for control of *Listeria monocytogenes* development on contaminated beef, however, this treatment did not yield an appreciable reduction due to the low MOI used. Also, phage-mediated reductions of bacterial cell viability have been reported in various food matrices like contaminated melon (Leverentz et al., 2003) and cheese (Carlton et al., 2005; Bueno et al., 2012).

Phages isolated in this study were characterized by their host range, electron microscopy (DT1 and DT6), and PCR analysis. The host range evaluation demonstrated that all O157:H7 strains, most non-O157 STEC and some EPEC isolated from diarrheic faeces or food, were sensitive to one or several bacteriophages. In contrast, no nonpathogenic strain was affected. These finding suggest the possibility of using phages for food conservation without altering the gastrointestinal tract normal microbiota. However, a broader host range needs to be assessed to ensure safety for commensal bacteria. Phages did not contain genes encoding *stx1*, *stx2*, *eaeA*, *LT1* and *ST1*, but other virulence factors and phage-encoded genes may contribute to bacterial virulence, so further sequencing and bioinformatic analysis are required to ensure they are benign prior to their use as biocontrol tools.

DT1 and DT6 on meat, gave statistically significant VC reductions, comparable to those previously reported (O'Flynn et al., 2004). EPEC920 was tested with both phages individually; at 5°C DT6 produces a minor reduction in VC count but a rapid and prolonged effect in time. This observation may be related to different adsorption rate and lysis time distinctively influenced by environmental conditions (Shao and Wang, 2008). Higher reductions were observed (for O157:H7 STEC) at the higher temperature (24°C), probably due to the active growth of bacteria allowing an efficient bacteriophage replication. Similar results at 5 and 24°C for *Salmonella* and *Campylobacter* were described by Bigwood et al. (2008). The biocontrol effect for all phages analyzed was found to be dose-dependent, with the highest phage concentration being the most effective, as was also found by Leverentz et al. (2001). Our findings showed that phage-bacteria interaction on meat surfaces was significantly influenced by the initial number of inoculated phage and bacteria. Atterbury et al. (2003) obtained marginal reductions of approximately 1 log CFU only with high initial bacterial density of *C. jejuni* (4–6 log CFU). The requirements for a relatively high threshold density

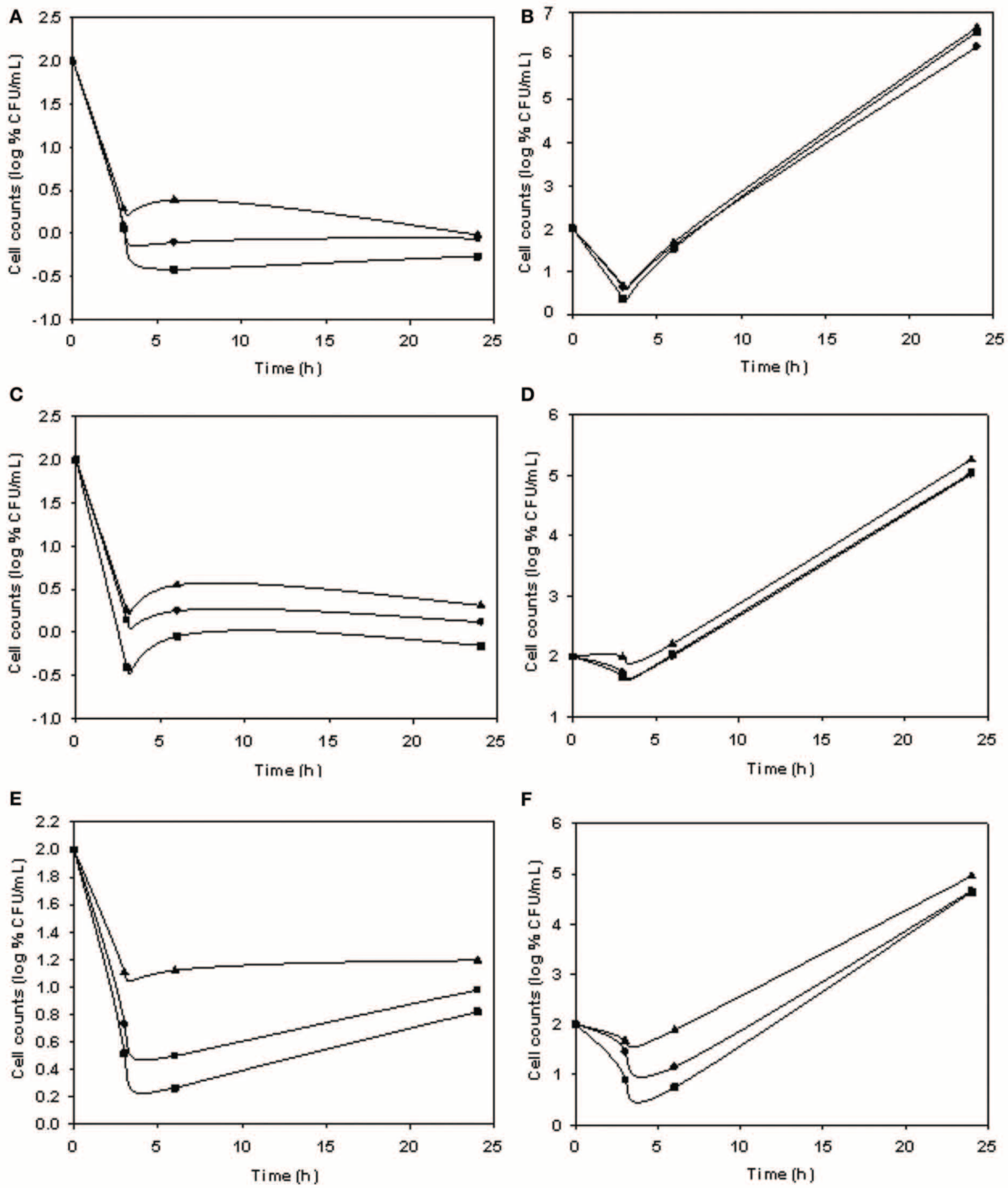

FIGURE 3 | Comparison of the lytic ability of phages at different MOI and temperatures. Symbols represent phage free controls (▲), low MOI (●), and high MOI (■). Cell count (CFU/mL) using as matrix inoculated meat with DT1/EPEC920 at 5°C **(A)** and 24°C **(B)**, DT6/EPEC920 at 5°C **(C)** and 24°C **(D)** and DT6/O157:H7 STEC at 5°C **(E)** and 24°C **(F)**.

of bacterial host cells may limit the impact of phages on bacteria, constituting an important impediment to phage biocontrol (Greer, 2005). In addition, there are several factors influencing inactivation on food, the most relevant in our experiments is the food matrix ability to absorb liquid from the phage suspension. This is a decisive parameter which physically limits the distribution of phage particles in order to reach all targeted bacteria. Therefore, decreased effectiveness of these treatments

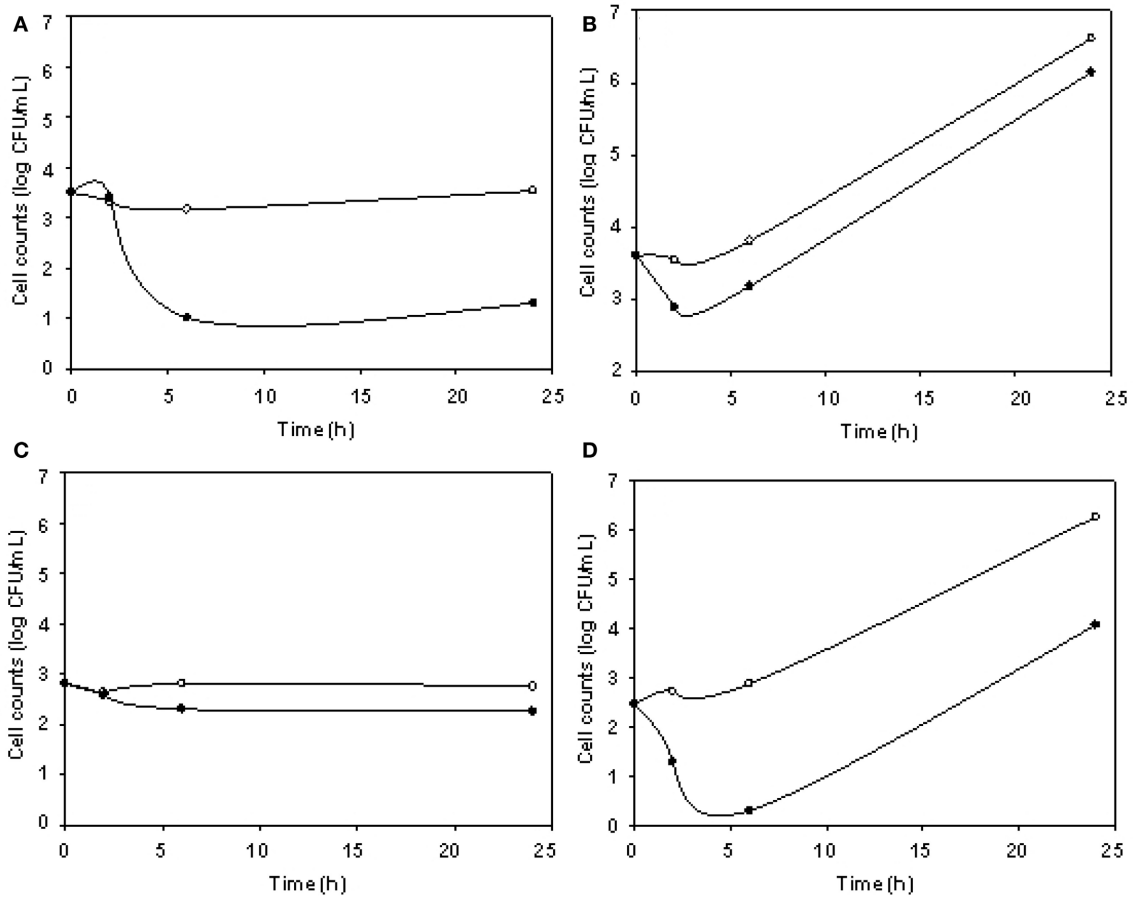

FIGURE 4 | *E. coli* viable cell counts (CFU/mL) in absence (○) and presence (●) of phages (cocktail) in meat products. Cocktail/DH5α at 5°C **(A)**, cocktail/DH5α at 24°C **(B)**, cocktail/O157:H7 STEC at 5°C **(C)**, and cocktail/O157:H7 STEC at 24°C **(D)** systems.

Table 5 | *In vitro* and meat BIM isolation and determination of BIM frequency.

Source of BIMs	Presumptive BIMs tested	Confirmed BIMs (%)	BIM frequency (mean ± SD)[a]
Secondary culture	8 EPEC	6 (75.0)	EPEC/DT1 = $8.7 \times 10^{-7} \pm 3.9 \times 10^{-8}$
			EPEC/DT6 = $6.5 \times 10^{-7} \pm 4.8 \times 10^{-8}$
Meat	8 O157:H7	2 (25.0)	O157:H7 STEC/DT1 = $1.8 \times 10^{-6} \pm 1.5 \times 10^{-7}$
	STEC		O157:H7 STEC/DT6 = $1.3 \times 10^{-6} \pm 9.4 \times 10^{-7}$

[a] *SD, standard deviation. Means and SD were calculated from duplicate experiments.*

EPEC, enteropathogenic E. coli; O157:H7 STEC: O157:H7 Shigatoxigenic E. coli.

may be partially due to limited diffusion and contact between bacteria and phage particles. Moreover, targeted bacteria may be embedded within the rather complex food matrix, thereby shielding them from phage particles. On these grounds, a greater biocontrol effect may be achieved by modifying phage application, e.g., by using larger liquid volumes to avoid total liquid absorption. While the results were statistically significant we cannot ascertain the practical use of these phages individually on meat products, mainly due to the 1.15 log reduction obtained at best. However, the phage cocktail (containing DT1 and DT6) at a higher MOI was able of further reduce viable cell counts of O157:H7 STEC (464) than individual phages. This indicates that phages may be of practical value if cocktails contain higher numbers of different phages (to reduce BIMs emergence and expand cocktail host range) and higher MOI values, since others authors report that working with higher phage concentrations generally resulted in greater inactivation (Guenther et al., 2009). In addition, DH5α indicator strain was only significantly reduced at 5°C, this reduction may be caused by lysis from without since DH5α has a shorter lipopolysaccharide (LPS), thereby being more permissive and susceptible to this mechanism, while for O157:H7 STEC (464) at 5°C this mechanism was not observed and

no reduction was obtained. This may rather be due to the absence of bacterial growth necessary for phage replication. At 24°C, DH5α cell number was not reduced (only after a 3 h of incubation a 0.66 log reduction was observed) and re-growth was observed. This recovery in cell number may be due to cells escaping phage treatment by limited phage diffusion and a subsequent multiplication in addition to BIMs emergence.

In our trials we found an increase in VC number after 24 h at 24°C, suggesting that there is a potential phage-resistant variant selection under these testing conditions. Thus, we propose to analyze the existence of *spontaneous bacteriophage-insensitive mutants (BIMs)*, naturally present in bacteria, in order to evaluate if they could prevent the use of phages to improve food safety. Other authors have also reported a subsequent bacterial growth during the *in vitro* challenge test (O'Flynn et al., 2004). BIMs emergence, which could compromise the efficacy of a phage treatment, is often associated with point mutations in genes encoding receptor molecules on the bacterial cell surface and commonly revert to phage sensitivity rapidly (Garcia et al., 2007). Kudva et al. (1999) propose that low temperatures and absence of bacterial growth favor phage adsorption and infection. In contrast, higher temperatures, cell growth, and the potential for phenotypic variability in expression of the O-antigen favor survival of phage-resistant cells. We were able to isolate *E. coli* BIMs from meat assays and, at a higher rate, when using the secondary culture method. BIMs isolated in this study were tested to evaluate resistance phenotype stability and reversion. As previously reported for other systems (O'Flynn et al., 2004), all these BIMs present low emergence frequency, variable stability, and reversion, namely, mutants resistant to phage DT6 revert while those resistant to DT1 show no reversion to phage sensitivity. These findings suggest that *E. coli* BIMs should not prevent the use of phages as biocontrol tools, mainly due to the low emergence frequency observed for all phages evaluated. In our trials phage cocktail reduced the appearance of presumptive-BIMs, however, further studies are required in order to evaluate the optimal conditions allowing the reduction of BIM emergence such as adding more different phages in the cocktail mixture.

We have isolated 10 bacteriophages belonging to the T-even type of the *Myoviridae* family. Phages isolated in this study were negative for all the virulence factors assayed and presented lytic activity for different *E. coli* virotypes. In addition, all non-pathogenic strains evaluated were not affected. BIMs isolated by exposure to DT1 and DT6 showed low emergence frequency and in *in vitro* challenge tests VC reductions were highly significant (up to 6.3 log units) (data not shown). However, on pieces of beef assayed, reduction obtained with individual phages was low and the phage cocktail showed greater reductions although lower than expected.

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Development of a multiplex PCR assay for detection of Shiga toxin-producing *Escherichia coli*, enterohemorrhagic *E. coli*, and enteropathogenic *E. coli* strains

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Escherichia coli O157:H7 and other pathogenic *E. coli* strains are enteric pathogens associated with food safety threats and which remain a significant cause of morbidity and mortality worldwide. In the current study, we investigated whether enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), and enteropathogenic *E. coli* (EPEC) strains can be rapidly and specifically differentiated with multiplex PCR (mPCR) utilizing selected biomarkers associated with each strain's respective virulence genotype. Primers were designed to amplify multiple intimin (*eae*) and long polar fimbriae *(lpfA*) variants, the bundle-forming pilus gene *bfpA*, and the Shiga toxin-encoding genes *stx1* and *stx2*. We demonstrated consistent amplification of genes specific to the prototype EHEC O157:H7 EDL933 (*lpfA1-3*, *lpfA2-2*, *stx1*, *stx2*, and *eae-γ*) and EPEC O127:H6 E2348/69 (*eae-α*, *lpfA1-1*, and *bfpA*) strains using the optimized mPCR protocol with purified genomic DNA (gDNA). A screen of gDNA from isolates in a diarrheagenic *E. coli* collection revealed that the mPCR assay was successful in predicting the correct pathotype of EPEC and EHEC clones grouped in the distinctive phylogenetic disease clusters EPEC1 and EHEC1, and was able to differentiate EHEC1 from EHEC2 clusters. The assay detection threshold was 2×10^4 CFU per PCR reaction for EHEC and EPEC. mPCR was also used to screen Argentinean clinical samples from hemolytic uremic syndrome and diarrheal patients, resulting in 91% sensitivity and 84% specificity when compared to established molecular diagnostic procedures. In conclusion, our mPCR methodology permitted differentiation of EPEC, STEC and EHEC strains from other pathogenic *E. coli*; therefore, the assay becomes an additional tool for rapid diagnosis of these organisms.

Keywords: Shiga toxin-producing *E. coli*, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, *E. coli* O157, diagnostics

INTRODUCTION

Rapid diagnosis of pathogenic *E. coli* strains is an increasingly important issue to address in public health. Infections with Shiga toxin-producing *E. coli* (STEC) and among those enterohemorrhagic *E. coli* (EHEC), can result in abdominal cramping and diarrhea (with or without blood). A small percentage of patients can progress to a more severe and often fatal condition called hemolytic uremic syndrome (HUS). STEC/EHEC strains are found in industrialized nations as well as developing countries and typical cases in the U.S. are associated with food-borne contamination. Enteropathogenic *E. coli* (EPEC) is frequently associated with outbreaks of infantile diarrhea in developing nations (Orskov et al., 1990), and is a contributor to diarrheagenic illnesses in human populations around the world (Ochoa et al., 2008).

Shiga toxin-producing *E. coli*/EHEC and EPEC strains encode a number of virulence factors in a chromosomally located pathogenicity island termed the locus for enterocyte effacement (LEE; McDaniel et al., 1995). Intimate adhesion of STEC/EHEC and EPEC to enterocytes is mediated in part by LEE-encoded intimin gene (*eae*), resulting in the formation of an attaching and effacing (A/E) lesion on the surface of the intestinal cells. In addition to virulence factors encoded in the LEE pathogenicity island, EHEC and EPEC possess one or more of the chromosomally encoded long polar fimbriae (*lpf*) loci. Together with intimin, Lpf is the only other well-characterized colonization factor of EHEC O157:H7 (Torres et al., 2002, 2004, 2007). Our group conducted an extensive study involving A/E-producing bacterial collections from Europe and South America and demonstrated a correlation between *lpf* genes and different genetic variants of the intimin genes in A/E-producing *E. coli* (AEEC; Torres et al., 2009). The *lpf* genes are also widely distributed throughout pathogenic and some commensal populations of *E. coli* and can be categorized

into distinct allelic variants (Galli et al., 2010; Gomes et al., 2011). Additionally, other groups have also reported the potential use of intimin for diagnostics based on the correlation of intimin type and lineage of STEC/EHEC and EPEC strains (Tarr and Whittam, 2002; Zhang et al., 2002; Jores et al., 2003). Due to the broad distribution of *lpf* and *eae* genes in AEEC and their association with these pathogenic *E. coli* strains, the *lpf* and *eae* subtypes could be used to genetically identify and distinguish diverse STEC/EHEC and EPEC serogroups (Torres et al., 2009). From a clinical point of view, the inclusion of primers for *stx* genes is critical, as the progression to HUS is strongly influenced by the presence of Shiga toxin (Friedrich et al., 2002; Brooks et al., 2005; Hedican et al., 2009). Further, the *bfpA* gene can be used as marker to detect typical (bfp^+) and atypical (bfp^-) EPEC strains (Nataro and Kaper, 1998).

Current proposed approaches for the specific detection of EHEC strains in clinical samples or food matrices are focused on the detection of genes present in a limited number of serotypes. For example, the inclusion of the *E. coli* O157:H7 O-antigen marker $rfbE_{O157}$ limits the detection of strains to O157 serogroups (Bai et al., 2010; Gordillo et al., 2011). Analysis of multiple O-group genes improves the detection capabilities of an assay, but does not remove the constraint of detecting only known serogroups (Madic et al., 2011). Similar constraints are present when targeting the H7 *fliC* flagellar antigen (Madic et al., 2010; Gordillo et al., 2011) or when employing O157 strain-specific methodologies (Ooka et al., 2009). A recent study utilized genes encoding intimin and Shiga toxin to detect EHEC and EPEC strains, yet the assay was not designed to specifically detect O157 strains (Pavlovic et al., 2010).

In developing countries, enteric pathogen identification is frequently time consuming and incomplete, resulting in potential misdiagnoses or mistreatments. Therefore, a rapid, specific assay designed to identify EHEC/STEC and EPEC strains in a public health setting would be advantageous to help ensure that a timely and proper response is initiated. Furthermore, assays like this one would accelerate the diagnosis and significantly reduce mortality in endemic areas. Specific identification of highly pathogenic EHEC would also be critical in the event of food-borne illness outbreaks or agroterrorism.

Therefore, our study addresses the aforementioned constraints on the detection of these diarrheagenic *E. coli* (DEC) categories by using genes that do not encode serogroup-specific antigens, yet can distinguish O157:H7 strains, as well as unknown serogroups. We examined the hypothesis that re-emerging and outbreak-associated *E. coli* strains can be rapidly, specifically, and easily distinguished using multiplex PCR (mPCR) amplification of specific biomarkers associated with each strain's respective virulence genotype. The results of our mPCR assay indicate that this approach can provide a rapid method for detection of pathogenic *E. coli* strains. We demonstrated that *lpfA* subtypes could distinguish between EHEC and EPEC groups and most importantly, inclusion of *lpfA* variants permitted detection of EHEC O157:H7 in 100% of the cases, further supporting the importance of *lpfA* in molecular diagnostics approaches.

MATERIALS AND METHODS

STRAINS

Enterohemorrhagic *E. coli* O157:H7 strain EDL933, EPEC O127:H6 strain E2348/69, *E. coli* K12 strain MG1655, *E. coli* HS, *Salmonella enterica* serovar Typhimurium 2157, *Shigella flexneri* M90T, adherent invasive *E. coli* O83:H1 NRG857c, enterotoxigenic *E. coli* H10407, enteroaggregative *E. coli* O42, and 78 isolates from the DEC Collection (Whittam et al., 1993) were grown in Luria–Bertani (LB) broth at 37°C with shaking. Genomic DNA (gDNA) was extracted from the cells using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA).

PCR

Single and mPCR reactions were carried out using REDTaq ReadyMix PCR Reaction Mix and REDTaq DNA Polymerase (Sigma, St. Louis, MO, USA) supplemented with the appropriate primers and template DNA. Oligonucleotide primer sequences were used from previously published work or manually designed to obtain amplicons of sufficiently different sizes to be resolved in the multiplex assay (**Tables 1** and **2**). Primer sets specifically designed in this study utilized sequences for *eae*-α (FM180568), *eae*-β (AF081186), *eae*-γ (AE005174), *eae*-δ (AJ875027), and *lpfA1-1* (NC_011601). Single PCR reactions using REDTaq ReadyMix (Sigma, St. Louis, MO, USA) and 0.8 mM of each primer were performed under the following conditions: 94°C for 5 min; 30 cycles of 94°C for 30 s, 42°C for 45 s, 72°C for 35 s; 72°C for 10 min; hold at 10°C. Multiplex reactions using an additional 0.8 units of REDTaq DNA polymerase (1.4 units total per reaction – supplementation with extra polymerase permitted an increase in specificity and band intensity) were performed under the following conditions using the primer concentrations indicated in **Table 1**: 94°C for 5 min; 40 cycles of 94°C for 30 s, 59°C for 1 min 30 s, 72°C for 40 s; 72°C for 10 min; hold at 10°C. Products were analyzed on 1.5% agarose gels.

THRESHOLD OF MULTIPLEX PCR ASSAY DETECTION

The assay was performed by first re-suspending cells (EHEC O157:H7 EDL933 or EPEC O127:H6 E2348/69) freshly streaked onto LB agar to an estimated concentration of $\sim 4 \times 10^9$ cells/ml by monitoring the OD_{600}. Ten-fold serial dilutions were made into sterile distilled water, which was then used as template directly in mPCR reactions.

ISOLATION AND MOLECULAR CHARACTERIZATION OF CLINICAL STRAINS

One hundred fecal samples (43 HUS, 36 non-bloody diarrhea, and 21 bloody diarrhea cases) submitted to the National Reference Laboratory (NRL) in Buenos Aires, Argentina were studied. Fecal samples were plated either directly onto sorbitol MacConkey agar or after enrichment at 37°C for 4 h in trypticase soy broth with or without cefixime (50 ng/ml) and potassium tellurite (25 mg/ml). Confluent growth zones were first screened for *stx1*, *stx2*, and *rfb*O157 genes by mPCR (Leotta et al., 2005). A single PCR targeting the *eae* gene was performed (Karch et al., 1993; Karch and Bielaszewska, 2001) as well as testing of the *eae* variants (Ramachandran et al., 2003), if mPCR for the *stx1*, *stx2*, and *rfb*O157 genes was negative. Isolates with *stx1*, *stx2*,

Table 1 | Virulence-associated genes and primers used in this study.

Target	Primer sequences	Primer conc. in multiplex PCR (mM)	Amplicon size (bp)	Reference
stx2-F	5′-ATCCTATTCCCGGGAGTTTACG-3′	1	587	Cebula et al. (1995)
stx2-R	5′-GCGTCATCGTATACACAGGAGC-3′	1		
eae-γ-F	5′-CAGGTTGGGGTAACGGACTTTAC-3′	1	472	This study
eae-γ-R	5′-TTGCTTGCGTTTGAGACTTACCGTTG-3′	1		
lpfA1-1-F	5′-GTGCTGGATTCACCACTATTCATCGC-3′	0.2	389	This study
lpfA1-1-R	5′-GCCTTGTCTGCACTGGCATTAACTTC-3′	0.2		
stx1-F	5′-CAGTTAATGTGGTKGCGAAGG-3′	1	348	Cebula et al. (1995)
stx1-R	5′-CACCAGACAATGTAACCGCTG-3′	1		
bfpA-F	5′-AATGGTGCTTGCGCTTGCTGC-3′	0.2	326	Aranda et al. (2007)
bfpA-R	5′-GCCGCTTTATCCAACCTGGTA-3′	0.2		
lpfA2-2-F	5′-CTACAGGCGGCTGATGGAACA-3′	0.2	297	Torres et al. (2009)
lpfA2-2-R	5′-GCTAATACCAGCGGCAGCATCGT-3′	0.2		
lpfA1-3-F	5′-GGTTGGTGACAAATCCCCG-3′	0.2	244	Torres et al. (2009)
lpfA1-3-R	5′-CGTCTGGCCTTTACTCAGA-3′	0.2		
eae-F	5′-CTTTGACGGTAGTTCACTGGACTTC-3′	0.2	166	This study
eae-R	5′-GAAGACGTTATAGCCCAACATATTTTCAGG-3′	0.2		

Table 2 | Gene profiles for differentiation of *E. coli* pathotypes.

Pathotype (serotype)	Gene targets in multiplex PCR assay			
	eae	***lpfA***	***stx***	***bfpA***
EHEC (O157:H7)	*eae-γ*	*lpfA1-3* and *lpfA2-2*	*stx1* and/or *stx2*	NA
STEC	various *eae*	Various *lpf*	*stx1* and/or *stx2*	NA
LEE$^-$ – STEC	NA	*lpfA1-2* and/or *lpfA2-1*	*stx1* and/or *stx2*	NA
Typical EPEC (O127:H6)	*eae-α*	*lpfA1-1*	NA	*bfpA*
Atypical EPEC	*eae-β*	*lpfA1-2* and/or *lpfA2-1*	*stx1* and/or *stx2*	NA

NA, not applicable.

and/or *eae* genes were identified by standard biochemical tests, serotyped, and characterized by phenotypic and genotypic techniques (Rivas et al., 2011). For comparison purposes, the same DNA templates were screened by the mPCR developed in the present study using Platinum Taq DNA Polymerase (Invitrogen, Brazil). This study was carried out in strict accordance with the Guidelines of the National Institutes of Health and the Ministry of Health, Argentina. The protocol was approved by the Institutional Review Board of the University of Texas Medical Branch (IRB#11-081).

RESULTS

MULTIPLEX PCR ANALYSIS OF STRAINS FROM A DIARRHEAGENIC *E. COLI* COLLECTION

The present work describes the development of an mPCR assay for the rapid detection of specific categories of pathogenic *E. coli*. The assay is based on the use of pathotype-specific genes for the detection of medically relevant *E. coli* strains, and is able to specifically detect EHEC, typical and atypical EPEC, and STEC strains.

We demonstrated robust amplification of genes specific to EHEC O157:H7 strain EDL933 (*lpfA1-3*, *lpfA2-2*, *stx1*, *stx2*, and *eae-γ*) and EPEC O127:H6 strain E2348/69 (*eae-α*, *lpfA1-1*, and *bfpA*) using an optimized mPCR protocol with purified gDNA. Amplification of genes encoding virulence factors specific to pathogenic *E. coli* (**Table 2**) was first tested in single PCR reactions. One amplification product was observed in each case (data not shown). During optimization of the multiplex reaction, the addition of REDTaq DNA polymerase to the polymerase already present in the 1× solution of REDTaq ReadyMix permitted an increase in the annealing temperature (thereby increasing specificity) and robustness of the assay. Modification of primer concentration, total number of cycles, and primer annealing time were also optimized. To assess the efficacy of this assay on clinical isolates, gDNA from strains in the DEC collection (Whittam et al., 1993), representing a variety of serotypes from the EHEC (clonal groups EHEC1 and EHEC2) and EPEC (clonal groups EPEC1 and EPEC2), was screened using mPCR (**Figure 1**). Amplicon sizes from each DEC sample were compared to those in the control strains to compose a genotype for each representative DEC group member. We determined that mPCR analysis using gDNA from DEC isolates was successful in predicting the correct pathotype in 75.6% (59/78) of the total number of isolates; however the assay was able to predict the pathotype of all EHEC1 (DECs 3, 4, and 5) and EPEC1 (DECs 1 and 2) and distinguish between EHEC1 and EHEC2 (DECs 8, 9, and 10) pathogroups.

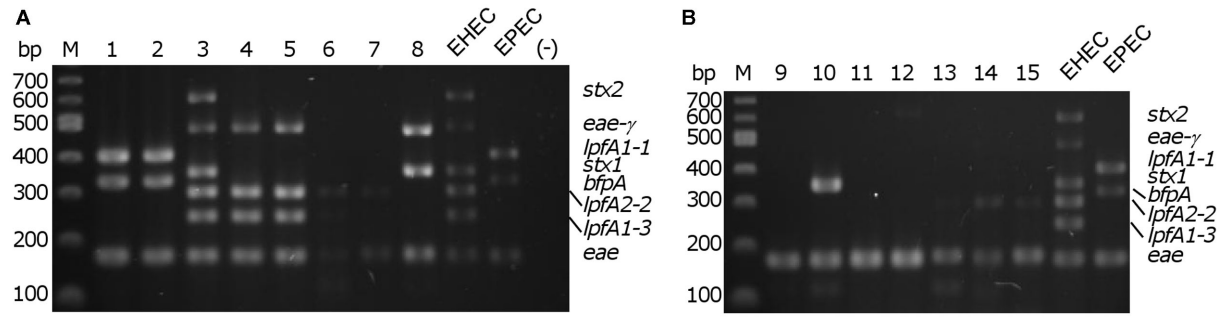

FIGURE 1 | Multiplex PCR screening of representatives from each diarrheagenic *E. coli* collection (DEC) group. (A) DEC 1–8; **(B)** DEC 9–15, M – 100 bp DNA markers (NEB). EHEC/EPEC, positive controls; (–), no template control.

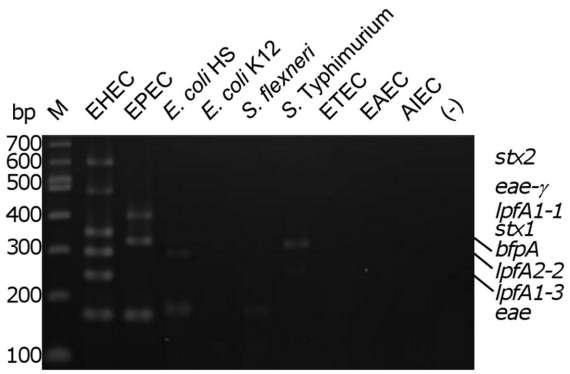

FIGURE 2 | Determination of biological assay specificity. Multiplex PCR assay was performed using gDNA from pathogenic and commensal enteric bacteria. M, 100 bp DNA markers (NEB); EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; *E. coli* HS and *E. coli* K12, human intestinal commensal and laboratory strains; *S. flexneri*, *Shigella flexneri*; *S. typhimurium*, *Salmonella enterica* serovar Typhimurium 2157; ETEC, enterotoxigenic *E. coli* H10407; EAEC, enteroaggregative *E. coli* O42; AIEC, adherent/invasive *E. coli* O83:H1; (–), no template control. The position of the amplicons in the EHEC and EPEC strains are indicated on the right of the figure.

ANALYTICAL SENSITIVITY OF MULTIPLEX PCR ASSAY

To assess the specificity of the assay, the multiplex reaction setup was tested using gDNA from commensal *E. coli* strains and pathogenic enteric bacteria. Relatively low intensity amplicons slightly larger than the size of the *eae* product were observed in three of the seven strains tested (two *E. coli* strains [one commensal and one laboratory isolate] and *Shigella flexneri*; **Figure 2**). With the *Salmonella* strain used, we observed a product of approximately 300 bp, possibly due to amplification of an uncharacterized *lpfA* variant in that strain. A smaller, faint amplicon was also observed using the *Salmonella* strain; however, its size does not correspond to any of the amplicons expected in our assay. Analysis of the commensal *E. coli* HS revealed another faint, non-specific product not corresponding to the size of a target amplicon (**Figure 2**).

ANALYTICAL SPECIFICITY OF MULTIPLEX PCR ASSAY

We then determined the *in vitro* threshold of detection with the EHEC and EPEC prototype strains. Colonies from freshly streaked LB agar plates were re-suspended in sterile distilled water to a concentration of $\sim 4 \times 10^9$ cells/mL. A 5 μl aliquot from each of the 10-fold serial dilutions were added to mPCR reactions to permit testing of a 10-fold range of template concentrations from 1×10^9 to 1×10^3 cells/mL in a 20 μl reaction. The threshold of detection for EHEC and EPEC *in vitro* was determined to be $\sim 2 \times 10^4$ CFU/reaction (corresponding to 1×10^6 cells/ml in each reaction), assessed by visibility of all six predicted amplicons for EHEC and all three amplicons for EPEC (**Figure 3**).

EVALUATION OF MULTIPLEX PCR ASSAY AT NRL (ARGENTINA)

Next, we tested the feasibility to implement our mPCR in a clinical setting, performing a trial study with samples received at the National Reference Laboratory (NRL), Argentina, and comparing the results to methods already optimized in that laboratory (**Table 3**). The clinical sensitivity and specificity of the assay was estimated to be 91% and 84%, respectively. Identification of at least one gene (*eae*, one of the *stx* genes, or *rfbO157*) was the basis for determining whether a given case was considered positive or negative.

Further analysis of non-O157 and non-O145 isolates revealed that although the mPCR assay typically identified *eae*-γ in these isolates, RFLP–PCR intimin typing (Ramachandran et al., 2003) at the NRL revealed *eae*-β or *eae*-θ in the non-O157 and non-O145 cases (data not shown). These findings likely represent a false positive indication of the presence of *eae*-γ, as strains that possess *eae*-γ typically contain *lpfA1-3* and *lpfA2-2* as well, both of which are absent in the *eae*-β and *eae*-θ strains.

In one positive case (**Table 3**, isolate 4), the mPCR assay was positive for both *stx1* and *stx2*, while the *stx*-negative result at the NRL was confirmed using RFLP–PCR (Tyler et al., 1991; Zhang et al., 2002). In two other cases, the standard methodology revealed eae^+ strains, whereas the mPCR assay indicated that the strains lacked intimin (**Table 3**, isolates 31 and 32). Interestingly, analysis of isolates 30–34 revealed that the mPCR produced amplicons for both *lpfA1-3* and *lpfA2-2*only (**Table 3**). Despite indicating a positive result for two of the markers for O157:H7 strains, that serotype was not confirmed in those isolates (**Table 3**).

In the 11 cases where the NRL indicated negative results and the mPCR assay indicated positive results (**Table 3**, isolates 90–96, 97, 100), nine of them were considered false positives for *eae*, as they were further confirmed to be negative for *eae* subtypes by

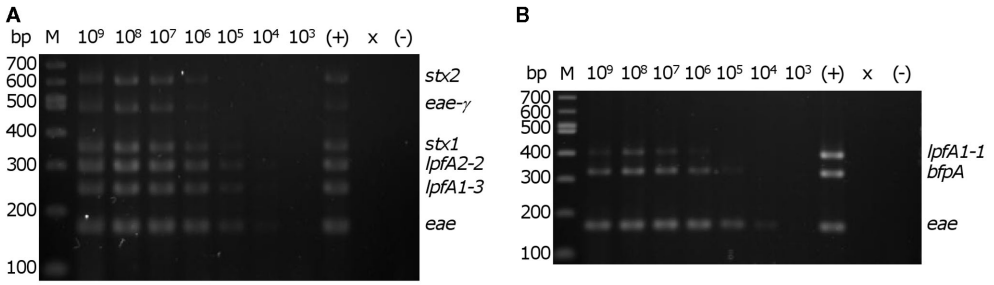

FIGURE 3 | Determination of detection threshold. Serial dilutions of re-suspended EHEC EDL933 or EPEC E2348/69 colonies were used as template in the multiplex PCR reaction. M – 100 bp DNA markers (NEB). Number above each lane indicates the concentration (cells/ml) of bacteria per reaction. Consistent detection of **(A)** all of six EHEC EDL933-specific bands and **(B)** all three EPEC E2348/69-specific bands was observed using 2×10^4 cells in a 20 μl PCR reaction. ×, lane was not loaded; (–), no template control. The position of the amplicons in the EHEC and EPEC strains are indicated on the right of each panel.

RFLP–PCR. Two strains identified by the described mPCR assay as either *stx1*$^+$ (**Table 3**, isolate 100) or *stx2*$^+$ (**Table 3**, isolate 97) were confirmed as *stx*-negative by RFLP–PCR.

While the assay can reliably detect EHEC, STEC, and typical or atypical EPEC strains, the repertoire of pathotype detection can be expanded by the inclusion of primers for *lpfA1-2* and *lpfA2-1*. The LEE$^-$ negative STEC typically possess one or both of these *lpfA* subtypes (Galli et al., 2010; Gomes et al., 2011). Additionally, detection of *lpfA1-2* and/or *lpfA2-1* permits the differentiation of strains from the EHEC2 (DECs 8, 9, and 10) and EPEC2 (DECs 11 and 12) clonal groups (**Figure 4**, data not shown). The EHEC and EPEC pathotypes were identified after examining a number of diarrheagenic strains by multilocus enzyme electrophoresis and serotyping, and appear to represent distinct clonal lineages of pathogenic *E. coli* (Whittam and McGraw, 1996; Reid et al., 2000).

The unique ability of the multiplex assay to specifically detect EHEC and EPEC clonal groups is predominantly conferred by *lpfA* subtype analyses (**Figure 4**). Using DEC collection isolates (Whittam et al., 1993), clonal group EHEC1 (DECs 3, 4, and 5) were detected, in part, by the inclusion of primers amplifying EHEC O157:H7-specific *lpfA* subtypes 1-3 and 2-2. EPEC1 isolates (DECs 1 and 2) and EPEC O127:H6 were specifically identified by the amplification of *lpfA1-1*. The addition of *lpfA* subtyping was advantageous since *lpfA* subtypes differ among EHEC and EPEC clonal groups (Torres et al., 2009). As such, it permitted us to differentiate the more common EHEC1 and EPEC1 clonal groups from members of the EHEC2 and EPEC2 categories, respectively. Further specificity was conveyed by the amplification of pathotype-specific intimin subtypes: γ-intimin was used to detect EHEC1 isolates and α-intimin was used to detect EPEC1 isolates.

DISCUSSION

A panel of eight genes was employed for the design of a sensitive and specific mPCR assay to facilitate detection of three pathotypes of *E. coli* that cause significant morbidity and mortality across the world – EHEC, STEC, and EPEC. The assay was also designed to be relatively low-cost, as compared to the financial burden of acquiring instrumentation and consumables to perform real-time PCR or multiplex bead-based assays. A collection of DEC strains (Whittam et al., 1993) was tested using the assay, and we demonstrated a relatively high degree of agreement between the mPCR results and strain information present in the DEC database. Evaluation of the specificity revealed no significant cross-reactivity of the primers with other *E. coli* pathotypes. The detection threshold of the assay was determined to be comparable to other PCR-based methods for detection of *E. coli* isolates (Aranda et al., 2007; Antikainen et al., 2009; Vidová et al., 2011). Of particular significance is the observation that defined combinations of *lpfA* subtypes permitted differentiation of EHEC and EPEC clonal groups.

Both EHEC and EPEC are AEEC strains, possessing the LEE-encoded gene products for development of the intestinal lesions (McDaniel and Kaper, 1997). The presence of the LEE-encoded adhesin intimin gene *eae* (Jerse and Kaper, 1991), is indicative of these strains, and; therefore, a first primer set was designed to amplify only *eae*-γ, previously demonstrated to be associated with the EHEC1 clonal group (Adu-Bobie et al., 1998; Reid et al., 1999). The second *eae* primer set was engineered to be more generic, amplifying the remaining major *eae* subtypes α, β, and δ (Adu-Bobie et al., 1998). Importantly, intimin α is associated with the EPEC1 clonal group, while intimin β is indicative of members of the EPEC2 clonal group (Adu-Bobie et al., 1998; Reid et al., 1999). Thus, the multiplex assay has the potential to detect a large number of AEEC strains.

In the multiplex strategy, the addition of primers for *lpfA* subtyping conferred the greatest increase in the ability of the assay to differentiate members of the EHEC and EPEC clonal groups. Lpf are elaborated appendages important for pathogenesis and adherence to cultured cells (Torres et al., 2002, 2004), persistence in animal models (Jordan et al., 2004; Torres et al., 2007), and tissue tropism in the human intestine (Fitzhenry et al., 2006). In addition, the combination of *lpfA* and *eae* subtyping can specifically detect EHEC O157:H7 (Torres et al., 2009), providing a distinct time advantage over more conventional culture- or immunoassay-based methodologies for the detection of O157:H7 strains.

Primer sets for both Shiga toxin 1 (*stx1*) and Shiga toxin 2 (*stx2*) were incorporated into the assay to facilitate identification of STEC strains, because early detection is critical for determining appropriate therapies for patients with suspected *E. coli* infections. Although there are a number of *stx2* variants, we included primers based on the *E. coli* O157:H7 *stx2* sequence due to the link

Table 3 | Comparison of results between molecular diagnostic assays at NRL and the current proposed mPCR methodology.

Results of assays[1] Isolate no. (serotype)	Diagnosis[2]	PCR (NRL)				mPCR (UTMB)					
		eae	*rfbO157*	*stx1*	*stx2*	*eae*	*eae-γ*	*stx1*	*stx2*	*lpfA1-3*	*lpfA2-2*
NRL POSITIVE/mPCR POSITIVE											
1 (ONT:motile)	BD	+	−	−	−	+	+	−	−	−	−
2 (OR:motile)	D	+	−	−	−	+	−	−	−	−	−
3 (O157:H7)	BD	+	+	−	+	+	+	−	+	+	+
4 ND	HUS	+	−	−	−	+	−	+	+	−	−
5 (O157:H7)	HUS	+	+	−	+	+	+	−	+	+	+
6 (O157:H7)	BD	+	+	−	+	+	+	−	+	+	+
7 (O145:NM)	HUS	+	−	−	+	+	+	−	+	−	−
8 ND	HUS	+	−	+	−	+	+	+	+	−	−
9 (O157:H7)	BD	+	+	−	+	+	+	−	+	+	+
10 (O157:H7)	HUS	+	+	−	+	+	+	−	+	+	+
11 (O157:H7)	HUS	+	+	−	+	+	+	−	+	+	+
12 (O145:NM)	D	+	−	−	+	+	+	−	+	−	−
13 (O157:H7)	HUS	+	+	−	+	+	+	−	+	+	+
14 (ONT:motile)	HUS	+	−	−	−	+	+	−	−	−	−
15 (O26:NM)	BD	+	−	−	−	+	−	−	−	−	−
16 (O26:H11)	D	+	−	−	+	+	+	−	+	−	−
17 (O145:NM)	HUS	+	−	−	+	+	+	−	+	−	−
18 (O145:NM)	HUS[3]	+	−	−	+	+	+	−	+	−	−
19 (O145:NM)	BD	+	−	−	+	+	+	−	+	−	−
20 ND	D	+	−	−	−	+	−	−	−	−	−
21 ND	D	+	−	−	−	+	+	−	−	−	−
22 (O145:NM)	D	+	−	−	+	+	+	−	+	−	−
23 (O145:NM)	D	+	−	−	+	+	+	−	+	−	−
24 (O157:H7)	HUS	+	+	−	+	+	+	−	+	+	+
25 (O157:H7)	BD	+	+	−	+	+	+	−	+	+	+
26 (ONT:H46)	BD	+	−	−	+	+	−	−	+	−	−
27 (O145:HNM)	HUS	+	−	−	+	+	+	−	+	−	−
28 (O157:HNT)	HUS	+	+	−	−	+	−	−	−	−	−
29 (O157:H7)	HUS	+	+	−	+	+	+	−	+	+	+
NRL POSITIVE/mPCR NEGATIVE											
30 (O157:HNT)	BD	−	+	−	−	−	−	−	−	+	+
31 ND	D	+	−	−	−	−	−	−	−	+	+
32 ONT:HNT	BD	+	−	−	−	−	−	−	−	+	+
NRL NEGATIVE/mPCR NEGATIVE											
33 ND	D	−	−	−	−	−	−	−	−	+	+
34 ND	HUS	−	−	−	−	−	−	−	−	+	+
35–89 ND	23 D	−	−	−	−	−	−	−	−	−	−
	9 BD	−	−	−	−	−	−	−	−	−	−
	23 HUS	−	−	−	−	−	−	−	−	−	−
NRL NEGATIVE/mPCR POSITIVE											
90 ND	D	−	−	−	−	+	−	−	−	−	−
91 ND	HUS	−	−	−	−	+	−	−	−	−	−
92 ND	HUS	−	−	−	−	+	−	−	−	−	−
93 ND	HUS	−	−	−	−	+	−	−	−	−	−
94 ND	BD	−	−	−	−	+	−	−	−	−	−
95 ND	HUS	−	−	−	−	+	−	−	−	−	−
96 ND	HUS	−	−	−	−	+	−	−	−	−	−
97 ND	BD	−	−	−	−	−	−	−	+	−	−

(Continued)

Table 3 | Continued

Results of assays[1]	Diagnosis[2]	PCR (NRL)				mPCR (UTMB)					
Isolate no. (serotype)		***eae***	***rfbO157***	***stx1***	***stx2***	***eae***	***eae-γ***	***stx1***	***stx2***	***lpfA1-3***	***lpfA2-2***
98 ND	D	–	–	–	–	+	–	–	–	–	–
99 ND	D	–	–	–	–	+	–	–	–	–	–
100 ND	D	–	–	–	–	–	–	+	–	–	–

[1] *Positivity is defined as genetic evidence for an O157, attaching/effacing-, or Shiga toxin-producing E. coli isolate.*

[2] *D, diarrhea; BD, blood diarrhea; HUS, hemolytic uremic syndrome.*

[3] *Patient died.*

ND serotype not determined (unable to isolate a strain).

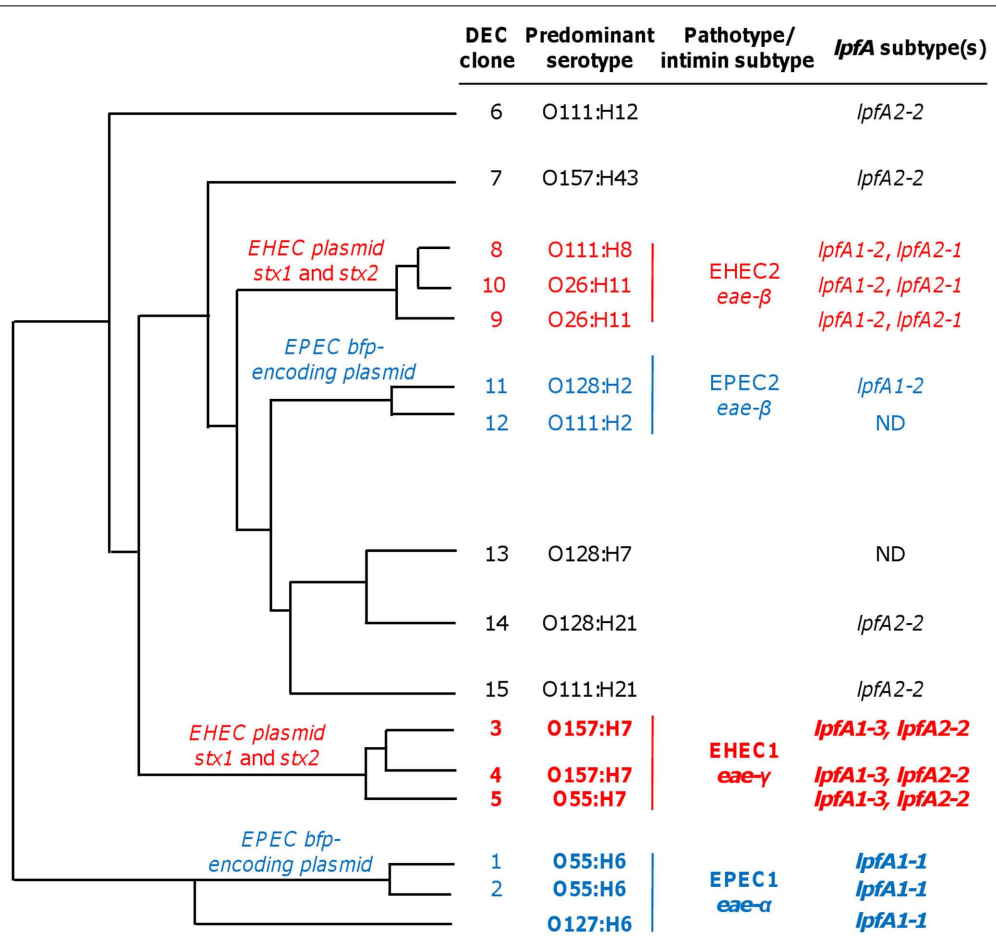

FIGURE 4 | Phylogeny and *lpfA* subtype of isolates in diarrheagenic *E. coli* (DEC) collection. DEC clone numbers (Reid et al., 1999), corresponding predominant serotype of the given group, pathotype/intimin subtype, and information regarding *lpfA* subtype(s) for each group are indicated (Adapted from Torres, 1999 Ph.D. Dissertation). The *lpfA1-2* and *lpfA2-1* were previously identified in EHEC2 and EPEC2 isolates (Torres et al., 2009). ND, not determined (none of the three *lpfA* subtypes included in the multiplex PCR were detected, and *lpfA* subtypes were not studied further during the multiplex PCR project).

between the development of HUS and the presence of *stx2*, particularly in O157:H7 strains (Friedrich et al., 2002; Brooks et al., 2005; Hedican et al., 2009). Conversely, the assay is capable of detecting non-O157 STEC, a group of under-diagnosed emerging pathogens (Coombes et al., 2011), and emerging LEE-negative STEC strains (Newton et al., 2009; Galli et al., 2010).

To further expand the detection capabilities, primers to amplify the bundle-forming pili subunit *bfpA* were incorporated into the

assay. Because the bundle-forming pilus is a central virulence factor of EPEC strains, playing a putative role in initial attachment to enterocytes (Cleary et al., 2004) and microcolony formation (Hicks et al., 1998), the *bfpA* gene can be used as a marker for identification of typical EPEC (Nataro and Kaper, 1998) in conjunction with *eae* (Giron et al., 1993).

Our mPCR results with DEC collection isolates suggest that this approach will prove useful for rapid identification of these pathogenic *E. coli* strains. The incidence of non-specific amplification was low, and in many cases, the bands were faint compared to the intensities of the primer-specific products and outside the size range of the target amplicons (data not shown). The presence of non-specific amplicons is not anticipated to result in the misidentification of strains as DEC, and is not uncommon in mPCR approaches (Antikainen et al., 2009).

The diagnostic sensitivity and specificity was calculated to be 91% and 84%, respectively, when comparing to the "standard" methodology used by the NRL (Argentina) for routine detection of highly virulent STEC strains and our mPCR assay. These data strongly suggest that the mPCR approach described here is a relatively low-cost and feasible screening methodology for clinical fecal samples within 24 h of obtaining a specimen. Because Argentina possesses the highest incidence of post-enteric HUS in infants and children in the world, and O157:H7 and O145:NM are the most prevalent serotypes (Rivas et al., 2010, 2011), this new mPCR approach permits rapid identification of STEC strains involved in the majority of the cases (>70%) received at the NRL. Further, this method can also be used in areas where other STEC or EPEC strains are prevalent.

Assay specificity was determined by screening additional *E. coli* pathotypes, commensal *E. coli*, and a limited number of non-*E. coli* intestinal pathogens. This analysis revealed that the assay is highly specific; none of the unexpected amplification products correspond to the size of a predicted amplicon (**Figure 2**). Minor cross-reactivity of the broad-range intimin primers with a putative intimin gene in *E. coli* K12 (*eaeH*) and *E. coli* HS (EcHS_A0351) may account for the observation of a band migrating at approximately the same size as the specific intimin target observed using EHEC or EPEC. However, *S. flexneri* does not appear to possess a putative intimin sequence.

The threshold detection of our multiplex approach was assessed and the template concentrations at which all expected amplicons were clearly visible on the gel was set as the limit for detection of that pathotype. EHEC and EPEC were detectable at or above 2×10^4 CFU per reaction (**Figure 3**). Compared to other mPCR methodologies (Aranda et al., 2007; Antikainen et al., 2009; Vidová et al., 2011), the sensitivities determined here are slightly higher, perhaps owing to the selection of DNA polymerase. The REDTaq Ready Mix was chosen based on the premixed nature of the components, thereby reducing pipetting errors and increasing reproducibility, and its relatively low-cost, a factor critical for adoption of this assay in developing countries. The balance between assay cost and sensitivity can be adjusted based on the financial resources of the testing facility, suggesting that the purchase of more costly polymerases could increase assay sensitivity.

The observation that *lpfA* and *eae* subtypes are related to specific EHEC and EPEC clonal groups provides evidence of the lineage of pathogenic *E. coli*, but also permitted us to design an assay exploiting these phylogenetic relationships (**Figure 4**). In its current form, the mPCR assay can reliably distinguish strains in the EHEC1 and EPEC1 clonal groups, atypical EPEC, and differentiate O157 and non-O157 STEC strains. Interestingly, isolates from the EPEC2 group (DEC 11 and DEC 12) were negative for *bfpA*. This result likely reflects the specificity of the *bfpA* primer set for the *bfpA*α1 allele present in the O127:H6 prototype EPEC strain (Blank et al., 2000). Therefore, strategies for detection of EPEC2 strains should also include primers to amplify the β4 (DEC 11) and α2 (DEC 12) *bfpA* alleles (Blank et al., 2000). While not included in the same mPCR assay, detection of additional *lpfA* subtypes would permit identification of EHEC2 and EPEC2 strains, as well as afford detection of LEE-negative STEC (*eae*$^-$, *stx*$^+$, *lpfA1-2*$^+$, and/or *lpfA2-1*$^+$) and atypical EPEC (*eae*$^+$, *bfpA*$^-$, *lpfA1-2*$^+$, and/or *lpfA2-1*$^+$). Recent data also supports this notion, as Gomes et al. (2011) demonstrated that of the *lpfA*$^+$ atypical EPEC strains tested, *lpfA1-2* and *lpfA2-1* were frequently present together in a given strain. Finally, the *lpfA1-1* variant was used to identify typical EPEC strains; however, this allele was present in only 35% (16/46) of the isolates tested. Currently, we are exploring the possibility to incorporate alternative *lpfA1* alleles identified in our initial screen (Torres et al., 2009) to increase the specificity of our assay.

In summary, we presented data supporting a mPCR approach that, with only eight virulence-associated genes, has the potential detect a wide range of pathogenic *E. coli* strains. The assay was tested with a collection of clinical isolates, resulting in a high degree of agreement between known strain information and the results of the mPCR. The specificity and sensitivity of the assay are such that diagnostic facilities in developing countries can easily incorporate this methodology into their workflow. The mPCR approach described here has potential for improving both diagnostics and epidemiological studies involving DEC.

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Detection of Shiga toxin-producing *Escherichia coli* in ground beef using the GeneDisc real-time PCR system

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Escherichia coli O157:H7 and certain non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serogroups have emerged as important public health threats. The development of methods for rapid and reliable detection of this heterogeneous group of pathogens has been challenging. GeneDisc real-time PCR assays were evaluated for detection of the stx_1, stx_2, *eae*, and *ehxA* genes and a gene that identifies the O157 serogroup followed by a second GeneDisc assay targeting serogroup-specific genes of STEC O26, O45, O91, O103, O111, O113, O121, O145, and O157. The ability to detect the STEC serogroups in ground beef samples artificially inoculated at a level of ca. 2–20 CFU/25 g and subjected to enrichment in mTSB or buffered peptone water (BPW) was similar. Following enrichment, all inoculated ground beef samples showed amplification of the correct set of target genes carried by each strain. Samples inoculated with STEC serogroups O26, O45, O103, O111, O121, O145, and O157 were subjected to immunomagnetic separation (IMS), and isolation was achieved by plating onto Rainbow agar O157. Colonies were confirmed by PCR assays targeting stx_1, stx_2, *eae*, and serogroup-specific genes. Thus, this work demonstrated that GeneDisc assays are rapid, sensitive, and reliable and can be used for screening ground beef and potentially other foods for STEC serogroups that are important food-borne pathogens worldwide.

Keywords: GeneDisc, real-time PCR, STEC, virulence genes, O-group, detection, isolation, *E. coli* O157:H7

INTRODUCTION

In addition to *Escherichia coli* O157:H7, numerous non-O157 Shiga toxin-producing *E. coli* (STEC) serogroups have caused outbreaks and severe illness, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) similar to STEC O157. Although *E. coli* O157:H7 causes many of the STEC-related outbreaks in the US, it is estimated that non-O157 STEC cause more than twice the number of infections overall compared to *E. coli* O157:H7 (Scallan et al., 2011). Furthermore, non-O157 STEC cause the majority of STEC-associated infections in some countries (Johnson et al., 2006). Data from the Centers for Disease Control and Prevention indicate that STEC serogroups O26, O45, O103, O111, O121, and O145 cause the majority of cases of illness due to non-O157 STEC in the US and are important STEC serogroups in other countries, as well (Brooks et al., 2005; Johnson et al., 2006; Gyles, 2007). These STEC serogroups are referred to as the "top six" or "big six" serogroups, and major outbreaks of HC and HUS associated with these non-O157 STEC have been reported (Bettelheim, 2007; Mathusa et al., 2010; Schaffzin et al., 2012). Other STEC serogroups including O91 and O113 have also been linked to cases and outbreaks of HC and HUS and are important STEC in many countries (Paton et al., 1999; Johnson et al., 2006; Bettelheim, 2007; EFSA, 2009; Mellmann et al., 2009).

It is likely that highly pathogenic STEC have acquired genetic elements encoding specific virulence factors through lateral gene transfer to a greater extent than strains with lesser virulence. STEC carry phage-encoded Shiga toxin genes, stx_1 and/or stx_2 or various genetic subtypes of these genes. STEC also possess a pathogenicity island, known as the locus of enterocyte effacement (LEE), which encodes proteins necessary for the formation of attaching and effacing lesions, including intimin (Eae, an outer membrane protein), a translocated intimin receptor (Tir), a type III secretion apparatus, and effector proteins translocated by the secretion system (Gyles, 2007; Bolton, 2011). The precise role of a plasmid-encoded enterohemolysin, EhxA, in STEC virulence remains to be resolved; however, the presence of the gene is a defining diagnostic epidemiological marker in outbreak strains and strains that cause severe disease (Schmidt and Karch, 1996; Boerlin et al., 1999; Brooks et al., 2005). Analysis of STEC belonging to different serotypes showed that there was a significant association between the presence of the *eae* gene and bloody diarrhea and the stx_2 gene with HUS (Brooks et al., 2005). STEC O113 and O91 that have caused outbreaks and severe disease lack the *eae* gene (Paton et al., 1999; Mellmann et al., 2009); however, these serogroups carry other genes, including *saa* (STEC autoagglutinating adhesion) (Paton et al., 2001; Gyles, 2007) that may allow attachment to host cells. A large outbreak that occurred in 2011 in Germany linked to sprouts was caused by *E. coli* O104:H4 that carried the stx_2 gene; however, the strain was an enteroaggregative *E. coli* (EAEC) that also carried adherence genes located on pAA, the large virulence plasmid of EAEC, that allowed attachment to cells (Bielaszewska et al., 2011), and the strain did not carry the *eae* gene. Thus, strains that lack *eae* may cause severe disease, and public health authorities should remain vigilant for these emerging STEC serogroups.

Similar to STEC O157, cattle and other ruminants are reservoirs for non-O157 serotypes, and surveys have demonstrated their presence in samples from cattle carcasses, retail beef, and raw milk, as well as other food (Hussein, 2007; Mathusa et al., 2010; Bosilevac and Koohmaraie, 2011). Food of bovine origin, food or water contaminated with bovine feces, and animal contact are important vehicles and modes of transmission of STEC infections (Kaspar et al., 2010). Due to the serious health concerns of non-O157 STEC infections, particularly due to specific *E. coli* serogroups, the United States Department of Agriculture's Food Safety and Inspection Service (USDA FSIS) declared the top six non-O157 STEC as adulterants in raw, non-intact beef products (Anonymous, 2011), and regulatory testing for these STEC serogroups began in June, 2012. Thus, there is a need for rapid methods that can be used for regulatory testing and for detection of the top six STEC serogroups by the food industry. There have been numerous reports on PCR-based methods for detection of food-borne pathogens, including STEC. Many of these PCR-based methods require the preparation of the individual components used for the reaction mixture, lengthy and cumbersome DNA extraction procedures, do not employ multiplexed detection, or do not include an internal amplification control (Hoorfar and Cook, 2003). A method that has flexibility is sensitive and is less time consuming and cumbersome would be more amenable for use by regulatory agencies and the food industry. One such method with these advantages is the GeneDisc Rapid Microbiology System manufactured by Pall GeneDisc Technologies that uses real-time PCR technology. The flexibility of the GeneDisc platform allows targeting specific STEC serogroups of interest and STEC virulence genes. The objective of the current study was to compare two different enrichment media and evaluate GeneDisc real-time PCR assays to detect nine important STEC serogroups: STEC O26, O45, O91, O103, O111, O113, O121, O145, and O157 in ground beef.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

STEC strains used in this study are listed in **Table 1** and were stored at −80°C in tryptic soy broth (TSB) (Becton Dickinson and Co., Sparks, MD) containing 10% glycerol. The bacteria were grown overnight in TSB at 37°C, and dilutions of the cultures were made in 0.1% sterile peptone (Bacto™ Peptone, Becton Dickinson and Co.).

INOCULATION AND ENRICHMENT OF GROUND BEEF

Ground beef was purchased from local supermarkets. One package of ground beef was divided into 25-g portions that were placed in sterile filter Stomacher bags (Seward Ltd., West Sussex, UK). The meat was inoculated with 1 mL of a diluted culture to achieve a target inoculum level of ca. 2–20 CFU/25 g, and then either 225 mL of mTSB enrichment medium or buffered peptone water (BPW) (Oxoid Ltd., Basingstoke, Hampshire, England) was added, followed by pummeling in a Stomacher apparatus (Seward Ltd.) for 1 min. The CFU/mL of the inoculum was determined by plating the cell dilutions onto tryptic soy agar (TSA) and enumerating the colonies after overnight growth at 37°C. An uninoculated control ground beef sample was included with each set of samples. mTSB was prepared as described previously (Fratamico et al., 2011) by adding cefsulodin (10 mg/L) and vancomycin (16 mg/L) (Sigma-Aldrich Corp., St. Louis, MO), and samples were incubated static for 6 h at 37°C. After this 6-h enrichment step, bile salts (1.5 g/L), rifampicin (2.0 mg/L), and potassium tellurite (1.0 mg/L) were added (Sigma-Aldrich Corp., St. Louis, MO) to the samples, and incubation was continued

Table 1 | Serotypes, source, and virulence gene profiles of STEC strains used in this study.

Serotype and strain	Source[a]		*stx*$_1$	*stx*$_2$	*eae*	*ehxA*
O26:H11 05-6544	PHAC	human	+[b]	−[b]	+	+
O45:H2 05-6545	PHAC	human	+	−	+	+
O91:H21 85-489	PHAC	human	−	+	−	+
O103:H25 03-2444	PHAC	human	+	−	+	+
O111:NM 00-4748	PHAC	human	+	+	+	−
O111:NM 98-8338	PHAC	human	+	−	+	+
O111:H8 01387	FDA	human	+	−	+	+
O111:H8 SJ14	CDC	human	+	+	+	+
O111:NM SJ15	CDC	human	+	+	+	+
O111:+[c] E05	University of Minnesota	cattle	+	−	+	+
O113:H21 04-1450	PHAC	human	−	+	−	+
O121:NM 03-4064	PHAC	human	−	+	+	+
O145:NM 03-4699	PHAC	human	+	−	+	+
O157:H7 380-94	FSIS	human	+	+	+	+

[a] *PHAC, Public Health Agency of Canada, Winnipeg, Manitoba, Canada; FDA, Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD; CDC, Centers for Disease Control and Prevention, Atlanta, GA; Dr. Francisco Diez-Gonzalez, University of Minnesota, St. Paul, MN; FSIS, Food Safety and Inspection Service, Athens, GA.*

[b] *"+," positive; "−," negative.*

[c] *The "+" indicates that the fliC gene was amplified by PCR in this strain; however, the restriction fragment length polymorphism pattern did not match with any of the known H types.*

static for 18 h at 42°C. Enrichment in BPW (pre-warmed to 42°C) was performed at 42°C for 18 h as recommended by the GeneDisc manufacturer.

DNA EXTRACTION

DNA extraction was performed using the GenDisc lysis reagent (Pall GeneDisc Technologies, Bruz, France). Following enrichment in mTSB or BPW, 50 μL of the enrichment were transferred to a lysis tube provided with the lysis solution and heated at 100°C for 10 min. The extracted DNA was either transferred to a GeneDisc for real-time PCR or stored at −20°C for testing at a later date.

GeneDisc REAL-TIME PCR ASSAY

Mastermix (37 μL) provided with the GeneDisc kits and 37 μL of template DNA were mixed in a sterile tube, and then 72 μL of the mixture for each sample were placed into the central reservoir of one of the GeneDisc sectors. Vacuum was applied using a pump provided with the GeneDisc Cycler to pull the mixture into the sector microchambers around the perimeter of the GeneDisc, which contained dessicated primers and probes labeled with either 6-FAM or ROX. The GeneDisc was inserted into the GeneDisc Cycler, following the manufacturer's instructions, and the reaction was started. The PCR consisted of 45 cycles, and the assay was completed in ca. 75 min. Two GeneDiscs were employed. The first disc (STEC and *E. coli* O157 GeneDisc) was composed of six sectors, consisting of real-time multiplex PCR assays targeting stx_1, stx_2, *eae*, *ehxA*, and O157. The microwells in each sector consisted of the following multiplex PCR assays with primers and labeled probes: microwell 1—negative control (FAM) and inhibition control (ROX), microwell 2—*ehxA* (FAM) only, microwell 3—stx_2 (FAM) and stx_1 (ROX), microwell 4—stx_2 (FAM) and stx_1 (ROX), microwell 5—O157 (FAM) and *eae* (ROX), and microwell 6—O157 (FAM) and *eae* (ROX). The second (GeneDisc 9 EHEC Identification) was also composed of six sectors and multiplex PCR assays targeted O-group-specific genes of serogroups O26, O45, O91, O103, O111, O113, O121, O157, and O145. The microwells in each sector consisted of: 1—only O145 (FAM), 2—negative control (FAM) and PCR inhibition control (ROX), 3—O157 (FAM) and O111 (ROX), 4—O26 (FAM) and O103 (ROX), 5—O91 (FAM) and O113 (ROX), and 6—O121 (FAM) and O45 (ROX). The decision support software installed in the GeneDisc Cycler processes the results, which can be exported as a table and are also presented as graphic images of the amplification curves.

IMMUNOMAGNETIC SEPARATION AND PLATING AND ISOLATION ON RAINBOW AGAR O157

The O26, O45, O103, O111, O121, O145, and O157 enrichments were also subjected to immunomagnetic separation (IMS) using Dynabeads EPEC/VTEC O26, O103, O111, O145, and O157 (Fratamico et al., 2011). For STEC O45 and O121, streptavidin-coated beads (Invitrogen Corp., Carlsbad, CA) were prepared using biotinylated polyclonal antibodies against *E. coli* serogroups O45 and O121 as described previously (Fratamico et al., 2011). IMS for STEC O91 and O113 was not performed. The beads–bacteria complexes were plated onto Rainbow Agar O157 (Biolog, Inc., Hayward, CA) containing 0.8 mg/L of potassium tellurite and 10 mg/L of novobiocin, and dilutions of enrichments from beef samples that had been inoculated with STEC O91 and O113 were plated directly onto the same agar. Two to five presumptive colonies were picked from the plates and confirmed by conventional PCR assays targeting stx_1, stx_2, *eae*, and the specific O-group *wzx* or *wbdI* genes (Fratamico et al., 2011). The primers and probe targeting the *E. coli* O157 *wzy* gene were described previously (Fratamico and DebRoy, 2010).

RESULTS AND DISCUSSION

In the US, *E. coli* O157:H7 and the top six non-O157 STEC (O26, O45, O103, O111, O121, and O145) cause the majority of the food-borne illnesses and outbreaks; however other emerging STEC serogroups including O113 and O91 have caused outbreaks and serious illness in the US and in other countries (Paton et al., 1999; Brooks et al., 2005; Johnson et al., 2006; Bettelheim, 2007; Mellmann et al., 2009). Cattle are an important reservoir for STEC O157:H7, as well as other STEC serogroups, and in September 2011, the FSIS announced that the top six non-O157 STEC would be declared as adulterants in beef similar to O157:H7. Thus, the availability of rapid screening methods for STEC of public health concern is critical. We evaluated the GeneDisc real-time PCR system developed by Pall GeneDisc Technologies. The GeneDisc for detection of specific O serogroups (O26, O45, O91, O103, O111, O113, O121, O145, and O157) was developed for evaluation in our laboratory in consultation with the manufacturer. The technology allows simultaneous detection of multiple targets by real-time PCR. The first GeneDisc screening assay targeted stx_1, stx_2, *ehxA*, *eae*, and O157, and the second was used to determine the presence of nine STEC O groups targeting serogroup-specific regions within the O-antigen gene clusters of these STEC. The first assay could be used to detect the presence of *E. coli* O157. A GeneDisc assay that contained primers and probe targeting the $fliC_{H7}$ gene has also been described (Beutin et al., 2009). These investigators evaluated GeneDisc assays for detection of STEC O26, O103, O111, O145, and O157 using DNA from colonies and from pure and mixed cultures. One GeneDisc assay targeted $rfbE_{O157}$, stx_1/stx_2, $fliC_{H7}$, and *eae*, and a second GeneDisc targeted wzx_{O26}, $wbdI_{O111}$, $ihpI_{O145}$, wzx_{O103}, and $ihpI_{O157}$. The assays were specific for the target organisms, and the sensitivity was <10 bacteria/sector. However, the stx_1/stx_2 assay required >100 bacteria for a positive result. Strains carrying the stx_{2f} variant and the *eae* rho variant were not detected. The sequences of the primers and probes in the GeneDisc assays used in the current study are proprietary; however, based on discussions with the manufacturer, the stx_{2f} and *eae* rho variants are not detected with the assays. The *stx* and *eae* subytpes of the strains tested in this study were not determined; however, PCR assays using primers described by Fratamico and DebRoy (2010) and Fratamico et al. (2011) were used to determine if the strains carried stx_1, stx_2, and *eae*.

In the current study, we compared enrichment in BPW (as recommended by GeneDisc) and mTSB for STEC detection in ground beef. mTSB contained selective agents, and the total enrichment time was 24 h (6 h at 37°C and 18 h at 42°C after addition of three other selective agents). BPW does not contain

selective agents and enrichment was for 18 h at 42°C. PCR results with mTSB and BPW were similar. Uninoculated ground beef samples were negative for all of the target genes. The cycle threshold (Ct) values for samples enriched in mTSB ranged from 18.5 ± 1.0 to 26.9 ± 4.8 (**Table 2**). The Ct values for PCR assays using DNA extracted from samples enriched in BPW ranged from 18.6 to 26.3 ± 5.9 (**Table 3**). Target genes in all of the STEC strains tested were correctly identified, and there were no notable differences in Ct values among the different target genes amplified. Some variability in initial inoculum levels, strain to strain variability in growth, and DNA extraction may have accounted for some of the differences observed in Cts. The standard deviations were based on results of at least three different experiments, except for STEC O111 in which there were three experiments done with strain 00-4748, which was negative for *ehxA*, and only one experiment each for strains 98-8338, 01387, SJ14, SJ15, and E05. Each experiment consisted on one sample inoculated with each serogroup. Several strains were used for

Table 2 | Average cycle thresholds ± STDEV of GeneDisc assays using mTSB for enrichment of ground beef samples.

Serogroup-strain[a]	*stx*$_1$	*stx*$_2$	*eae*	*ehxA*	O157[b]	O group[b]
O26	24.8 ± 0.8	–	26.5 ± 0.6	24.7 ± 0.8	–	25.3 ± 1.1
O45	22.1 ± 3.4	–	24.0 ± 3.1	22.4 ± 2.9	–	22.7 ± 2.6
O91	–	21.0 ± 1.5	–	20.4 ± 1.5	–	20.8 ± 1.3
O103	20.0 ± 2.9	–	21.8 ± 2.3	20.5 ± 2.0	–	21.8 ± 2.4
O111						
00-4748	25.0 ± 4.4	25.2 ± 4.2	26.9 ± 4.8		–	26.0 ± 4.7
98-8338	23.6	–	24.3	23.0	–	23.5
01387	25.2	–	25.7	24.0	–	25.4
SJ14	21.4	22.0	22.2	20.6	–	20.9
SJ15	21.0	19.7	23.4	20.0	–	24.4
E05	20.9	–	21.4	19.7	–	22.2
O113	–	18.8 ± 0.8	–	19.1 ± 0.9	–	18.5 ± 1.0
O121	–	23.2 ± 2.1	24.2 ± 2.3	22.5 ± 2.1	–	21.7 ± 1.9
O145	20.3 ± 3.4	–	22.6 ± 3.9	21.0 ± 3.2	–	21.9 ± 5.5
O157	18.9 ± 0.4	19.5 ± 0.2	20.9 ± 0.5	20.0 ± 0.7	19.9 ± 0.4	21.0 ± 0.6

[a] *Results represent averages from at least three separate experiments except for O111 strains 98-8338, O1387, SJ14, SJ15, and E05 for which only one experiment was performed.*

[b] *Results in the column labeled O157 represent data from the STEC and E. coli O157 GeneDisc that targeted virulence genes, as well as O157. The column labeled O group shows data from the GeneDisc 9 EHEC Identification that targeted the 9 O serogroups.*

Table 3 | Average cycle thresholds ± STDEV of GeneDisc assays using BPW for enrichment of ground beef samples.

Serogroup-strain[a]	*stx*$_1$	*stx*$_2$	*eae*	*ehxA*	O157[b]	O group[b]
O26	22.7 ± 3.2	–	24.8 ± 4.1	23.2 ± 3.2	–	22.5 ± 3.1
O45	20.9 ± 3.7	–	23.6 ± 4.3	21.6 ± 3.7	–	22.6 ± 3.4
O91	–	22.7 ± 3.7	–	22.0 ± 4.5	–	22.4 ± 3.5
O103	22.2 ± 3.1	–	23.8 ± 3.2	22.4 ± 3.2	–	23.3 ± 2.7
O111						
00-4748	22.8 ± 4.6	23.0 ± 6.0	23.2 ± 2.6	–	–	21.9 ± 4.2
98-8338	21.7	–	22.7	21.7	–	20.4
01387	21.1	–	22.2	20.7	–	20.3
SJ14	20.0	20.8	22.0	20.6	–	20.3
SJ15	21.4	18.6	23.2	19.0	–	21.2
E05	20.0	–	21.2	19.4	–	19.4
O113	–	20.7 ± 2.8	–	21.1 ± 3.2	–	21.2 ± 3.0
O121	–	24.6 ± 6.7	26.3 ± 5.9	24.2 ± 5.9	–	24.6 ± 4.4
O145	22.9 ± 3.7	–	25.2 ± 4.7	23.5 ± 3.9	–	25.3 ± 3.7
O157	18.8 ± 2.3	19.2 ± 2.3	19.6 ± 0.9	20.0 ± 1.8	19.1 ± 1.0	20.9 ± 3.3

[a] *Results are averages from at least three separate experiments except for O111 strains 98-8338, O1387, SJ14, SJ15, and E05 for which only one experiment was performed.*

[b] *Results in the column labeled O157 represent data from the STEC and E. coli O157 GeneDisc that targeted virulence genes, as well as O157. The column labeled O group shows data from the GeneDisc 9 EHEC Identification that targeted the 9 O serogroups.*

STEC O111 because it was more difficult to isolate this serogroup on the Rainbow Agar O157 plates compared to the other strains, and we wanted to verify if there was strain to strain variation in the ability to identify STEC O111 colonies on Rainbow Agar O157. The STEC O91 and O113 strains tested lacked the *eae* gene, as expected. Several other O91 and O113 strains in our culture collection were also tested to determine the presence of the *eae* gene, and all were negative (data not shown).

Screening assays based on detection of *stx* and *eae* followed by PCR assays targeting genes in the O-antigen gene clusters of the top six non-O157 STEC serogroups are useful for detection of these serogroups; however, since some serogroups, including STEC O91 and O113 lack the *eae* gene, they would not be identified using screening assays that rely on samples to be positive for both stx_1/stx_2 and *eae*. Therefore, an assay that targets additional virulence genes such as *ehxA* and O-group-specific genes of the *eae*-negative STEC, such as serogroups O91 and O113, are useful for detection of these pathogens. A number of assays targeting STEC virulence genes and O-group-specific genes have been reported. Perelle et al. (2004) developed real-time PCR assays to detect STEC serogroups (O26, O55, O91, O103, O111, O113, O145, and serotype O157:H7). The PCR assays that were designed targeted the stx_1, stx_2, and $fliC_{H7}$ genes, as well as O-group-specific genes. These same investigators used a PCR-ELISA (enzyme-linked immunosorbent assay) targeting stx_1 and stx_2 for initial screening of food samples, and samples were further tested by a multiplex PCR assay targeting O-group-specific genes, the O103 *eae* gene, and an O-island gene of O145 (Perelle et al., 2007). This was followed by PCR assays to identify the specific O-group and *stx* type, and this method was used to screen raw milk and minced meat samples for STEC O26, O103, O111, O145, and O157. The results revealed that ca. 2.6 and 4.8% of minced meat and raw milk samples, respectively, were contaminated with the targeted STEC. This protocol is rather arduous and time consuming, and use of a method such as the GeneDisc system targeting all of the genes of interest in an easy to use format would be more rapid and less laborious.

It is important to recognize that detection of specific genes by PCR in food samples does not necessarily imply that the genes are carried by the same bacterium and that the bacterium is viable; therefore, isolation of the target pathogen is necessary. There are no commercially available plating media that are very suitable for differentiation and isolation of *E. coli* O157:H7 and important non-O157 STEC serogroups. Furthermore, there are selective agents used in agar media, which may inhibit some STEC strains. We described the use of Rainbow Agar O157 for isolation of non-O157 STEC in an earlier report (Fratamico et al., 2011); therefore, this agar was also used in current study. The typical color of the non-O157 STEC colonies on Rainbow Agar O157 was purple, magenta/mauve, blue-violet, gray, pink with dark pink center, and violet/light purple, for *E. coli* O26, O45, O103, O111, O121, and O145, respectively. *E. coli* O157:H7 appears as black or dark gray colonies on this agar. For the STEC that were subjected to IMS, typically 2–5 colonies were picked for confirmation by the PCR. *E. coli* O91 and O113 enrichments were plated directly without performing IMS; typical O91 and O113 colonies were blue-gray and cream in color, respectively. *E. coli* O91 and O113 colonies from enrichments that were not subjected to IMS were more difficult to identify on Rainbow Agar O157 due to the higher level of background colonies that were present; therefore, no colonies were picked for confirmation. Thirty-one to 100 percent of the presumptive STEC O26, O45, O103, O111, O121, O145, and O157 colonies picked from Rainbow Agar O157 were confirmed as the correct STEC serogroup, and generally the isolation rate was similar with colonies picked from mTSB and BPW enrichments (**Table 4**). The correct STEC O121 and O145 colonies were somewhat easier to pick and confirm from mTSB (100 vs. 55% confirmed for O121, respectively, and 90 vs. 31% for O145) compared to BPW; however, more work must be done to validate this observation.

Table 4 | Number (percentage) of colonies confirmed by real-time PCR assays out of total presumptive colonies picked from Rainbow Agar O157.

STEC serogroup	mTSB	BPW
O26	8/10 (80%)	11/11 (100%)
O45	7/9 (78%)	12/12 (100%)
O91[a]	Not available	Not available
O103	9/9 (100%)	11/11 (100%)
O111	9/24 (37.5%)	20/39 (51%)
O113[a]	Not available	Not available
O121	9/9 (100%)	6/11 (55%)
O145	9/10 (90%)	4/13 (31%)
O157	7/9 (78%)	7/7 (100%)

[a]*IMS was not performed on samples inoculated with STEC O91 and O113; therefore, colonies were not picked for confirmation by PCR.*

Results of the current study demonstrate that the GeneDisc real-time PCR technology is rapid and simple to use, and it is a promising method for testing for STEC in food samples. The versatility of the GeneDisc system is evidenced by reports on the use of a GeneDisc array for detection of various STEC virulence and O-group- and H-group-specific genes (Bugarel et al., 2010a,b), for genotyping *Salmonella* (Bugarel et al., 2011), and for detection and genotyping of *Clostridium botulinum* (Fach et al., 2011; Sevenier et al., 2012; Woudstra et al., 2012). New types of assays can be designed by incorporating primers and probes for new gene targets. In the future, GeneDisc assays can be designed for detection of other STEC virulence genes and emerging STEC serogroups, including O104, O55, or others. The current study demonstrated that GeneDisc assays for STEC detection are rapid, simple, and accurate and can be used for screening for these pathogens in ground beef and potentially other foods, as well.

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O-antigen and virulence profiling of Shiga toxin-producing *Escherichia coli* by a rapid and cost–effective DNA microarray colorimetric method

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Shiga toxin-producing *Escherichia coli* (STEC) is a leading cause of foodborne illness worldwide. The present study developed the use of DNA microarrays with the ampliPHOX colorimetric method to rapidly detect and genotype STEC strains. A low-density 30-mer oligonucleotide DNA microarray was designed to target O-antigen gene clusters of 11 *E. coli* serogroups (O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, and O157) that have been associated with the majority of STEC infections. In addition, the DNA microarray targeted 11 virulence genes, encoding adhesins, cytotoxins, proteases, and receptor proteins, which have been implicated in conferring increased ability to cause disease for STEC. Results from the validation experiments demonstrated that this microarray-based colorimetric method allowed for a rapid and accurate genotyping of STEC reference strains from environmental and clinical sources and from distinct geographical locations. Positive hybridization signals were detected only for probes targeting serotype and virulence genes known to be present in the STEC reference strains. Quantification analysis indicated that the mean pixel intensities of the signal for probes targeting O-antigen or virulence genes were at least three times higher when compared to the background. Furthermore, this microarray-based colorimetric method was then employed to genotype a group of *E. coli* isolates from watershed sediment and animal fecal samples that were collected from an important region for leafy-vegetable production in the central coast of California. The results indicated an accurate identification of O-type and virulence genes in the tested isolates and confirmed that the ampliPHOX colorimetric method with low-density DNA microarrays enabled a fast assessment of the virulence potential of STEC using low-cost reagents and instrumentation.

Keywords: DNA microarrays, photopolymerization, Shiga toxin, *Escherichia coli*, foodborne pathogen, ampliPHOX, genotyping, STEC

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a food- and water-borne enteric pathogen known to cause human gastrointestinal illnesses with diverse clinical manifestations, ranging from diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome (HUS; Rangel et al., 2005; Gyles, 2007). The varying disease severity in humans has been associated with more than 100 serotypes of STEC (Johnson et al., 1996; Gould et al., 2009; Mathusa et al., 2010). In particular, *E. coli* O157:H7 is the STEC most-commonly reported in the United States (Brooks et al., 2005; Hoefer et al., 2011), and more than 20 outbreaks due to O157:H7 contamination, occurring between 1995 and 2008, were associated with consumption of produce items that were grown in or near the Salinas Valley of California (Mandrell, 2011). Although the O157 serogroup has been associated frequently with hemorrhagic colitis and HUS (Karmali, 1989; Karmali et al., 2003), the reporting of non-O157 infections by public health laboratories has increased considerably in recent years (Brooks et al., 2005; Gould et al., 2009; Hoefer et al., 2011; Stigi et al., 2012). Epidemiological studies have indicated that six non-O157 serogroups, O26, O45, O103, O111, O121, and O145, have been associated with outbreaks and severe disease in the United States (Johnson et al., 1996; Brooks et al., 2005). Additionally, STECs of serogroups, O91, O104, O113, and O128 have been reported to be significant causes of human infections worldwide (Brooks et al., 2005; Bettelheim, 2007; Gyles, 2007; Mathusa et al., 2010). These findings have led to the proposal that certain non-O157 STEC strains are potentially as virulent as O157:H7 strains (Bettelheim, 2007; Coombes et al., 2011).

The onset of life-threatening disease symptoms caused by STEC has been associated with the production of Shiga toxins (Stx), which are considered the primary virulence determinant in STEC (Karmali et al., 1983; Karmali, 1989). However, epidemiological studies revealed that not all STEC strains that produce Stx are clinically significant. The observed difference in virulence among distinct serotypes has been ascribed to genes encoding virulence factors other than Stx and has been

linked to genes on pathogenicity islands, such as the locus for enterocyte and effacement (LEE) and the non-LEE (Bettelheim, 2007; Bolton, 2011; Coombes et al., 2011). In addition to these genomic regions, other chromosomal and plasmid-borne virulence genes, encoding adhesins, cytotoxins, proteases, and receptor proteins, are proposed to also contribute to STEC pathogenesis by allowing these bacterial pathogens to attach to and colonize the human epithelium. Screening for these virulence genes in STEC isolates would facilitate an expanded characterization through the identification of risk factors that could lead potentially to sporadic- and outbreak-related human illness. Thus, the development of rapid and cost–effective genotyping tools is imperative for determining the genetic composition of STEC isolates that may pose a threat to food safety and human health.

In contrast to established culturing methods, which only assess a small number of markers, the use of DNA microarrays as a genotyping method enables the examination of multiple gene targets in a single assay (Call, 2005; Uttamchandani et al., 2009). However, development of sensitive and rapid procedures with cost–effective instrumentation has been challenging with current DNA microarray platforms. Fluorescent microarray methods are commonly used procedures but can be inconsistent and highly variable. These methods also utilize expensive and non-portable scanners for data acquisition and analysis (Call, 2005; Kuck and Taylor, 2008). Thus, improvements in the cost–effectiveness and reliability of procedures are required for routine pathogen surveillance. To implement a simpler, rapid, and inexpensive method for identifying and characterizing pathogenic STEC strains, the present study developed the use of the ampliPHOX colorimetric method, based on light-initiated signal amplification through polymerization (Kuck and Taylor, 2008; Sikes et al., 2008), with a low-density microarray to simultaneously detect multiple STEC serotypes and virulence genes. This microarray-based genotyping method was employed to characterize a collection of STECs, recovered from environmental and clinical sources and from distinct geographical locations.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

The characteristics and sources of the STEC reference strains that were used in this study are described in **Table 1**. For the isolation of STEC isolates from environmental sources, cloacal bird swabs or 10 g samples of watershed sediment/animal feces were subjected to a non-selective enrichment step in 50 or 90 ml of tryptic soy broth (Beckton Dickinson, Sparks, MD, USA), respectively, at 25˚C for 2 h, 42˚C for 8 h with shaking, and 4˚C until the following morning (Cooley et al., submitted). The enrichments were further screened for *stx1* and *stx2* by real-time PCR, as in previous studies (Cooley et al., 2007). STEC isolates were obtained after plating samples from positive enrichments on Rainbow O157 agar (Biolog, Inc., Hayward, CA, USA) containing 20 μg/ml novobiocin and 0.8 μg/ml potassium tellurite solution (Sigma-Aldrich, St. Louis, MO, USA; NT-Rainbow) or on CHROMagar O157 media (DRG International, Mountainside, NJ, USA) after immunomagnetic separation with Dynabeads®anti-*E. coli* O157 (Invitrogen, Carlsbad, CA, USA) as reported previously (Cooley et al., 2007). Suspect colonies were selected from NT-Rainbow or CHROMagar plates based on colony colors and morphologies, as recommended by the manufacturer's product insert. Bacterial cultures were propagated on Luria–Bertani (LB) agar (Difco, Detroit, MI, USA).

CONSTRUCTION OF THE DNA MICROARRAY

To simultaneously evaluate multiple virulence determinants and serotypes of STEC strains, a 30-mer DNA oligonucleotide microarray was constructed. The serotype-specific probes were

Table 1 | Shiga toxin-producing *Escherichia coli* reference strains used in this study.

Strain	Other strain designations	Serotype	Source	Location	Provider or reference[a]
RM1697	Feedlot strain 42	O157:H7	Cattle	United States	Kimura et al. (2000)
RM2016	3323-61; DEC 9a	O26:H11	Human	United States	ECRC (Reid et al., 1999)
RM2028	C142-54; DEC 6b	O111:H12	Human	Germany	ECRC (Reid et al., 1999)
RM2048	A9619-c2; DEC 11c	O45:H2	Human	United States	ECRC (Reid et al., 1999)
RM2084	EDL-933; DEC 4f	O157:H7	Meat	United States	ECRC (Reid et al., 1999)
RM3642	CPK175	O121:NM	Human	Canada	CPKDRC
RM3655	CPK117	O113:H21	Human	Canada	CPKDRC
RM6011	WI_06BC005150	O157:H7	Human	United States	Tim Monson (Cooley et al., 2007)
RM6765	ATCC 23982; H515b	O103:H8	Human	Denmark	ATCC (Ørskov et al., 1977)
RM7006	B2F1; TW01393	O91:H21	Human	Canada	The STEC Center (Ito et al., 1990)
RM7007	T4/97; TW09358	O128:H2	Pigeon	Germany	The STEC Center (Schmidt et al., 2000)
RM7519	F260-H2	O113:H21	Cattle	United States	Laboratory collection
RM12761	EH1605	O145:H28	Ice cream	Belgium	Buvens et al. (2011)
RM13368	3024-94	O104:H21	Human	United States	CDC (1995), Dean-Nystrom et al. (2003)

[a] *Contact information of strain providers: ECRC, E. coli Reference Center, College of Agricultural Sciences, The Pennsylvania State University, University Park, PA, USA; CPKDRC, Canadian Pediatric Kidney Disease Research Centre, Ottawa, Ontario, Canada; Tim Monson, Wisconsin State Laboratory of Hygiene, Madison, WI, USA; ATCC, American Type Culture Collection, Manassas, VA, USA; The STEC Center, National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI, USA.*

designed to target either *wzx* or *wzy* genes in the O-antigen gene clusters of 11 serogroups and to target the flagellin H7 antigen (*fliC*; **Table 2**). The virulence-specific probes targeted genes encoding Stx (*stx1* and *stx2*), intimin (*eae*), non-enterocyte effacement effectors (*nleA* and *ent/espL2*), extracellular serine protease (*espP*), catalase peroxidase (*katP*), autoagglutinating adhesin (*saa*), subtilase cytotoxin (*subA*), enterohemolysin (*ehxA*), and perosamine synthetase (*per*; Wang et al., 2003; Bettelheim, 2007; Bolton, 2011; Quiñones et al., 2011; **Table 2**). The *gadB* gene, encoding glutamate decarboxylase (Smith et al., 1992), was included as a positive control to confirm that the DNA tested was derived from *E. coli* (**Table 2**). The probes were designed using PRIMER3 software (Rozen and Skaletsky, 2000), were purchased from Eurofins MWG Operon (Huntsville, AL, USA) with a 5′-amino-C6 modification for covalent binding to the slide surface, and were spotted in duplicate at a final concentration of 50 μM on ArrayIt®SuperEpoxy 2 microarray slides (Arrayit Corporation, Sunnyvale, CA, USA). As a positive control for the ampliPHOX reaction, a synthetic, 24-mer oligonucleotide probe with a 5′-amino-C6 and 3′-biotin modification (InDevR, Inc., Boulder, CO, USA) was spotted at a final concentration of 500 nM; this biotinylated control oligonucleotide does not have any sequence homology to any *E coli* genes. The microarrays were manufactured with an approximate spot diameter size of 200 μm and a center-to-center spacing of 700 μm (Arrayit Corporation). After printing, an adhesive microarray well (9 mm diameter, InDevR, Inc.) was placed in the center of the printed array, and the microarray slides were stored in a desiccator until further use.

POLYMERASE CHAIN REACTION

The detection of serotyping and virulence genes was achieved by performing a multiplex PCR amplification of biotinylated fragments, ranging in size between 154 and 342 base pairs (**Table 3**). PCR primers were purchased from Eurofins MWG Operon (Huntsville, AL, USA) with a 5′-phosphorylated modification for the forward primers and a 5′-biotin modification for the reverse primers. The target screen was divided into four groups, and each multiplex PCR reaction amplified six different targets (**Table 3**). As template for the PCR reaction, bacterial cultures of the reference strains and environmental isolates were grown aerobically in LB broth (Difco) for 24 h with constant shaking (200 rpm) at 37°C, and 100 μl aliquots of the bacterial cultures were collected by centrifugation at 2000 × *g* for 5 min. Cell pellets were resuspended in 100 μl of HyPure™ molecular biology-grade water (HyClone Laboratories, Inc., Logan, UT, USA) and incubated at 95°C for 20 min. The lysates were centrifuged at 2000 × *g* for 5 min, and the supernatants were

Table 2 | List of DNA oligonucleotides probes used in this study for microarray construction.

Target gene[a]	Probe sequence (5′ → 3′)	Accession number	Reference
GROUP 1			
wzy_{O26}	ATCAAGACTATGAAGCGTATGTTGATATAT	AF529080	This study
wzy_{O45}	GTATACCGACATATCATGGGATAGTTAATA	AY771223	This study
wzy_{O103}	CTAAGATTCATCGAGGATGAGGGGTTATTT	AY532664	This study
wzy_{O111}	CTTTCATTGTTGTAAGTTGTTTGTTACTGT	AF078736	This study
wzy_{O121}	CCTTTGTCATACTCTTTTTGATATTATCAC	AY208937	This study
wzy_{O145}	GCCTGATTATTCCTCTATTTTCTTATAATA	AY647260	This study
GROUP 2			
wzy_{O104}	GTTCAAAGTTATTTTGGAGAGAAAGATATT	AF361371	This study
wzx_{O91}	GAGAGATTAAACAAGGATATCTGTTTCTTC	AY035396	This study
wzy_{O113}	GATGATATGTTTTATTATATGGTTGTTAAG	AF172324	This study
wzy_{O128}	TTTATATCAATGTGCTCATTAATTACACTG	AY217096	This study
wzy_{O157}	CAGGGAATAAAGCATCAAGACTTATTTTAT	AF061251	This study
gadB	AGATTATCAATGACGAATTATATCTTGATG	M84025.1	This study
GROUP 3			
ent	TATAATAAACCTGACAGCATATGATTTTCT	AAG58110	This study
espP	CGGATATAATCAGTCTTTCAATAAAATTGC	X97542	This study
katP	GGGACTTATTTATGTCAATCCTGAAGGCCC	X89017	This study
nleA	TACAGATATTCCCTCTGCATGATGAAATAG	BAF96541	This study
saa	TAAATAAAAATAGAAAACGTTCAGATGCCG	AF325200	This study
subA	GCAAGATCCTGTGATAAGAGTCGCTGCCCT	AF399919	This study
GROUP 4			
eae	CATTGATCAGGATTTTTCTGGTGATAATAC	M58154	This study
ehxA	CAAAACAGGCTATGTTTGAGCACGTTGCAG	EF204929	This study
$fliC_{H7}$	ATCTGAAAACCGAAAATACCTTGTTAACTA	AF169323	This study
per	TAGGCTACAATTATAGGATGACAAATATCT	AAG57096	Quiñones et al. (2011)
stx1	GTTCTTATGTAATGACTGCTGAAGATGTTG	AAG57228	This study
stx2	GGTTTCCATGACAACGGACAGCAGTTATAC	AAG55587	This study

Table 3 | List of DNA oligonucleotides used in this study for multiplex PCR amplification of biotinylated fragments.

Target gene[a]	Primers for PCR			Amplicon (bp)	Accession number	Reference
	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)	Amount[b]			
Group 1						
wzy_{O26}	GGTTTATGGATTATTGCATTC	CCAATTATGACTTCATGGGT	1.0	221	AF529080	This study
wzy_{O45}	CGTCTGGATGAAATTATGCC	GTCTGAAGACCAGCATTTCCC	0.5	289	AY771223	This study
wzy_{O103}	ATACAAATGGCGTGGATTGG	GCCAGTAATTGACGTAACTG	0.5	280	AY532664	This study
wzy_{O111}	GGTGTGATAGGAGCATTGGT	CAACTCCAACTGTAAGCCCG	0.5	192	AF078736	This study
wzy_{O121}	CCCGATATTCTAGTAGCCGT	CTTCAATGAGTGCAGGCAAA	1.0	235	AY208937	This study
wzy_{O145}	GCCCTTTTCAGTGGTGCG	GCAGCCCAATATGAAACCAT	0.5	206	AY647260	This study
GROUP 2						
wzy_{O104}	GTTCATTAGATCGAGGTT	CTTGTCTGATACGGTCAAGTG	0.4	162	AF361371	This study
wzx_{O91}	CAAGCAGGTATTTGGGATGG	TCCCCCATAGATACGAATGA	1.0	248	AY035396	This study
wzy_{O113}	TGAGCGTTTCTGACATATGGA	GTTTCGCTGGCATATTACTG	0.4	296	AF172324	This study
wzy_{O128}	TGGATTTGATTGGGGGAAT	TCTTGCAAAACACCGCATAC	1.0	190	AY217096	This study
wzy_{O157}	CCGACACCAGAGTTAGAAAAG	CAGTTCTGCTCCATACGTAG	0.4	156	AF061251	This study
gadB	CACGTTTTGGTGCGAAGTCT	TTGTGGACATTTTCGTCGTC	0.2	175	M84025.1	This study
GROUP 3						
ent	CACATCATTAGAAGTTCATT	AGTCCTGCTCCCATAGCAAA	0.5	342	AAG58110	This study
espP	GCACTGTCTGGCGGTGAATA	CGTCCAGATTCCCGTTTATG	0.1	202	X97542	This study
katP	GCGGAAGAGAAGATGACTGG	GCACCATGTGCTTTACCAAA	1.0	277	X89017	This study
nleA	TATGGTGTCCAGCTAAACAT	CCAGTCGATGCAATAGTCGA	0.1	185	BAF96541	This study
saa	CCAATCAACAGTTTCGTCAA	GCAATAGCCTGTTCATCACG	0.1	166	AF325200	This study
subA	CGGCTTATCATCCTGTCAGC	TATAGCTGTTGCTTCTGACG	0.1	233	AF399919	This study
GROUP 4						
eae	CGTTACATTGACTCCCGCTT	CTCATGCGGAAATAGCCGTT	0.2	188	M58154	This study
ehxA	GAGTTCTGTATCTGCGGGAG	GCAAGTTCACCGATCTTCTC	0.75	339	EF204929	This study
$fliC_{H7}$	CAACAAAGCTGCAACGGTAA	GCCGCCAACTGTGACTTTAT	0.2	154	AF169323	This study
per	GGTGAAGGTGGAATGGTTGTC	TCAGCAATTTCACGTTTTCGTG	0.2	206	AAG57096	Quiñones et al. (2011)
stx1	GGATGCAGATAAATCGCCAT	GAGTCTTGTCCATGATAGTC	0.4	274	AAG57228	This study
stx2	GATTTYDCACATATWTCAKTGCC	AACTCCATTAAMKCCAGATATGA	0.75	155	AAG55587	This study

[a] *Gene targets examined in each multiplex group.*

[b] *Final micromolar concentration of primer used for each multiplex group in a 50-μl PCR reaction.*

collected and frozen until further use. For each multiplex group (**Table 3**), the PCR amplification was performed in a 50-μl reaction mixture, containing 3 μl of bacterial crude lysate, 25 μl of 2× GoTaq®Colorless Master Mix (Promega Corporation, Madison, WI, USA), and 5 μl of 10× multiplex group primer mix, as shown in **Table 2**. The reaction mixtures were placed in a Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following settings: 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 60°C (group 1) or 1 min at 55°C (groups 2–4), 1 min at 72°C, and a final extension time of 7 min at 72°C. Identification of serotype and virulence genes in the *E. coli* isolates was confirmed by a single PCR assay, and each reaction consisted of 12.5 μl of 2× GoTaq®Green Master Mix (Promega Corporation), 0.5 μM of each primer (**Table 3**), and 3 μl of the bacterial crude lysate in a total volume of 25 μl. Amplified products were analyzed in 2% agarose gels containing 0.04 μl/ml GelRed Nucleic Acid Stain (Phenix Research, Candler, NC, USA).

MICROARRAY HYBRIDIZATION

For each hybridization reaction, 45 μl of PCR amplicons were purified by using the MinElute®PCR purification kit (Qiagen, Valencia, CA, USA). To achieve a rapid microarray hybridization (Boissinot et al., 2007), single stranded DNA targets were produced after a lambda exonuclease digestion of the PCR-amplified targets, consisting of 10 μl of the eluate, 10 U of lambda exonuclease and 1× lambda exonuclease reaction buffer (Epicenter Biotechnologies, Madison, WI, USA) in a final volume of 23 μl for 15 min at 37°C, followed by addition of 23 μl of 2× Hybridization Buffer (InDevR, Inc.). The hybridization mixture was applied to each microarray, and the slides were incubated in a humidified chamber (InDevR, Inc.) for 1 h at room temperature. Following hybridization, the slides were transferred to a slide drying tray (Evergreen Scientific, Los Angeles, CA, USA) and were rinsed with Microarray Wash Buffers A thru D (InDevR, Inc.) in the following order: wash Buffer D for 5 s, Wash Buffer A for 1 min, Wash Buffer D for 5 s, Wash Buffer B for 5 min, and Wash Buffer C for 5 min. The slides

were then dried by centrifugation at 200 × *g* for 2 min prior to labeling.

MICROARRAY LABELING, SIGNAL AMPLIFICATION, AND ANALYSIS

The hybridized microarrays were labeled after incubation with 40 μl of a streptavidin-conjugated ampliTAG™ labeling solution (InDevR, Inc.) for 5 min at room temperature in a dark humidity chamber, and immediately after labeling, microarrays were rinsed with Wash Buffer D for 5 s, Wash Buffer C for 5 min, distilled water for 5 min, and then dried by centrifugation at 200 × *g* for 2 min. Positive hybridization signals on each microarray were detected by incubation with 40 μl ampliPHY™ solution (InDevR, Inc.), followed by photoactivation for approximately 1–2 min with the ampliPHOX Reader™ and the associated ampliVIEW™ software 2.0 (InDevR, Inc.), as recommended by the manufacturer. Polymer formation was visualized after a 1-min staining with ampliRED™ solution. Color digital images of the stained arrays were acquired with the ampliPHOX Reader, and for each spot, quantification of signal and background mean pixel intensities were determined with the ampliVIEW software.

RESULTS

A RAPID AND INEXPENSIVE COLORIMETRIC MICROARRAY METHOD FOR GENOTYPING STEC

To implement using a rapid and cost–effective method to detect a selected set of genes in STEC isolates, recovered from an important food production region in the central coast of California, the present study explored an alternative procedure for genotyping STEC using DNA microarrays. This novel and innovative colorimetric method is based on light-initiated signal amplification through polymerization (Kuck and Taylor, 2008; Sikes et al., 2008). Instead of using a fluorophore, a streptavidin-conjugated photoinitiator specifically binds to biotin-labeled amplified targets, hybridized to oligonucleotide probes on the microarray (**Figure 1**). After a short light exposure, a colorless polymer forms where the probe and target sequences hybridized exclusively on the microarray. Polymer formation is easily visualized after a short staining step (**Figure 1**). The process of photopolymerization, also referred to as ampliPHOX colorimetric detection method, replaces expensive dyes and scanners required for fluorescent-based detection with microarrays (Kuck and Taylor, 2008; Moulton et al., 2011).

SPECIFICITY OF STEC IDENTIFICATION

As a proof-of-principle method for routine pathogen surveillance, a low-density microarray was designed to target relevant serotypes and virulence genes, associated with pathogenic STEC. To validate the specificity of using DNA microarrays with the ampliPHOX colorimetric method, several STEC reference strains, recovered from environmental and clinical samples with different genotypes, were examined (**Table 1**). The results of the validation experiments of group 1 and group 2 probes indicated this microarray method was accurate in detecting the O-antigen types of the STEC reference strains (**Figure 2**). Polymer formation was observed exclusively where the probe sequences were spotted on the microarrays, and the patterns of photopolymerization correlated with the O-type of the reference strains. For example, analysis of the O26 strain RM2016 resulted in polymer formation exclusively where O26 *wzy* gene probes were spotted on the microarray. Moreover, analysis of group 2 probes resulted in the accurate O-antigen identification in the reference strains as well as the *gadB* gene, included as a positive control for detecting all *E. coli* isolates (**Figure 2**).

Similar results were obtained when assessing the virulence profile of the STEC strains. In particular, analysis of O157:H7 strain RM2084 resulted in polymer formation for probes targeting *ent*, *nleA*, *espP*, and *katP* genes and for probes targeting *eae*, *per*, *ehxA*, *stx1*, *stx2*, and $fliC_{H7}$ when testing the multiplex group 3 or group 4, respectively (**Figure 2**). Further examination of the virulence profiles of the O113:H21 strain RM7519 and the O157:H7 strain RM6011 demonstrated that the patterns of polymer formation correlated with their genotype that was determined previously by conventional PCR. For reactions lacking a DNA template, polymer formation was observed only with the biotinylated oligonucleotide probes spotted on the slide as a control for the ampliPHOX polymerization reaction (**Figure 2**).

High mean pixel intensities of the signal were obtained for spots where polymer formation was detected. Positive signals for O157 O-antigen *wzy* gene spots from analysis of several experiments for O157:H7 strain RM1697 had mean pixel intensity values ranging from 84.4 ± 4.8 to 97.5 ± 4.5 with background values ranging from 25.2 ± 1.4 to 25.6 ± 1.5. Further, analysis of O157:H7 strains RM2084 and RM6011 and of O113:H21 strain RM7519 indicated positive signal values for all virulence spots ranged from 82.4 ± 2.9 to 108.3 ± 2.5 for the group 3 probes, and for the group 4 probes, mean pixel signal intensities ranged from 70.9 ± 6.1 to 112.8 ± 3.4. These results for the positive signals contrasted with background values, ranging from 25.2 ± 1.4 to 25.9 ± 1.8. Similar values were obtained for other arrays used in several experiments for genotyping reference strains with different O-antigens and virulence profiles. These findings indicated a significant difference between signal intensities and background noise, facilitating microarray data analysis, and accurate interpretation of results with this DNA microarray-based genotyping method.

GENOTYPING STEC ISOLATES FROM A PRODUCE PRODUCTION REGION IN CALIFORNIA

The high level of specificity of the microarray colorimetric method, obtained in the validation experiments, prompted us to expand the study to genotype *E. coli* isolates from produce production regions in California. Enrichment broths were prepared from watershed sediment or from wildlife and livestock fecal samples, and the recovery of *E. coli* isolates was achieved after plating an aliquot of the enrichment broth on selective solid media. A subset of recovered *E coli* isolates, identified initially to be positive for various relevant O-types by a conventional PCR assay, were selected for analysis with the microarray colorimetric method. As shown in **Table 4**, the results indicated that this microarray colorimetric method accurately determined the O-antigen serogroup of the tested environmental STEC isolates. The formation of polymer on spots targeting a particular O-type correlated with the O-antigen that was determined previously by a conventional PCR assay (**Table 4**). Quantification analysis revealed that the mean pixel intensity values of the O-antigen spot signals for all tested isolates ranged from 75.6 ± 6.3 to 110.1 ± 4.8; however, values ranging from 25.1 ± 1.6 to 25.9 ± 2.7 were detected for the background.

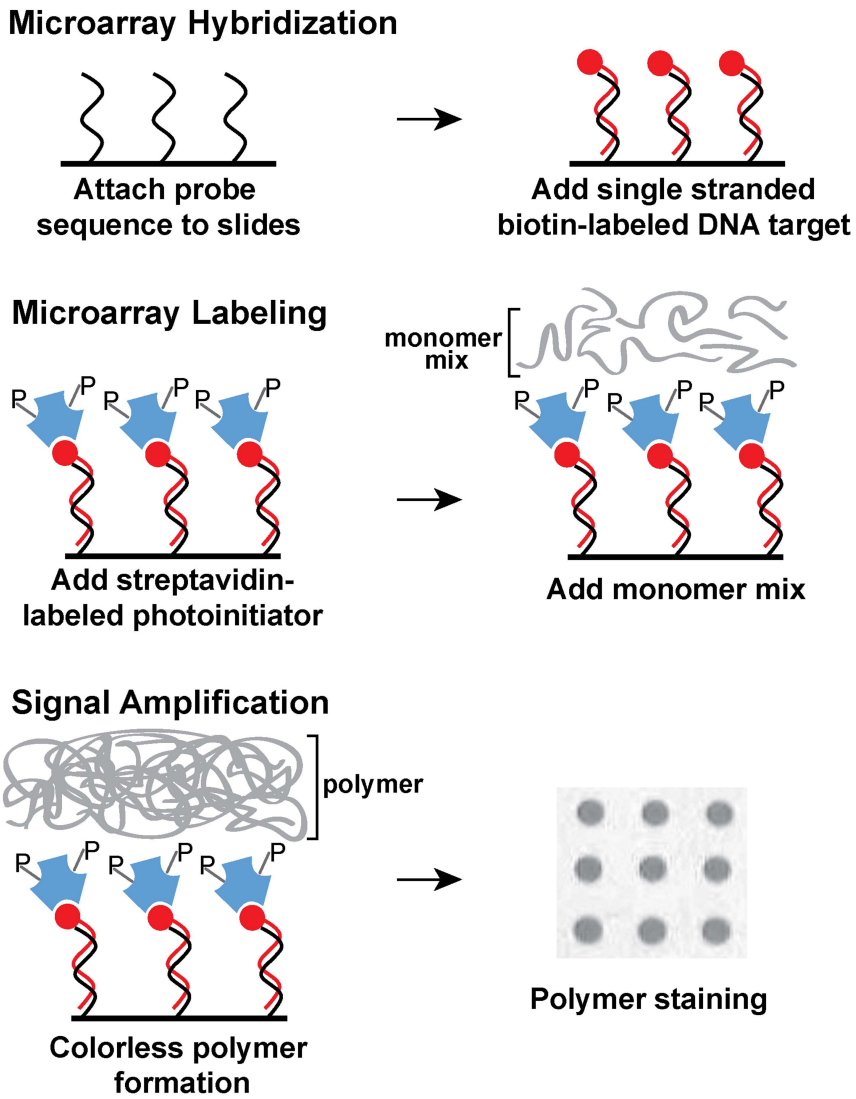

FIGURE 1 | Schematic diagram of ampliPHOX colorimetric method with DNA microarrays. First, DNA microarrays are hybridized with DNA target labeled with biotin (red circles). Second, the microarray is labeled with a photoinitiator (letter P) that is conjugated to streptavidin (blue polygons). Third, a short light-initiated polymerization reaction results in a colorless polymer localized exclusively where the probe and target sequences hybridized on the microarray. Polymer formation is visualized after a quick staining step.

The virulence profile of the isolates was then analyzed by this microarray method. The analysis indicated that more than 50% of the tested isolates were positive for the virulence factors *eae*, *ehxA*, *ent*, *espP*, and *stx1*, and STEC isolates harboring the same O-type were also similar in virulence gene profiles (**Table 4**). Similar to the O-antigen analysis, the virulence profiles were confirmed by a separate analysis using conventional PCR assay (data not shown). Further quantification of the detected microarray signals in the analysis of O45 cattle isolate RM10729 resulted in average signal intensity values of 99.3 ± 4.7, 106.7 ± 6.3, and 78.8 ± 3.8 for spots targeting *espP*, *saa*, and *stx1* genes, respectively with a background value of 25.3 ± 2.4 for this microarray examined. Moreover, quantification of the mean pixel intensity of *stx2* spots had a signal value of 103.1 ± 3.8 and a background value of 25.4 ± 3.5 when analyzing feral pig isolate RM10740. These quantification results were typical for all environmental isolates examined by this microarray method. These results revealed a clear distinction between positive signals, where polymer formation was observed on microarray spots, when compared to the background, and this facilitated accurate genotyping of the tested environmental isolates.

DISCUSSION

The growing concern about sporadic and outbreak illnesses associated with STEC has prompted the development of methods for more efficient detection of STEC in the United States (Johnson et al., 1996; Brooks et al., 2005; Gould et al., 2009; Hoefer

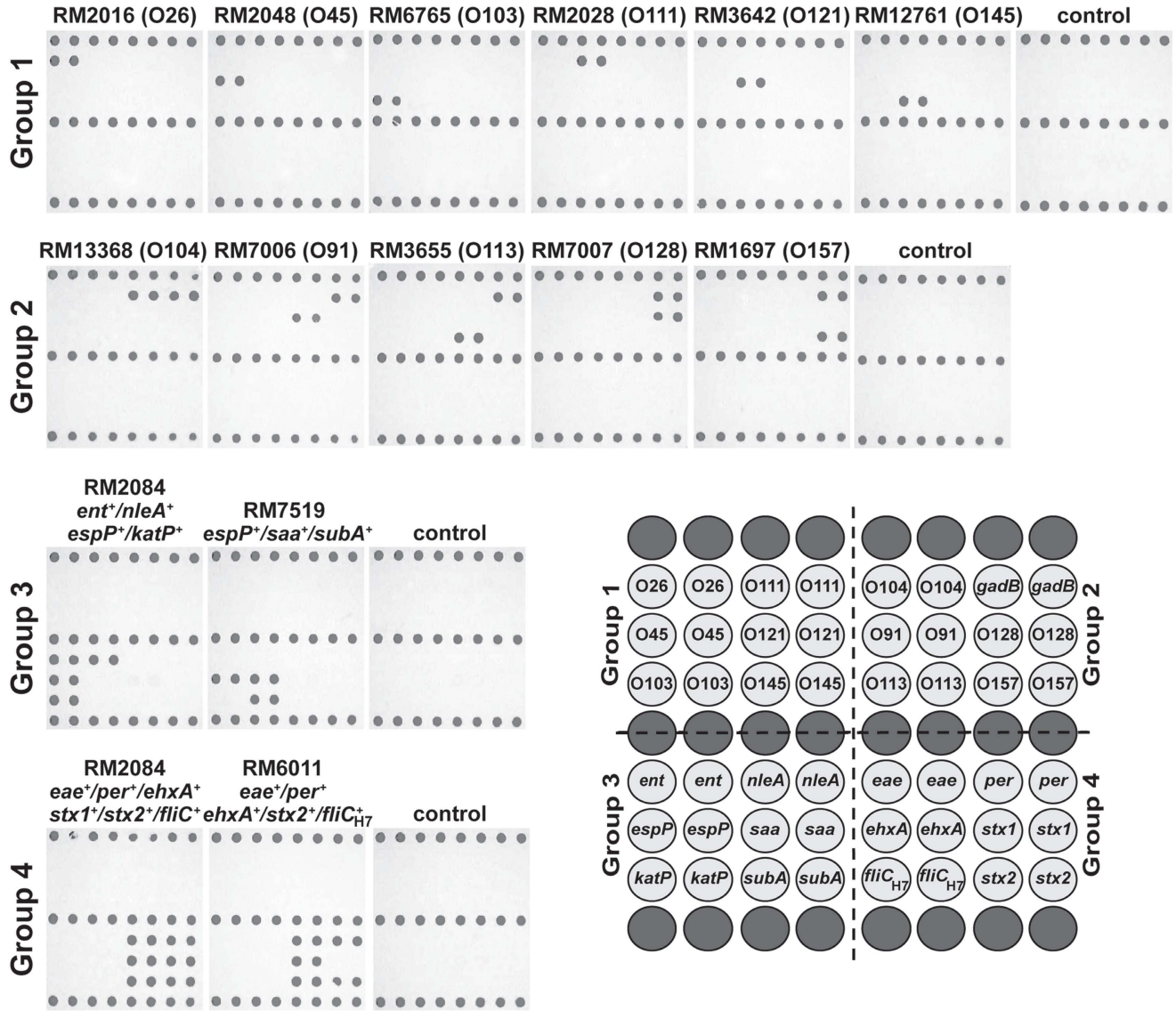

FIGURE 2 | Genotyping Shiga toxin-producing *Escherichia coli* (STEC) with low-density DNA microarrays and the ampliPHOX colorimetric method. STEC reference strains from clinical or environmental sources with different genotypes (**Table 1**) were analyzed by 30-mer oligonucleotide DNA microarrays and the ampliPHOX colorimetric method. The panels, shown under strain designations, illustrate polymer formation on spotted probes for microarrays hybridized with amplification reactions testing a particular STEC reference strain or controls lacking a DNA template. The array layout shows the duplicate spots with probes targeting the O-antigen and virulence genes that are represented in groups 1–4. Biotinylated probes spotted along the top, middle and bottom rows (dark gray) of the array layout correspond to positive controls for polymer formation.

et al., 2011; Stigi et al., 2012). To address this issue, the present study developed a simple, rapid, cost–effective, and quantitative colorimetric method for the assessment of molecular risk factors associated with STEC pathogenesis. The advantage of using the DNA microarray platform in conjunction with ampliPHOX colorimetric method was successfully demonstrated by simultaneously detecting highly relevant genes on a selected group of STEC recovered from clinical and environmental sources. Previously, a low-density DNA oligonucleotide microarray was developed to target *E. coli* O157 (Quiñones et al., 2011), the STEC that has been most-commonly associated with produce outbreaks that traced back to leafy vegetables from the United States (Cooley et al., 2007; Mandrell, 2011). In the present study, additional probes were designed to target six non-O157 serogroups, reported previously to be associated with the majority of STEC infections in the United States and worldwide as well as another set of non-O157 serogroups, linked to sporadic disease and diarrhea (Brooks et al., 2005; Bettelheim, 2007). The selected virulence-specific probes targeted *stx*, *eae*, *nle*, and *ent* genes, located on pathogenicity islands and implicated in conferring *E. coli* strains with an increased ability to cause disease (Coombes et al., 2008; Bolton, 2011). In addition to *katP*, *exhA*, *espP*, and s*ubA* gene detection, this microarray

Table 4 | Genotyping Shiga toxin-producing *Escherichia coli* environmental isolates with DNA microarray-based ampliPHOX colorimetric method.

Isolate number	Source	O-antigen PCR assay	Microarray-ampliPHOX genotyping assay genes																							
			wzy$_{O26}$	*wzy*$_{O45}$	*wzx*$_{O91}$	*wzy*$_{O103}$	*wzy*$_{O104}$	*wzy*$_{O111}$	*wzy*$_{O113}$	*wzy*$_{O121}$	*wzy*$_{O128}$	*wzy*$_{O145}$	*wzy*$_{O157}$	*eae*	*ehxA*	*ent*	*espP*	*fliC*$_{H7}$	*katP*	*nleA*	*per*	*saa*	*stx1*	*stx2*	*subA*
RM7847	Cattle	O26	+	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−
RM7914	Coyote	O26	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	+	−	−	+	−	−
RM9913	Feral pig	O26	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	+	−	−
RM9414	Cattle	O45	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−
RM9408	Cattle	O45	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−
RM10729	Cattle	O45	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−
RM7190	Feral pig	O91	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−
RM7191	Sediment	O91	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−
RM7933	Cattle	O91	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
RM9882	Cattle	O103	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	+	−	−
RM10408	Crow	O103	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	+	−	−
RM10804	Feral pig	O103	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	−	+	+	−	−	+	−	−
RM8799	Cattle	O104	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−	+	−	+	+
RM9387	Cattle	O104	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−	+	−	+	+
RM11037	Cattle	O104	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−	+	−	+	+
RM9322	Sediment	O111	−	−	−	−	−	+	−	−	−	−	−	+	+	+	−	−	−	+	−	−	+	−	−
RM9881	Cattle	O111	−	−	−	−	−	+	−	−	−	−	−	+	+	+	−	−	−	+	−	−	+	−	−
RM9907	Feral pig	O111	−	−	−	−	−	+	−	−	−	−	−	+	+	+	−	−	−	+	−	−	+	−	−
RM7513	Cattle	O113	−	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	+
RM7806	Feral pig	O113	−	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	+
RM10410	Crow	O113	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
RM10046	Cattle	O121	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+	−	−	+	−	−	−	+	−
RM10068	Sediment	O121	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+	−	−	+	−	−	−	+	−
RM10740	Feral pig	O121	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
RM7408	Deer	O128	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	+	+	−
RM10461	Cattle	O128	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	+	+	−	−
RM10743	Sediment	O128	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	+	+	−	−
RM9303	Cattle	O145	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	−	−	−	−	−	+	−	−
RM9320	Soil	O145	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	−	−	−	−	−	+	−	−
RM9917	Feral pig	O145	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	−	+	−	−	−	+	−	−
RM9471	Cattle	O157	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+	−
RM9908	Soil	O157	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	+	−
RM10058	Crow	O157	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	+	+	−	−	+	−

[+] *Signals are provided with grey shade.*

method targeted *saa*, encoding an attachment factor that has been found in non-O157 STEC strains isolated from patients suffering from severe disease symptoms (Bettelheim, 2007; Bolton, 2011). An important advantage of the ampliPHOX colorimetric method is the relatively short 30-mer oligonucleotide probes used, thus facilitating the specific detection of target genes without any cross-hybridization of non-target genes. In contrast, the commonly used fluorescent assays for microarray detection require longer probes (70 or 100-mer) to distinguish signals from the background noise (Letowski et al., 2004). Quantification of the colorimetric positive signals in this study revealed that mean pixel intensities were at least three times higher than the colorimetric signals for spots without any specific hybridization, and consequently, positive signal intensities were discriminated easily from background noise.

The use of DNA oligonucleotide arrays on glass slides in conjunction with traditional fluorescence detection assays for genotyping STEC has been reported previously (Call et al., 2001; Chizhikov et al., 2001; Chen et al., 2005; Bruant et al., 2006). The disadvantage of traditional fluorescent assays for microarray-based detection is that the labeling of target DNA with fluorescent Cy dyes can be inconsistent, resulting consequently in decreased assay sensitivity (Call, 2005; Kuck and Taylor, 2008; Vora et al., 2008). Furthermore, the use of traditional fluorescence requires the use of expensive and non-portable confocal scanners for data analysis and quantification (Kuck and Taylor, 2008; Moulton et al., 2011). Recent reports have documented the development of microtube-based DNA arrays as more cost–effective than standard fluorescent methods (Anjum et al., 2007; Ballmer et al., 2007). However, the use of the microtube-based arrays requires microgram amounts of purified genomic DNA from the tested strain to reliably detect all expected genes (Anjum et al., 2007), which is not amenable for high-throughput sampling of a large number of bacterial isolates. In contrast, the ampliPHOX colorimetric detection method, employed in the present study, uses a small, portable, and inexpensive scanner that is at least tenfold lower in cost, compared with confocal microarray scanners (Moulton et al., 2011). Moreover, this colorimetric assay uses 2–3 μl of crude/unpurified bacterial lysates to successfully detect all target genes on the microarray with a rapid assay time. The inclusion of an amplification step coupled with an exonuclease digestion of target DNA allowed for rapid and accurate genotyping of 25–30 STEC isolates in approximately 3–4 h.

The microarray-based colorimetric assay was then employed to examine the genetic composition of environmental isolates from an important produce production region in the United States located on the central coast of California. Our findings indicated that this assay simultaneously detected relevant O-antigens and virulence genes in isolates recovered from multiple types of animal fecal samples (livestock and wildlife) and watershed sediment samples present on or near farms or ranches in this agricultural region. The genotyping results demonstrated that different isolates with the same O-antigen gene had a similar virulence gene profile. Still to be determined is whether the similar virulence gene profiles in non-O157 and in O157 strains represents a stable and predominant genotype in this major produce production region. Future work is aimed at assessing molecular risk factors associated with STEC pathogenesis in a larger and diverse set of environmental isolates recovered from ranches, farms, and watershed sites in produce production regions in California for assessing pathogen incidence, movement, and virulence potential. Thus, the DNA microarray-based ampliPHOX colorimetric system, using low-cost reagents and instrumentation, proved to be a simple and quantitative method that allowed for rapid and high-throughput O-antigen and virulence factor typing of STEC isolates.

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Detection of Shiga toxin-producing *Escherichia coli* by sandwich enzyme-linked immunosorbent assay using chicken egg yolk IgY antibodies

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Enterohemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin producing *E. coli* (STEC) is associated with a spectrum of diseases that includes diarrhea, hemorrhagic colitis and a life-threatening hemolytic-uremic syndrome (HUS). Regardless of serotype, Shiga toxins (Stx1 and/or Stx2) are uniformly expressed by all EHEC, and so exploitable targets for laboratory diagnosis of these pathogens. In this study, a sandwich ELISA for determination of Shiga toxin (Stx) was developed using anti-Stx2B subunit antibodies and its performance was compared with that of the Vero cell assay and a commercial immunoassay kit. Chicken IgY was used as capture antibody and a HRP-conjugated rabbit IgG as the detection antibody. The anti-Stx2B IgY was harvested from eggs laid by hens immunized with a recombinant protein fragment. Several parameters were tested in order to optimize the sandwich ELISA assay, including concentration of antibodies, type and concentration of blocking agent, and incubation temperatures. Supernatants from 42 STEC strains of different serotypes and *stx* variants, including $stx_{2EDL933}$, stx_{2vha}, stx_{2vhb}, stx_{2g}, $stx_{1EDL933}$, and stx_{1d} were tested. All Stx variants were detected by the sandwich ELISA, with a detection limit of 115 ng/ml Stx2. Twenty three strains negative for *stx* genes, including different bacteria species, showed no activity in Vero cell assay and produced negative results in ELISA, except for two strains. Our results show that anti-Stx2B IgY sandwich ELISA could be used in routine diagnosis as a rapid, specific and economic method for detection of Shiga toxin-producing *E. coli*.

Keywords: *Escherichia coli*, Shiga toxin, ELISA, IgY, egg yolk, chicken, STEC

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) causes a spectrum of human diseases ranging from mild non-bloody diarrhea through hemorrhagic colitis to the extraintestinal manifestation hemolytic-uremic syndrome (HUS) (Griffin and Tauxe, 1991). The incidence of HUS in Argentina is one of the highest in the world, with approximately 500 new cases being observed each year in under-5-year-old children (Rivas et al., 2010). Also, it is the leading cause of acute renal failure in pediatric age and the second for chronic renal failure (Exeni, 2001). Prompt and accurate diagnosis of Shiga toxin producing *E. coli* (STEC) infection is important to achieve an appropriate and early supportive treatment in the course of infection to decrease renal damage and improve overall patient outcome (Ake et al., 2005).

Shiga toxins (Stxs) are thought to be the major virulence factor of STEC strains (Tarr et al., 2005) and comprise a family composed of Stx1, Stx2, and their variants, which can be found in STEC strains isolated from either humans or animals (Ito et al., 1990). Stx2, which is 56% homologous to Stx1 at the amino acid sequence level, is clinically the most important Stx type, because it is associated with severe outcomes of human infections including HUS (Friedrich et al., 2002; Brooks et al., 2005). Stxs consist of a single A subunit, with catalytic activity, linked to a ring of five B subunits, responsible for specific cell binding of the toxin (O'Brien and Holmes, 1987).

The expression of Stx is characteristic of STEC strains and so, exploitable targets for laboratory diagnosis of these pathogens. Numerous assays for the diagnosis of STEC have been developed including microbiological, immunological, and genetical methods (Bettelheim and Beutin, 2003). Cytotoxicity assays are the most sensitive methods for detecting active Stxs (Paton and Paton, 1998) and have been used as "gold standard" for evaluation of immunological tests. However, this technique is expensive, labor-intensive, and time consuming and so, not often established

for routine diagnosis. Stx-specific PCR detects gene sequences whether or not they are expressed (Bettelheim and Beutin, 2003). Stx-specific ELISA is a rapid, easy to perform and applicable technique for routine diagnosis, with a growing number of Stx-detection test kits offered by several companies (Scheutz et al., 2001). Compared to cytotoxicity assays or PCR, ELISAs are less sensitive (Beutin et al., 1996, 1997; Gerritzen, 1998) and not suitable to evaluate samples where low amounts of Stx are expected, such as mixed cultures and certain Stx2 variants (Ball et al., 1996; Beutin et al., 1996, 2007). These commercial kits are also economically unaffordable for use in developing countries. A lower cost alternative are ELISA assays based on the use of egg yolk antibodies (IgY) from laying hens and obtained in a non-invasive way. IgY is the typical low-molecular-weight egg yolk antibody of birds, reptiles, amphibians, and lungfish, whereas IgG occurs in mammals (Hardin et al., 2001). Because of the evolutionary distance between birds and mammals, a chicken is often a better choice for antibody production than a mammal when the antigen is of human or other mammalian origin (Schade et al., 2005). IgY also has the advantage to avoid the interference caused by the complement system, rheumatoid factors, anti-mouse IgG antibodies or human and bacterial Fc receptors in immunological assays. In addition, there is a minimal or no cross-reaction with mammalian IgG (Ambrosius and Hadge, 1987; Larsson and Sjoquist, 1990). Therefore, this study was intended to develop a sandwich ELISA using IgY as the capture antibody and a rabbit IgG as the detection antibody for determination of Shiga toxins in culture supernatants as a potential affordable research and diagnostic tool.

MATERIALS AND METHODS

PRODUCTION OF RECOMBINANT Stx2B

Stx2B subunit was produced as previously described (Parma et al., 2011). Briefly, a DNA fragment encoding Stx2B was obtained by PCR amplification using DNA extracted from the reference strain *E. coli* EDL933. For expression of recombinant Stx2B subunit, transformed BL21–AI™ *E. coli* cells were grown in Luria-Bertani (LB) broth supplemented with 100 μg/mL ampicillin (Sigma Aldrich) and induced overnight with 0.2% L (+) arabinose (Sigma Aldrich). The Stx2B was purified by affinity chromatography using a Ni-NTA column (Qiagen). The purity and identity of the protein was checked by SDS-PAGE and Western blotting, respectively. The protein content was determined by Bradford assay using BSA as the standard protein.

PREPARATION OF Stx2-SPECIFIC IgY AND IgG ANTIBODIES

Anti-Stx2 polyclonal IgY and IgG antibodies were obtained as described in Parma et al. (2011) by immunization of hens and rabbits with recombinant Stx2B. This procedure was reviewed by the Animal Care and Use Committee at The National Institute of Agriculture Technology. Briefly, the egg yolk was physically separated from the egg white and mixed with four volumes of cold distilled water and kept at −20°C for at least 72 h and thereafter thawed at 4°C. The disrupted emulsion was centrifuged at 8000 × g, 15 min at 4°C. The liquid phase containing the IgY was filtered through a gauze tissue and ammonium sulphate was added (0.24 g/ml supernatant). After centrifugation at 10,000 × g, 15 min at 4°C, the pellet was dissolved with ammonium sulphate 2 M and centrifuged again. Finally, the pellet was resuspended and dialyzed against PBS (pH 7.4) at 4°C. For rabbit sera antibodies, blood samples were kept overnight at room temperature, sera were separated by centrifugation and thereafter stored at −20°C until use. IgG was precipitated with ammonium sulphate (45% saturation) and stirred overnight at 4°C. After centrifugation at 10,000 × g for 3 min at 4°C, supernatants were discarded and pellets were dissolved and dialyzed against PBS (pH 7.4). The specificity and titer of the antibodies was checked against Stx2B recombinant protein by Dot Blot and Western Blot assays. Also, the cytotoxin-neutralizing activity of anti-Stx2B IgY and IgG antibodies was assessed by incubation of 4 CD_{50} of the holotoxin with serial dilutions of the antibodies. Preimmune antibodies were included as a negative control (Parma et al., 2011). Anti-Stx2B IgG antibodies were conjugated to horseradish peroxidase by using EZ-Link Plus activated peroxidase (Thermo Scientific).

TEST SAMPLES

The STEC strains analyzed in this study have been previously described regarding the serotype, cytotoxicity and other virulence factors (Parma et al., 2000; Padola et al., 2004; Sanz et al., 2007; Krüger et al., 2011). The 42 STEC strains were selected in order to include strains harboring different *stx* variants as well as different origins (feedlot cattle, grazing cattle, cattle at abattoir, ground beef, and hamburgers) (**Table 1**). To assess the specificity of the anti-Stx2B IgY sandwich ELISA, non-STEC strains (23 isolates) were also collected (**Table 2**). Supernatants were prepared from the selected strains and tested by ELISA and Vero cell assay. Briefly, bacterial cultures were grown at 37°C in LB medium until OD 600 nm reached 0.3. The culture was then supplemented with mitomycin C (0.5 μg/ml) and incubated overnight. Supernatants were obtained by centrifugation of bacteria at 10,000 × g for 10 min at 4°C. Samples were filter-sterilized and stored in aliquots at −20°C.

PURIFICATION OF WILD TYPE Stx2 HOLOTOXIN

The supernatant of wild type Stx2 was prepared from strain 59-2 isolated from cattle (Parma et al., 2000) as in Section "Test Samples." This preparation was applied to the HiTrap NHS-activated HP (GE) column coupled with affinity purified anti-Stx2B IgG obtained as described previously (Parma et al., 2011). The column was washed with binding buffer (75 mM Tris-HCl pH 8.0) until the absorbance (λ: 280 nm) of the wash solution returned to baseline values. The Stx2 bound to the column was eluted with three volumes of elution buffer (100 mM glycine, 0.5 M NaCl pH 2.7) in collection tubes and immediately neutralized with 1 M Tris-HCl (pH 9.0). This procedure was repeated three times and the fractions were pooled and concentrated using an Amicon ultrafiltration unit fitted with an YM 10 membrane. The purity and concentration of Stx2 holotoxin in the eluted fraction were determined by SDS-PAGE. Briefly, recombinant Stx2B (4.34 μg), Stx2 supernatant and Stx2 in the eluate (13.5 μl of each one) were separated by 12.5% SDS-PAGE and stained with Coomasie Blue. The percentage of purity and the concentration of Stx2 were quantified by densitometry using NIH Image software

Table 1 | STEC strains tested in this work.

		Result		
***Stx* genotype**	**Serotype**	**Vero cell**	**Ridascreen-EIA**	**IgY-ELISA**
$stx_{2EDL933}$	O91:H21	+	3+	+
$stx_{2EDL933}$	O145:H–	+	4+	+
$stx_{2EDL933}$	O20:H19	+	4+	+
$stx_{2EDL933}$	O145:H–	+	4+	+
$stx_{2EDL933}$	O145:H–	+	4+	+
$stx_{2EDL933}$	O113:H21	+	4+	+
$stx_{2EDL933}$	O113:H21	+	4+	+
$stx_{2EDL933}$	O26:H11	+	4+	+
$stx_{2EDL933}$	O91:H21	+	2+	+
$stx_{2EDL933}$	O39:H49	+	4+	+
$stx_{2EDL933}$	ONT:H21	+	4+	+
$stx_{2EDL933}$	O39:H49	+	4+	+
$stx_{2EDL933}$	ONT:H19	+	4+	+
$stx_{2EDL933}$	O145:H–	+	4+	+
$stx_{2EDL933}$	O39:H49	+	4+	+
$stx_{2EDL933}$	O39:H49	+	4+	+
stx_{2vha}	O113:H21	+	4+	+
stx_{2vha}	O162:H7	+	4+	+
stx_{2vha}	ONT:H21	+	4+	+
stx_{2vha}	ONT:H7	+	4+	+
stx_{2vha}	O113:H21	+	1+	+
stx_{2vha}	O171:H2	+	2+	+
stx_{2vha}	O117:H7	+	4+	+
stx_{2vha}	O178:H19	+	1+	+
stx_{2vhb}	O91:H21	+	1+	+
stx_{2vhb}	O20:H19	+	3+	+
stx_{2vhb}	O79:H19	+	4+	+
stx_{2vhb}	O174:H21	+	4+	+
stx_{2vhb}	O2:H–	+	2+	+
stx_{2vhb}	O171:H–	+	4+	+
stx_{2vhb}	O117:H7	+	2+	+
stx_{2vhb}	O171:H2	+	4+	+
stx_{2g}	O15:H21	+	–	+
stx_{2g}	O175:H8	+	–	+
stx_{2g}	O175:H8	+	–	+
stx_{2g}	O2:H25	+	4+	+
$stx_{1EDL933}$	O174:H21	+	4+	+
$stx_{1EDL933}$	O8:H16	+	4+	+
$stx_{1EDL933}$	O5:H–	+	4+	+
$stx_{1EDL933}$	O145:H–	+	4+	+
$stx_{1EDL933}$	O26:H11	+	4+	+
stx_{1d}	ONT:H8	+	1+	+

Table 2 | Non-STEC strains tested in this work.

	Result	
Strain	**Vero cell**	**IgY-ELISA**
Proteus mirabilis	–	–
Proteus vulgaris	–	–
Morganella morganii	–	–
Citrobacter freundii	–	–
Enterococcus faecalis	–	–
Campylobacter coli	–	–
Shigella flexneri	–	+
Shigella sonnei	–	–
Campylobacter jejuni	–	–
Staphylococcus aureus	–	–
Enterobacter cloacae	–	–
Salmonella typhimurium 09/63	–	–
Salmonella typhimurium 09/67	–	–
Salmonella enteritidis PT II	–	+
Salmonella gallinarum 1982	–	–
Klebsiella pneumoniae	–	–
Clostridium difficile	–	–
Clostridium perfringens tipo D	–	–
Clostridium perfringens	–	–
Clostridium perfringens tipo A	–	–
E. coli DH5 α	–	–
Enteropathogenic E. coli	–	–
E. coli XL1	–	–

(Image J). Then, proteins were transferred from SDS-PAGE to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) blocked overnight at 4°C with 5% skimmed milk in PBS-T 0.1% and incubated with a 1:200 dilution of anti-Stx2B IgY in PBS-T for 1 h at 37°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-chicken IgY (1:4000) for 1 h at 37°C. Finally, membrane was revealed using DAB/H_2O_2 system. The integrity and biological activity of the holotoxin was checked by ELISA and Vero cell assay, respectively.

ANTI-Stx2B IgY SANDWICH ELISA

Microplates (Nunc, Maxisorp) were coated overnight at 4°C with a 1:500 dilution of specific IgY antibodies in carbonate/bicarbonate buffer pH 9.6. After washing with PBS-T 0.05%, plates were blocked with 5% skimmed milk in PBS-T for 1 h at 37°C. Culture supernatants were diluted 1:5 in PBS-T and incubated at 37°C for 1 h. Plates were washed and then incubated with a 1:500 dilution of horseradish peroxidase-conjugated anti-Stx2B IgG antibodies in 5% skimmed milk -PBS-T. Plates were incubated 1 h at 37°C, and after washing developed with TMB (Sigma)/peroxidase substrate solution. The reaction was stopped with 2 N H_2S0_4 and absorbance was read at 450 nm. Stx2-containing supernatant from strain 59-2 was used as a positive control. A non-STEC *E. coli* DH5α supernatant was included as a negative control. All samples were processed by duplicate.

Receiver operating characteristic (ROC) curve analysis (MedCalc Software 8.1.0.0) was performed on the ELISA results to determine the optimal cut-off point (at which the sum of the sensitivity and specificity values is highest) for distinguishing between positive and negative results. Specimens were considered Shiga toxin positive when the optical density was >0.26. Test results were recorded as positive (+) or negative (−). The detection limit was determined with twofold serial dilutions of the purified Stx2 holotoxin and calculated from the mean + 3 SD of the blank control. The intra-assay test was performed using three different culture supernatants (with high, medium, and

low OD values), in replicates of six across the microplate. The inter-assay test was performed using three different culture supernatants (with high, medium, and low OD values) in duplicates repeated on three different microplates on different days. Coefficients of variation were calculated for each sample as the SD/mean × 100.

THE RIDASCREEN-EIA

This assay (Ridascreen® Verotoxin, R-Biopharm, Germany) is provided as a kit containing a microtitre plate coated with mouse monoclonal antibodies directed against Stx1 and Stx2, as well as enzyme-conjugate, washing buffer, enzyme substrate, and stop solution. The assay was performed following the instructions of the manufacturer (http://www.r-biopharm.de/). Inactivated Stx is provided as positive and non-inoculated growth medium was taken as negative control. The cut-off value for recording results as positive was calculated by adding 0.1 OD to the value obtained with the negative control as described for the Ridascreen-EIA. Test results were recorded as weak (1+) positive if the extinction was >0.1–0.5 above the negative control, moderate (2+) (extinction >0.5–1.0 above negative control), strong positive 3+ (>1.0–2.0) to 4+ (>2.0) and negative (−).

CYTOTOXICITY TEST

African green monkey kidney (Vero) cells were plated at 10^4/well on 96-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and incubated overnight at 37°C under 5% CO_2. Serial twofold dilutions of STEC supernatants in DMEM medium were prepared. Non-STEC supernatants were also included in order to discard the presence of Stx in species other than *E. coli*. Dilutions were added to the cell monolayer (100 µl/well) and incubated for 48 h at 37°C under 5% CO_2. The viability of the Vero cells was determined by crystal violet staining (Gentry and Dalrymple, 1980). All data represent the average of triplicate assays.

RESULTS

IgY AND IgG ANTIBODY YIELD

Highly purified total antibodies preparations were obtained from rabbit sera and chicken egg yolk by the ammonium precipitation method. The final concentration of total IgY was 0.84 mg/ml of egg yolk (equivalent to 8.4 mg of total IgY per egg). The concentration of total IgG was 1.35 mg/ml of sera. Both polyclonal antibodies, IgY and IgG neutralized the cytotoxic effects of Stx2 holotoxin on Vero cells. However, IgG antibodies were four times more efficient than IgY antibodies, since 1.09 µg/ml were sufficient to neutralize the effect of the toxin *in vitro*, compared with IgY antibodies that obtained the same effect with 4.38 µg/ml (Parma et al., 2011).

PURIFICATION OF Stx2 HOLOTOXIN

Densitometry analysis of the band patterns obtained by SDS-PAGE, showed approximately a 20% purity of the Stx2 in the eluate (**Figure 1**, lane 2) representing an enrichment of 50% compared with the Stx2 in the supernatant of strain 59-2 (**Figure 1**, lane 3). Band intensity corresponding to the 2B subunit from Stx2 in the eluate (**Figure 1**, lane 2) was compared with the one produced by a known amount of 2B recombinant protein (**Figure 1**, lane 1) using NIH Image software (Image J). We concluded that the concentration of Stx2 in the eluted fraction was around 75 µg/ml. In addition, the purification procedure preserved the biological activity of the holotoxin, as this solution was cytotoxic on Vero cell monolayer.

DETERMINATION OF THE DETECTION LIMIT AND THE REPRODUCIBILITY OF ANTI-Stx2B IgY SANDWICH ELISA

Serial twofold dilutions of purified Shiga toxin were tested by the IgY sandwich ELISA to evaluate its sensitivity (**Figure 2**). The ELISA developed was able to detect 115 ng/ml of purified wild type Stx2. A good dose-response correlation was observed between 0.93 and 7.5 µg of toxin per ml.

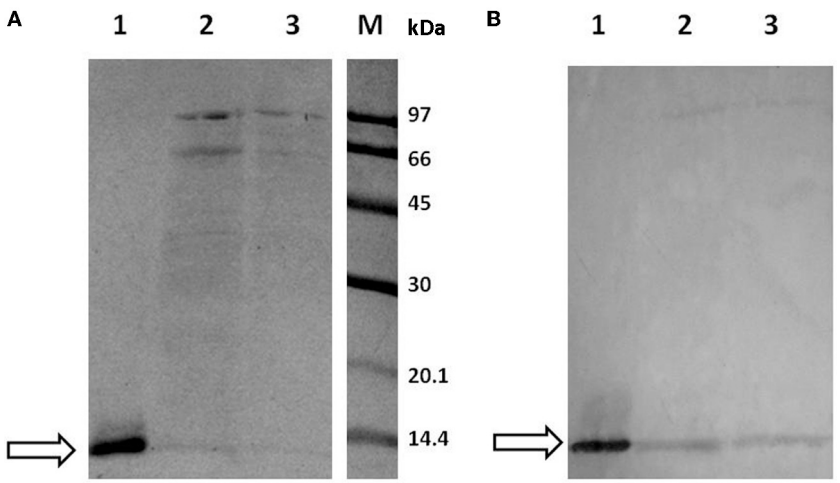

FIGURE 1 | Analysis of Stx2 holotoxin by SDS-PAGE and Western blot. **(A)** SDS-PAGE (12.5% acrylamide, under reducing conditions) Lane 1: Purified recombinant Stx2B (4.34 µg) Lane 2: Stx2 holotoxin in the eluate (13.5 µl) Lane 3: Stx2 supernatant from strain 59-2 (13.5 µl). Gel was stained with Coomassie Blue. **(B)** Western blot Lane 1: Purified recombinant Stx2B (4.34 µg) Lane 2: Stx2 holotoxin in the eluate (13.5 µl) Lane 3: Stx2 supernatant from strain 59-2 (13.5 µl). Membrane was incubated with anti-Stx2B IgY and anti-IgY HRP. Arrow: Stx2B subunit position.

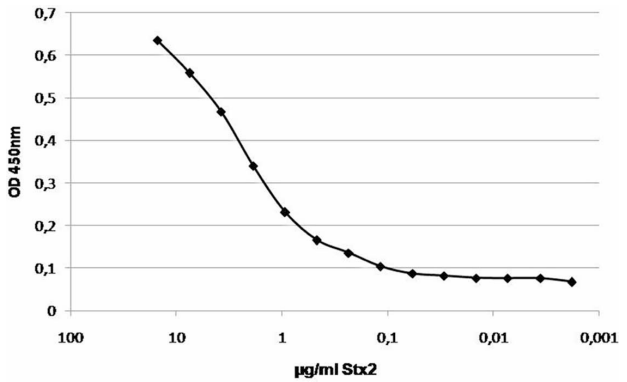

FIGURE 2 | Titration curve of purified Stx2 by anti-Stx2B IgY sandwich ELISA. The reaction was carried out using anti-Stx2B IgY followed by serial twofold dilutions of purified Stx2 and incubation with horseradish peroxidase-conjugated anti-Stx2B IgG. The detection limit was calculated from the mean + 3 SD of the blank control.

Coefficients of variation among the wells (intra-assay) ranged from 5.5 to 5.7% and coefficients of variation among the plates (inter-assay) ranged from and 3.2 to 7.7%.

COMPARISON OF THE PERFORMANCE OF IgY-ELISA, RIDASCREEN EIA AND VERO CELL ASSAY FOR THE DETECTION OF SHIGA TOXINS

The IgY-ELISA assay detected all *stx* gene variants tested in this work: $stx_{2EDL933}$, stx_{2vha}, stx_{2vhb}, stx_{2g}, $stx_{1EDL933}$, stx_{1d} (**Table 1**), although IgY antibodies were prepared by immunization with recombinant Stx2B obtained from a $stx_{2EDL933}$ variant. These samples were collected from STEC strains of various serotypes and origins. The positive results in the IgY-ELISA were in accordance to those of the Vero cell assay and the Ridascreen-EIA, with the exception of three samples containing the stx_{2g} variant, which were negative in the Ridascreen-EIA.

Only two supernatants from a total of 23 non-STEC samples showed an OD above the cut off. These samples were negative for cytotoxicity on the Vero cell assay (**Table 2**).

RELATIVE SENSITIVITY AND SPECIFICITY OF THE IgY-ELISA

The relative sensitivity and specificity were determined considering the cut off value calculated by an interactive dot diagram (MedCalc Software 8.1.0.0) that divided the population into Stx-positive and Stx-negative strains (**Figure 3**). The relative sensitivity was defined as the number of positive strains in the assay divided by the total number of STEC strains, and resulted in 100% for the IgY-ELISA compared with 92.8% obtained with Ridascreen-EIA.

The relative specificity, defined as the number of negative strains in the assay divided by the total number of non-STEC strains, showed a percentage for IgY-ELISA of 91.3.

DISCUSSION

Chickens, as a host for the production of egg yolk antibodies against *E. coli* Stx2B, showed a remarkable ability to rapidly and efficiently generate an abundant supply of high-titer antibody, which is able to bind to the wild type holotoxin and also to neutralize their biological activity both *in vitro* and *in vivo* (Parma et al., 2011). The present report describes a sensitive antigen capture-ELISA for Shiga toxin which is based on antibodies produced in chicken and rabbits immunized with a purified non-toxic recombinant Stx2B subunit. All STEC strains tested in this work, which produced Stx (as confirmed by the Vero cell assay) were specifically recognized in the IgY-ELISA, although they belong to different origins and serotypes. One disadvantage of ELISA assays are false positives, in this case IgY antibodies recognized 2/23 non-STEC supernatants, one from *Salmonella enteritidis* and another from *Shigella flexneri* which showed negative results on Vero cell assay. Non-STEC supernatants were included in the cytotoxicity assay because it has been reported that other members of the family *Enterobacteriaceae* are known

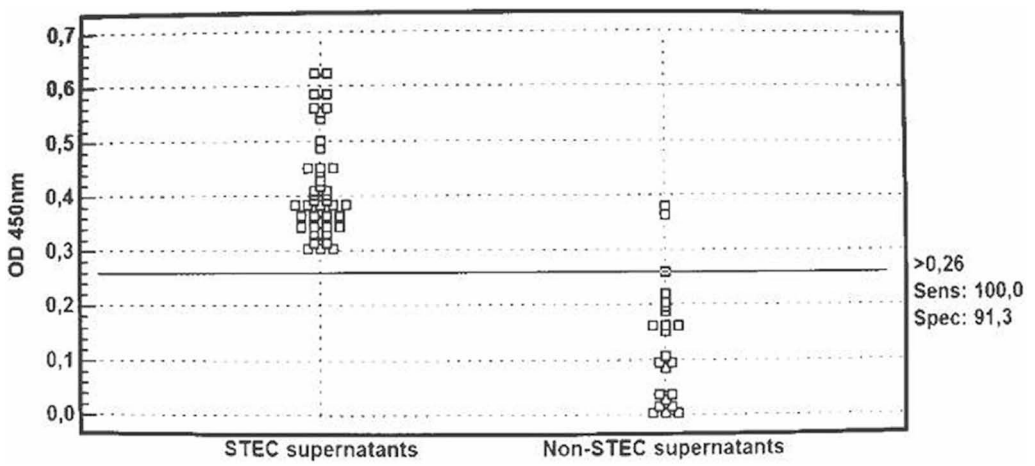

FIGURE 3 | Interactive dot diagram (MedCalc Stadistical Software). In the graph the data of the Stx-positive and Stx-negative strains are displayed as dots on two vertical axes. A horizontal line indicates the cut-off point with the best separation (minimal false negative and false positive results) between the two groups. The corresponding test characteristics sensitivity and specificity are shown at the right side of the graph.

to produce Stx and to cause serious gastrointestinal disease and HUS in humans (Paton and Paton, 1998). *S. dysenteriae* type 1, the causative agent of bacillary dysentery, is the principal cause of HUS in parts of Africa and Asia (Azim et al., 1997; Bhimma et al., 1997). Stx2-producing *Citrobacter freundii* also causes diarrhea and HUS in humans, including one outbreak in a German child care centre (Schmidt et al., 1993; Tschäpe et al., 1994). *Enterobacter cloacae* has also been associated with transient expression of an stx_2-related gene, although its role in disease is unproven (Paton and Paton, 1996). In addition, samples containing *Staphylococcus aureus* can produce false positives in the ELISA since protein A binds the Fc region of IgG antibodies. This interference is not observed with IgY antibodies since they do not recognize this protein (Mizutani et al., 2012). This fact was confirmed in our lab when we run an ELISA assay with a *S. aureus* supernatant, where IgG instead of IgY was coated to the plate (data not shown).

Several ELISAs employed monoclonal antibodies, such as the Ridascreen-EIA (R-Biopharm AG, Germany) and Premier EHEC assay (Meridian Diagnostics Inc.) to improve the specificity and reduce false positives. Monoclonal antibodies being directed against single epitopes are homogeneous, highly specific and can be produced in unlimited quantities. However, producing a high quality monoclonal antibody is often difficult, laborious and expensive. Polyclonal antibodies present some advantages compared to monoclonal antibodies, as they recognize multiple epitopes on the antigen, so they become more tolerant to minor changes on their structure. Their production is relatively inexpensive, the technology and skills required for production are low, and the production time scale is comparatively short. However, the use of polyclonal antibodies has certain limitations such as batch to batch variation, inconsistent yields of antibodies and scanty serum obtained from individual animals. The IgY derived from hyperimmune chicken egg yolk has been recognized as an excellent, alternative source of polyclonal antibodies (Hodek and Stibororá, 2003). A single chicken can produce an enormous amount of antibody, up to 3 g of IgY per month, which is 10–20 times the amount of a rabbit (Mine and Kovacs-Nolan, 2002). Furthermore, compared to rabbits, chickens produce antibodies in shorter time periods given that high-titer antibodies can be available from eggs as early as day 25. To our knowledge, it is the most humane way to produce polyclonal antibodies because there is no need to bleed the chicken, simply collecting the eggs. In addition, we have previously described (Parma et al., 2011) that the same amount of specific anti-Stx2B antibodies can be obtained from five eggs yolks than from the exsanguination of one rabbit.

Analyzing mitomycin C treated cultures, we determined a relative sensitivity of 100% for the IgY-ELISA, with a detection limit of around 115 ng/ml. Law et al. (1992) described that mitomycin C enhanced the yield of Shiga toxins in O157:H7 cultures about 100-fold compared with growth without mitomycin C. This group also described an ELISA with a detection limit of around 1.6 ng/ml. In this case, plates were coated with crude hydatid cyst material and toxins were detected adding specific rabbit antibodies and revealed with anti-rabbit conjugated to alkaline phosphatase. Kongmuang et al. (1987) developed a sandwich ELISA for Shiga toxin detection, using antibodies from rabbits immunized with the complete inactivated toxin. This ELISA also detected several nanograms of purified Shiga toxin per milliliter. The Premier EHEC assay was found to be more sensitive than conventional sorbitol-Mac Conkey culture for the detection of *E. coli* O157:H7 (100 and 60%, respectively) (Sue Kehl et al., 1997). Beutin et al. (2007) evaluated the suitability of Ridascreen-EIA for detection of Stx1, Stx2, and their variants with STEC reference strains and STEC isolates from sources such as food, human feces, surface water, and animal feces, resulting in a relative sensitivity and specificity of 95.7 and 98.7%, respectively. However, this assay was less applicable for testing samples where low amounts of Stx are expected, such as mixed cultures or presence of stx_{2g} variant. This fact was also observed in our work, were IgY-ELISA could detect all STEC containing stx_{2g} variant while Ridascreen-EIA only showed positive in one of four cases.

In STEC infections, a rapid diagnosis is necessary in order to establish a proper supportive treatment. In many laboratories, diagnosis is made by fecal culture on sorbitol-Mac Conkey agar to isolate strains of *E. coli* serotype O157:H7 which are sorbitol non-fermenters, followed by agglutination with specific antisera. However, several drawbacks limit the utility of culture, including slow turnaround, false negative results in antibiotic-treated patients, and false STEC negative results due to emerging serotypes of non-O157 STEC that ferment sorbitol (Bettelheim, 1998). Although, Vero cell assay is more sensitive than ELISA, with detection limits of around picograms per milliliter of toxin (Kongmuang et al., 1987) this assay requires that specificity of any cytotoxic activity be confirmed with appropriate neutralizing antibodies. In addition, it is a time-consuming and expensive technique. It is however essential for reference laboratories to continue to use this test, because it will reveal the presence of unknown variants of Stxs (Bettelheim and Beutin, 2003). ELISAs do offer relative speed, greater availability, are suitable for large-scale screening, and easily applicable in routine diagnostic laboratories without the need for expensive equipment.

The results of this study indicate that polyclonal IgY antibodies anti-Stx2B are a useful alternative to detect most of the variants of Shiga toxin among supernatant cultures of STEC strains belonging to different serotypes. Although further studies with clinical samples must be done, we propose the use of polyclonal IgY antibodies in order to reduce the cost of the assay in comparison to commercial kits that include monoclonal antibodies. EIA and other quick non-culture tests are useful tools for diagnosis of STEC infection, constituting an important complement to microbiological, molecular characterization and serological methods for detecting and controlling STEC outbreaks (CDC, 2009).

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Subtyping of STEC by MLVA in Argentina

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Shiga toxin-producing *Escherichia coli* (STEC) causes serious human illness such as hemolytic uremic syndrome (HUS). Argentina has the world's highest rate of this syndrome, which is the leading cause of acute renal failure among children. *E. coli* O157:H7 is the most common cause of HUS, but a substantial and growing proportion of this illness is caused by infection due to non-O157 strains. Multiple-locus variable-number tandem repeat analysis (MLVA) has become an established technique to subtype STEC. This review will address the use of routine STEC subtyping by MLVA in order to type this group of isolates and to get insight into the genetic diversity of native STEC. With regard to these objectives we modified and adapted two MLVA protocols, one exclusive for O157 and the other, a generic *E. coli* assay. A total of 202 STEC isolates, from different sources and corresponding to 20 serotypes, have been MLVA genotyped in our laboratory. In our experience, MLVA constitutes a very sensitive tool and enables us to perform an efficient STEC subtyping. The diversity found in many serotypes may be useful for future epidemiological studies of STEC clonality, applied to O157 as well as to non-O157 isolates.

Keywords: STEC, MLVA, genotyping, O157:H7, non-O157

Shiga toxin-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), is the most important recently emerged group of foodborne pathogens. STEC can produce serious human illness linked to the consumption of contaminated foods, mainly of bovine origin. Argentina has the highest rate of hemolytic uremic syndrome (HUS) globally and HUS is the leading cause of acute renal failure among children (Cobeñas et al., 2007). *E. coli* serotype O157:H7 is the most common cause of HUS, but a substantial and growing proportion of this illness is caused by infection due to non-O157 strains (Johnson et al., 2006).

Bacterial typing methods generate strain specific molecular fingerprints to assess the epidemiological relationship among isolates. Lately, our laboratory is focused on implementing multiple-locus variable-number tandem repeat analysis (MLVA) as genotyping method, which will be reviewed here. With regard to this objective we modified and adapted two previously described MLVA protocols, one exclusive for O157:H7 (Lindstedt et al., 2003; Keys et al., 2005; Noller et al., 2006), based on polymorphism in nine variable number of tandem repeats (VNTR) loci – named $MLVA_{O157}$ in the present manuscript (Bustamante et al., 2009a), and the other, a generic *E. coli* assay (Lindstedt et al., 2007) based on seven VNTR loci – named $MLVA_G$ (Bustamante et al., 2010).

A total of 202 STEC isolates, tested previously for selected virulence factors, has been MLVA genotyped in our laboratory between 2006 and 2010. They have been isolated from bovines, foods, and patients in previous studies (Parma et al., 2000; Padola et al., 2004; Sanz et al., 2007; Fernández et al., 2010a; Rivero et al., 2010). Twenty-eight isolates belonged to O157:H7 serotype and the remaining ones were non-O157:H7 belonging to 19 serotypes. In order to name the VNTR alleles we used a nomenclature where the actual number of repeats at each locus is reported. Alleles which presented partial repeats were rounded to the nearest complete repeat number. If no amplification product was detected, the allele was designated with an arbitrary number (30). All genotyping data were stored as allelic number strings which is an easy way of comparing isolates.

In the case of $MLVA_{O157}$, we carried out two different studies. In the first one, we analyzed a set of 15 STEC O157:H7 mostly isolated from cattle. The isolates could be grouped according to MLVA profiles in two main clusters, one that grouped all the bovine isolates from the same farm and the other one, the rest of the isolates. Within the cluster of STEC isolated from the same farm ($n = 10$) it was possible to identify four profiles which shared alleles for two loci (TR3 and O157-37). In concordance with the origin of the samples, the differences between unrelated isolates were greater than those presented by isolates from the same farm (Bustamante et al., 2009a).

A second study included 13 STEC O157:H7 of human origin. They have been isolated from children with diarrhea and/or HUS living in Tandil and its surroundings (province of Buenos Aires). We detected as many profiles as examined isolates, which highlights a great O157:H7 genetic diversity in a same geographic region (Rivero et al., 2008). There were no epidemiological associations between the isolates from the first and the second studies and, as expected, the profiles obtained in the second were different from those of the first one. Taking into account both studies, the results revealed 22 different profiles, from which 20 were unique. A similar proportion was detected by other authors (Lindstedt et al., 2003) studying a larger number of samples. We observed variation at all nine loci and the most variable locus was TR2, coincidently with the results of Hyytiä-Trees et al. (2006) and Noller et al. (2006).

In relation with the $MLVA_G$ we applied it in order to analyze both non-O157:H7 and O157:H7 isolates (Bustamante et al., 2009b, 2010; Fernández et al., 2010b; Franci et al., 2011). In a total of 174 samples we detected 66 (37.9%) different MLVA profiles, being 41 of them unique. To our knowledge, we subtyped by MLVA for the first time 14 out of the 20 serotypes studied: O8:H19, O20:H19, O91:H21, O112:H2, O113:NM, O117:H7, O130:H11; O145:NM, O171:H2, O174:H21, O171:NM, ONT:H7, ONT:H19, and ONT:H21. Also, we observed several alleles which have not been previously described. The locus CVN014 was the most variable among serotypes and among isolates from a same serotype (**Table 1**), coincidently with the results of Lindstedt et al. (2007) and Gorgé et al. (2008). Among non-O157:H7 serotypes, the loci which presented the lowest variability were CVN002, CVN007, CVN015, and CVN003. Furthermore, this last locus presented null alleles (no PCR amplification) in all isolates except for those belonging to O157:H7 and O145:NM serotypes (Bustamante et al., 2010). Similarly, Løbersli et al. (2012) found this locus was absent in several serotypes and they only confirmed the presence of this locus among *E. coli* O145, O157, and O55:H7 isolates.

The results obtained performing $MLVA_{O157}$ and $MLVA_G$ showed a high genetic diversity in the STEC isolates analyzed, and five or more MLVA profiles were found in the serotypes O20:H19, O117:H7, O157:H7, O171:H2, O174:H21, and O178:H19 (**Figure 1**). On the contrary, preliminary data in regard to O130:H11 serotype, showed a unique profile in all the studied isolates which could be indicating that it is an emergent serotype or, on the contrary, that the chosen VNTR loci are not variable enough (Fernández et al., 2010b).

Another laboratory from Argentina has begun to evaluate the use of the MLVA for the epidemiological surveillance of *E. coli* O157:H7, as a complementary technique to pulsed-field-gel-electrophoresis (PFGE) in order to solve difficult cases (Chinen et al., 2010). The chosen protocol implies the study of eight VNTRs described by Hyytiä-Trees et al. (2010), some of which are also analyzed in the $MLVA_{O157}$. Using that MLVA approach they were able to distinguish between sporadic cases and outbreaks, with higher discrimination than PFGE. Other authors who also applied MLVA for STEC typing obtained a higher number of MLVA than PFGE profiles and observed that MLVA was particularly useful to discriminate epidemiologically unrelated isolates (Keys et al., 2005; Hyytiä-Trees et al., 2010; Izumiya et al., 2010; Konno et al., 2011).

$MLVA_G$ worked well with the majority of STEC serotypes. However, in the case of some serotypes it was not possible to discriminate enough and, in consequence, this method could be

Table 1 | Alleles detected by $MLVA_G$: distribution by serotype and locus.

Serotypes	*Loci*						
	CVN001	***CVN002***	***CVN003***	***CVN004***	***CVN007***	***CVN014***	***CVN015***
O8:H19	7, 9	1	NA	12	6	9, 10, 11, 12	5
O20:H19	7, 9	1	NA	10, 12	6	7, 11, 16	5
O22:H8	7	1	NA	12	6	7	5
O91:H21	7	1	NA	12	6	5, 6, 7	5
O112:H2	7	1	NA	12	6	8	5
O113:H21	7	1	NA	9, 12	NA, 6	6, 7, 9, 10	NA, 5
O113:NM	7	1	NA	12	NA, 6	8	NA, 5
O117: H7	7	1	NA	12	6	6, 8, 10, 11	5
O130: H11	5	2	NA	9	8	NA	6
O145:NM	8	1	2	12	6	3	5
O157:H7	7	5, 7, 8, 9	3, 4, 5	10, 13	8, 9, 10	5, 6, 8, 9, 10, 11	8, 10
O171:H2	1, 8	1	NA	12	6	2, 6, 8, 9, 10, 11	5
O171:NT	8	1	NA	12	6	7	5
O171:NM	1	1	NA	12	6	7	5
O174:H21	6, 7, 8, 9	1	NA	12	6	5, 7, 8, 9, 11, 12, 14, 15, 16, 18	5
O178:H19	7, 9	1	NA	11, 12	6	7, 14, 15	5
ONT:H7	7, 9	1	NA	12	6	5, 9, 10	5
ONT:H19	9	1	NA	12	6	5	5
ONT:H21	7	NA	NA	12	6	8, 11	5
ONT:NT	7, 9	1	NA	12	6	5, 7, 10	5

NA, null allele.

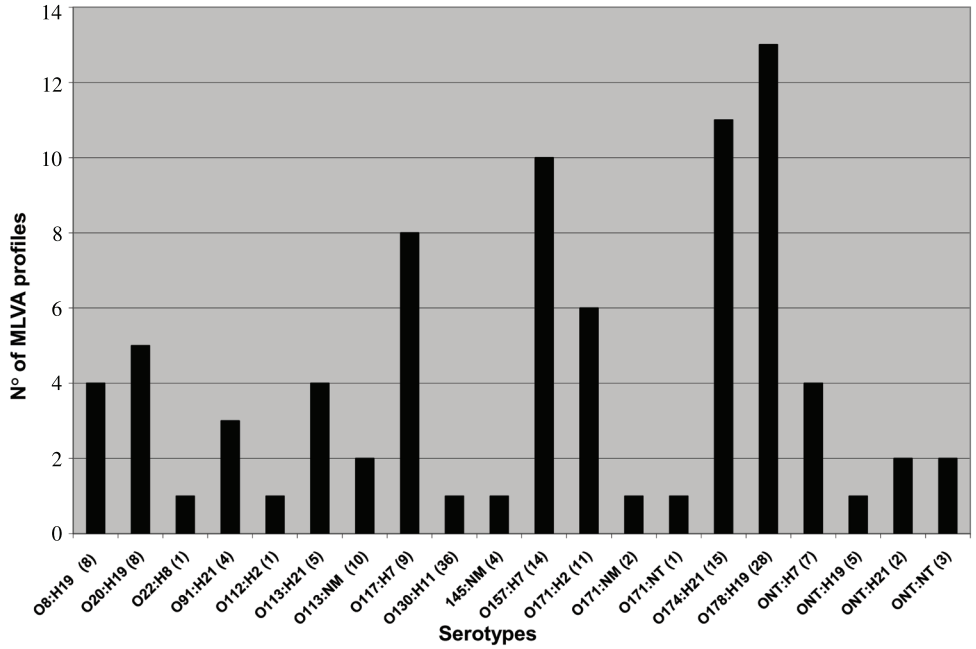

FIGURE 1 | Number of MLVA profiles obtained from each serotype typed by $MLVA_G$. Number of isolates is indicated between parentheses.

improved by incorporating more loci. Recently, Løbersli et al. (2012) improved that method by adding three new repeat-loci to a total of 10. They applied it and observed a considerable increase in resolution, of 71%, using the three new loci. Now, we are in process of adapting this proposed method in our laboratory and using it to subtype STEC. Regarding O157:H7 serotype, both MLVA protocols allowed to find high genetic diversity. In addition, they showed variability in all the VNTR loci analyzed in the $MLVA_{O157}$. This protocol was the one that better reflected the epidemiological relationship among the isolates.

CONCLUDING REMARKS

In our experience, MLVA works well at our laboratory and enables us to perform an efficient O157:H7 and non-O157 STEC subtyping. The obtained results showed a high genetic diversity in the analyzed STEC isolates. The approach also allowed us to stablish possible associations between MLVA genotypes and parameters such as source and virulence characteristics. The diversity found in many serotypes may be useful for future epidemiological studies of STEC strains, of both O157 as well as non-O157 serogroups.

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