Virulence profiling of Shiga toxin-producing Escherichia coli recovered from domestic farm animals in Northwestern Mexico

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Shiga toxin-producing Escherichia coli (STEC) is a zoonotic enteric pathogen that causes human gastrointestinal illnesses. The present study characterized the virulence profiles of O157 and non-O157 STEC strains, recovered from domestic animals in small rural farms within the agricultural Culiacan Valley in Mexico. Virulence genes coding for adhesins, cytotoxins, proteases, subtypes of Shiga toxin (Stx), and other effectors were identified in the STEC strains by PCR. The genotyping analysis revealed the presence of the effectors nleA, nleB, nleE, and nleH1-2, espK, and espN in the O157:H7 and O111:H8 STEC strains. Furthermore, the genes encoding the autoagglutinating adhesin (Saa) and subtilase (SubA) were exclusively identified in the O8:H19 eae-negative strains. The adhesin (iha) and the silent hemolysin (sheA) genes were detected in 79% of the O157 and non-O157 strains. To examine the relative toxicities of the STEC strains, a fluorescent Vero cell line, Vero-d2EGFP, was employed to measure the inhibition of protein synthesis by Stx. Analysis of culture supernatants from serotype O8:H19 strains with the stx gene profile stx1a, stx2a, and stx2b, and serotypes O75:H8 and O146:H8 strains with the stx gene profile stx1a, stx1c, and stx2b, resulted in a significant reduction in the Vero-d2EGFP fluorescent signal. These observations suggest that these non-O157 strains may have an enhanced ability to inhibit protein synthesis in Vero cells. Interestingly, analysis of the stx2c-positive O157:H7 strains resulted in a high fluorescent signal, indicating a reduced toxicity in the Vero-d2EGFP cells. These findings indicate that the O157 and non-O157 STEC strains, recovered in the Culiacan Valley, display distinct virulence profiles and relative toxicities in mammalian cells and have provided information for evaluating risks associated with zoonotic STEC in this agricultural region in Mexico.

Keywords: Escherichia coli, STEC, virulence genes, Shiga toxin, Vero cells, Mexico, zoonosis, foodborne pathogen

INTRODUCTION

Shiga toxin-producing Escherichia coli (STEC) is considered to be a major cause of foodborne disease and can cause a wide variety of disease symptoms in humans, ranging from watery and bloody diarrhea to the life-threatening diseases such as hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Tarr et al., 2005; Gyles, 2007; Karmali et al., 2010; Scallan et al., 2011; Melton-Celsa et al., 2012). Cattle are considered to be the major carrier of STEC strains (Gyles, 2007; Ferens and Hovde, 2011). However, additional studies that examined important animal reservoirs for these bacterial pathogens have indicated that small domestic ruminants, including sheep and goats, have been implicated as carriers of STEC (Ogden et al., 2005; Gyles, 2007; La Ragione et al., 2009; Ferens and Hovde, 2011; Mandrell, 2011). Furthermore, STEC strains have been also detected in other domestic and wild animals, including cats, dogs, rodents, deer, birds, feral pigs, chickens, and insects (Cooley et al., 2007; Ferens and Hovde, 2011; Mandrell, 2011).

Severe disease in humans has been associated with more than 100 serotypes of STEC (Gould et al., 2009; Mathusa et al., 2010). Serotype O157:H7 is responsible for most outbreaks in the United States (Karmali, 2009; Hoefer et al., 2011; Melton-Celsa et al., 2012). Additional epidemiological studies have indicated that six non-O157 serogroups, O26, O45, O103, O111, O121, and O145, have been associated with severe disease symptoms in North America (Johnson et al., 2006; Gould et al., 2009; Stigi et al., 2012). Additionally, STEC of serogroups, O91, O104, O113, and O128 have been reported to be significant causes of human infections worldwide (Brooks et al., 2005; Bettelheim, 2007; Mathusa et al., 2010; Beutin and Martin, 2012). Thus, these findings have indicated that strains with certain non-O157 serogroups may be potentially as virulent as strains with the O157:H7 serotype (Bettelheim, 2007; Coombes et al., 2011; Beutin and Martin, 2012; Stigi et al., 2012).

The production of Shiga toxins (Stx) by STEC contributes to the development of the life-threatening disease symptoms in humans (Karmali et al., 1983; Karmali, 1989). The Stx family...
has been associated with pathogenic STEC strains. The activity of the Stx subtypes, expressed by the tested STEC strains, was also further examined to obtain more detailed information on their ability to inhibit protein synthesis in mammalian cells.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS AND GROWTH CONDITIONS**

A subset of 29 STEC strains, previously recovered from various animal reservoirs in the Culiacan Valley in Mexico (Amézquita-López et al., 2012), was studied (Table 1). The subset of strains was selected based on serotype and on the recovery from distinct dates, sampling sites and animal reservoirs (Amézquita-López et al., 2012).

The method employed to isolate the STEC strains (Table 1) from fecal samples from various domestic animals was previously described (Amézquita-López et al., 2012). The characteristics and

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### Table 1 | List of E. coli O157 and non-O157 strains analyzed in this study.

| Strain | Serotype | Sampling date | Source | Region |
|--------|----------|---------------|--------|--------|
| RM8744 | O157:H7  | 18-Nov-08     | Cattle | Iraguato |
| RM8745 | O73:H4   | 02-Dec-08     | Sheep  | El Castillo |
| RM8747 | O15:NT   | 22-Jul-08     | Cattle | Agua Caliente |
| RM8748 | O73:NT   | 22-Jul-08     | Cattle | Agua Caliente |
| RM8749 | O20:H4   | 12-Aug-08     | Sheep  | El Castillo |
| RM8752 | O75:H8   | 07-Oct-08     | Sheep  | Cofradia de Navolato |
| RM8753 | O157:H7  | 02-Dec-08     | Sheep  | Cofradia de Navolato |
| RM8755 | O111:H8  | 20-Jan-09     | Sheep  | Cofradia de Navolato |
| RM8756 | O146:H21 | 20-Jan-09     | Sheep  | Jotagua |
| RM8759 | O157:H7  | 20-Jan-09     | Sheep  | Cofradia de Navolato |
| RM8760 | O75:H8   | 20-Jan-09     | Sheep  | Cofradia de Navolato |
| RM8761 | O146:H21 | 20-Jan-09     | Sheep  | El Castillo |
| RM8762 | O146:H8  | 20-Jan-09     | Sheep  | El Castillo |
| RM8763 | O75:H8   | 03-Feb-09     | Sheep  | Cofradia de Navolato |
| RM8768 | O157:H7  | 20-Jan-09     | Cattle | Cofradia de Navolato |
| RM8772 | O8:H19   | 17-Feb-09     | Cattle | El Castillo |
| RM8776 | O8:H19   | 03-Feb-09     | Cattle | Iraguato |
| RM8778 | O75:H8   | 25-Feb-09     | Sheep  | Cofradia de Navolato |
| RM8781 | O157:H7  | 25-Feb-09     | Sheep  | Cofradia de Navolato |
| RM8922 | O157:H7  | 10-Mar-09     | Sheep  | Cofradia de Navolato |
| RM8923 | O75:H8   | 10-Mar-09     | Sheep  | Cofradia de Navolato |
| RM8928 | O157:H4  | 10-Mar-09     | Sheep  | El Castillo |
| RM8929 | O75:H8   | 10-Mar-09     | Sheep  | Cofradia de Navolato |
| RM9450 | O157:H7  | 10-Mar-09     | Sheep  | Cofradia de Navolato |
| RM9452 | O157:H7  | 26-May-09     | Sheep  | Cofradia de Navolato |
| RM9454 | O157:H7  | 26-May-09     | Sheep  | Cofradia de Navolato |
| RM9456 | O157:H7  | 07-Apr-09     | Cattle | Cofradia de Navolato |
| RM9458 | O157:H7  | 24-Mar-09     | Chicken | Agua Caliente |
| RM9462 | O157:H7  | 03-Jun-09     | Cattle | Iraguato |
| RM13865| O75:H8   | 07-Apr-09     | Cattle | Cofradia de Navolato |

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*NT, Non-typeable H-antigen.*

*Sampling sites correspond to regions in the Culiacan Valley, Sinaloa, Mexico (Amézquita-López et al., 2012).*

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sources of the STEC reference strains that were used in this study are described in Table 2. Bacteria were routinely propagated under aerobic conditions at 37°C on Luria-Bertani (LB) agar (Difco, Detroit, MI).

**POLYMERASE CHAIN REACTION FOR AMPLIFICATION OF stx SUBTYPES AND OTHER VIRULENCE GENES**

For the detection of stx subtypes and other virulence genes, the following STEC reference strains (Table 2) were used as a control for the PCR amplification of ent/espL2, espK, espN, espP, etpD, iha, katP, nleA, nleB, nleE, nleH1-2, shcA, stx1a, and stx2a (RM2084); stx2c (RM7004); stx2d (RM7005); sua and stx2d (RM7006); stx2 (RM7007); stx2e (RM7110); stx2g (RM7203); stx1d (RM7369); stx1c (RM7508); hlyA (RM10227). All PCR amplifications were performed by using primers as shown in Table 3. As template for the PCR reaction, cultures of the STEC strains were grown aerobically in tryptic soy broth (Beckton Dickinson, Sparks, MD) for 24 h with constant shaking (200 rpm) at 37°C. Immediately, 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) and incubated at 95°C for 20 min, as in previous studies (Quiñones et al., 2011, 2012). The lysates were centrifuged at 2000 × g for 5 min, and the supernatants were collected and frozen until further use. The PCR amplifications consisted of a 25 µl reaction mixture, each containing 5 µl of the bacterial crude lysate, 0.5 µM of each primer (Eurofins MWG Operon, Huntsville, AL), and 12.5 µl of 2 × GoTaq® Green Master Mix (Promega Corporation, Madison, WI). The reaction mixtures were placed in a Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), as in previous studies (Amézquita-López et al., 2012). The virulence genes were amplified with PCR cycling conditions, as described in the references listed in Table 3. Amplified products were analyzed in 2% agarose gels containing 0.4 µg/ml GelRed Nucleic Acid Stain (Phenex Research, Candler, NC).

**VERO CELL-BASED METHOD TO DETECT STX ACTIVITY**

The Stx activity of the STEC strains was measured using a Vero cell line, Vero-d2EGFP, that harbored a destabilized variant (t1/2 = 2 h) of the enhanced green fluorescent protein (EGFP) (Quiñones et al., 2009; Quiñones and Swimley, 2011). To monitor the Stx-induced inhibition of protein synthesis, the tested STEC strains (Table 1), the Stx-expressing O157:H7 strain RM2084 (positive control) (Table 2), and the Stx-negative O157:H4 strain RM8928 (Amézquita-López et al., 2012) (negative control) were inoculated in 1 ml of sterile LB broth (Difco, Detroit, MI). All E. coli strains were grown aerobically for 24 h at 37°C with shaking at 200 rpm and were then centrifuged at 2000 × g for 15 min. The culture supernatants were filter-sterilized using 0.45 µm polyvinylidene fluoride syringe filters (Durapore® membranes, Millipore Corporation, Billerica, MA) and were frozen at −20°C until further use (Quiñones and Swimley, 2011). One day prior to intoxication, the Vero-d2EGFP cells were seeded at 10,000 cells per well in Greiner black 96-well microplates with clear bottoms (VWR International, Aurora, CO) and were grown at 5% CO2 and 37°C under humidified conditions in Ham’s F-12 complete medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY) (Quiñones et al., 2009; Quiñones and Swimley, 2011). The Vero-d2EGFP cells were then exposed to Ham’s F-12 complete medium containing a tenfold dilution of the cell-free supernatants from each strain and were incubated for 16 h at 37°C in a 5% CO2 humidified incubator. The EGFP fluorescence from the Vero-d2EGFP cells was measured using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT) with the 485/20 nm excitation filter and the 528/20 nm emission filter (Quiñones et al., 2009; Quiñones and Swimley, 2011). All measurements were performed in triplicate, and the results were expressed as percentages of the fluorescence values obtained for culture supernatant-treated Vero-d2EGFP cells when compared to the fluorescence values from control Vero-d2EGFP cells incubated without culture supernatants. To determine statistical differences in the Stx activity among the STEC strains, the fluorescence values from control Vero-d2EGFP cells were set as 100% and the percentage of the fluorescence values from culture supernatant-treated Vero-d2EGFP cells were calculated by comparing with control Vero-d2EGFP cells.

**Table 2 | Shiga toxin-producing Escherichia coli reference strains used in this study.**

| Strain | Other strain designations | Serotype | Source | Location | Provider or reference |
|--------|--------------------------|----------|--------|----------|----------------------|
| RM2084 | EDL933; DEC 4f           | O157:H7  | Meat   | United States | ECRC (Reid et al., 1999) |
| RM7004 | E32511; TW02883          | O157:H   | Human  | United States | The STEC Center (Schmitt et al., 1991) |
| RM7005 | Eh250; TW081611          | O118:H12 | Human  | Belgium     | The STEC Center (Picard et al., 1998) |
| RM7006 | B2F1; TW01393            | O9:1:H21 | Human  | Canada      | The STEC Center (Ito et al., 1990) |
| RM7110 | NADC2228; S1191          | O139:NM  | Pig    | United States | Evelyn Dean-Nystrom (Weinstein et  al., 1988) |
| RM7203 | SC-0012                  | O168:H8  | Coyote | United States | Michael B. Cooley (Cooley et al., 2013) |
| RM7369 | SP-0082-G9               | ONT:H7   | Pig    | United States | Michael B. Cooley (Cooley et al., 2013) |
| RM7508 | MA146B-A7                | O128:H2  | Deer   | United States | Michael B. Cooley (Cooley et al., 2013) |
| RM7519 | F260-H2                  | O113:H21 | Cattle | United States | Michael B. Cooley (Cooley et al., 2013) |

*Contact information of strain providers: ECRC, E. coli Reference Center, College of Agricultural Sciences, The Pennsylvania State University, University Park, PA, USA; The STEC Center, National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI, USA; Michael B. Cooley, USDA/ARS, Western Regional Research Center, Produce Safety and Microbiology Research Unit, Albany, CA, USA; Evelyn Dean-Nystrom, Iowa State University, National Animal Disease Center, Veterinary Microbiology and Preventive Medicine, Ames, Iowa, USA.*
the results were analyzed by performing a k-means clustering using the Hartigan and Wong algorithm (Hartigan and Wong, 1979) with the R Statistical Software (version 3.0.1; R Foundation for Statistical Computing, Vienna, Austria) (R-Core Team, 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013). The distinct clusters were further validated by measuring the Dunn Index with the clValid R Package (Brock et al., 2013).

### RESULTS

#### VIRULENCE GENE PROFILES OF STEC STRAINS FROM DOMESTIC FARM ANIMALS

To further characterize the virulence potential of STEC strains recovered from domestic farm animals in the agricultural Culiacan Valley region in Mexico, the presence of genes, associated with pathogenic STEC strains, was identified. Our initial analysis focused on the identification of the subtypes of Stx, a virulence factor that has been attributed to the development of serious disease symptoms in humans (Karmali et al., 2010; Bolton, 2011; Melton-Celsa et al., 2012). The results indicated that 97% (28/29) of the O157 and non-O157 STEC strains, recovered from sheep, cattle and chickens, were PCR-positive for genes encoding stx subtypes (Table 4). By contrast, stx1 subtypes were only identified in the non-O157 strains from sheep and cattle. Interestingly, the stx2c subtype was detected in 51% (15/29) of the strains selected from the different animal sources and was predominantly identified in strains with the O157:H7 serotype (Table 4). Furthermore, the stx2d subtype was only identified in the ovine strain RM8749 belonging to serotype O20:H4. Our results also demonstrated that 45% (13/29) of the recovered STEC strains were positive for more than one gene encoding stx subtypes in the same strain. In particular, strains with serotypes O73:H4, O75:H8, O146:H8, and O111:H8 were found to harbor the stx1a, stx1c, and stx2b (Table 4). Moreover, the stx gene profile stx1a, stx2c, and stx3d was exclusively found in the O8:H19 strains recovered from cattle. None of the STEC strains recovered from domestic animals in rural farms in the Culiacan Valley were PCR-positive for the stx subtypes stx1a, stx2e, stx2f, or stx3c.

Given that Stx is not the only virulence determinant that is responsible for full pathogenicity (Karmali et al., 2010; Bolton, 2011; Melton-Celsa et al., 2012), the STEC strains isolated from this agricultural region were further screened for the presence of additional markers encoding adhesins, cytotoxins, proteases, and other effectors. The virulence typing analysis revealed the presence of the non-LEE encoded effectors, nleA, nleB, nleE, and nleH1-2, in the recovered O157:H7 strains from sheep, cattle, and chicken as well as in the recovered O111:H8 strains from sheep.

### Table 4

| Target gene | Forward sequence (5′ → 3′) | Reverse sequence (5′ → 3′) | Amplicon size (bp) | Reference |
|-------------|---------------------------|---------------------------|--------------------|-----------|
| ent/espL2   | CATTCATGAGAATTCA          | GTTCGCTGCCCA             | 342                | Quiñones et al., 2012 |
| espK        | GTAGGCACCCAGCA           | ATCCCGGTGGCCTCA          | 242                | Kyle et al., 2012   |
| espN        | TTTCTTGTACGTAG            | GCACCGGGAATCATCGT        | 155                | Kyle et al., 2012   |
| espP        | GACCTGTCTGGCTGAAA         | CGTCCAGTCCCGTGTATG       | 202                | Quiñones et al., 2012 |
| etpD        | TGTGATTGAGGGCAAC          | AGTAGATACGCTTGTTGGAAG    | 85                 | Bugarel et al., 2010b |
| hlyA        | GTCTGCAAAGCACTCCGTCGAATAA| CTGTTCCACAGTTGGTGTGATTAG| 561                | Kerényi et al., 2005 |
| iha         | GTGATGATCTGCTGCA          | GATACTGCTGGCACTTCC       | 207                | Kyle et al., 2012   |
| katP        | GGCGGAGAGAAAGAATGACCTG   | GCACCATGTGTTCA         | 277                | Quiñones et al., 2012 |
| nleA        | TTGGATTAACDGTGCTGATG     | GTATTGGAAGYARGC         | 267                | Kyle et al., 2012   |
| nleB        | GGAAGTTGTTCTACAGACG      | AAAATCCTGCTGTGATCC       | 297                | Coombes et al., 2008 |
| nleE        | GTAAACCCAGGAGATG         | GATCCTACAACAAATGTC      | 260                | Coombes et al., 2008 |
| nleH1-2     | GCCTGATAATCGTGTTTATC     | CGCATAATCCACTGGAAGTAA   | 295                | Kyle et al., 2012   |
| saa         | CCAACACATGTTGGTCA         | GCAATAGCCTGTGTCAGC      | 166                | Quiñones et al., 2012 |
| sheA        | GAGGCGAATGTATGACCTG      | ACTTCAGGTACCTCAAGAG     | 920                | Kerényi et al., 2005 |
| stx1a       | CAGCTGATAGCCCTGGTCA      | GGGGCCACTGAGATCCTC      | 219                | Kyle et al., 2012   |
| stx1c       | AGACCGAAATTAATATTATGT    | CCTGATGTAGTAATTTCT      | 555                | Koch et al., 2001   |
| stx1d       | CTTCTCAGTTAATGCTGATC     | AACCCCGATATGCTGATCC     | 192                | Bürk et al., 2003   |
| stx2a       | AGATATCGACCCGCTTGGG      | GTCGAATCTCCACGTGAATG    | 969                | Nakao et al., 2002  |
| stx2b       | TATACGATGACACGGGAAGAG    | CCTGCGATCAGAAAGACG      | 300                | Nakao et al., 2002  |
| stx2c       | TTTTATATACACGGGTA        | GGGCCACTTATCTGTGGAATGA  | 163                | Nakao et al., 2002  |
| stx2d       | CTTTATACACGGGTA          | CGTATGTCGACAGATGTC      | 359                | Zheng et al., 2008  |
| stx2e       | CAGAAGTTATATATCTGGTAAGG  | GTATTCTTCTTCTGACACTTC   | 911                | Nakao et al., 2002  |
| stx2f       | TTATCGTGGATTCCTTCGGC     | TGATGATTGAGCGGGTGCC     | 875                | Nakao et al., 2002  |
| stx2g       | GTATTATCTCCTGATAC        | GAATACCGCTACAGTA        | 573                | Leung et al., 2003  |
| subA        | CCGCTTATACCTGGTCACGC     | TATAGCTGTGTCTTCTGACG    | 233                | Quiñones et al., 2012 |

*The stx1 and stx2 subtypes are listed with new stx nomenclature, as recently described (Feng et al., 2011; Scheutz et al., 2012)*
The ovine strains belonging to serotypes O20:H4 and O73:H4 present in 79% (23/29) of the non-O157 and O157 strains. Furthermore, saa and subA genes were exclusively identified in the O8:H19 cattle strains (Table 4). Finally, the iron-regulated adhesion gene (iha) and the cytolsin A gene (sheA) were both present in 79% (23/29) of the non-O157 and O157 strains. The ovine strains belonging to serotypes O20:H4 and O73:H4 were found to be negative for the presence of the accessory virulence determinants that were tested in the present study (Table 4).

### Table 4 | Identification of virulence genes in E. coli O157 and non-O157 strains used in this study.

| Serotype | Strain | Source | Virulence profile |
|----------|--------|--------|------------------|
| O8:H19   | RM8772 | Cattle | espP, saa, stx1a, stx2a, stx2c, subA |
|          | RM8776 | Cattle |                  |
| O15:NT   | RM8747 | Cattle | ent/espL2, espP, katP, stx2c |
| O20:H4   | RM8749 | Sheep  | stx2a |
| O73:NT   | RM8748 | Cattle | ent/espL2, espP, katP, stx2a |
| O73:H4   | RM8745 | Sheep  | stx1a, stx1c, stx2b |
| O75:H8   | RM8752 | Sheep  | iha, sheA, stx1a, stx1c, stx2b |
|          | RM8760 | Sheep  |                  |
|          | RM8763 | Sheep  |                  |
|          | RM8778 | Sheep  |                  |
|          | RM8923 | Cattle |                  |
|          | RM8929 | Cattle |                  |
|          | RM13865| Cattle |                  |
| O111:H8  | RM8755 | Sheep  | ent/espL2, espK, espN, iha, nleA, nleB, nleE, nleH1-2, sheA, stx1a |
| O146:H8  | RM8762 | Sheep  | iha, sheA, stx1a, stx1c, stx2b |
| O146:H21 | RM8756 | Sheep  | iha, sheA, stx1a, stx1c, stx2b |
|          | RM8761 | Sheep  |                  |
| O157:H7  | RM8744 | Cattle | ent/espL2, espK, espN, espP, etpD, iha, katP, nleA, nleB, nleE, nleH1-2, sheA, stx2c |
|          | RM8753 | Sheep  |                  |
|          | RM8759 | Sheep  |                  |
|          | RM8768 | Sheep  |                  |
|          | RM8781 | Sheep  |                  |
|          | RM8922 | Cattle |                  |
|          | RM9450 | Sheep  |                  |
|          | RM9452 | Sheep  |                  |
|          | RM9454 | Cattle |                  |
|          | RM9456 | Cattle |                  |
|          | RM9458 | Chicken|                  |
|          | RM9462 | Cattle |                  |

**STX ACTIVITY IN STEC STRAINS ISOLATED FROM DOMESTIC FARM ANIMALS**

A quantitative and sensitive cell-based assay was further employed to examine the activity of the Stxs expressed by the O157 and non-O157 strains recovered from domestic animals in the Culiacan Valley. A Vero cell line, Vero-d2EGFP, was employed to measure the inhibition of protein synthesis by Stx in mammalian cells (Quiñones et al., 2009; Quiñones and Swimley, 2011). Consequently, incubation with active Stx results in a reduction of the EGFP fluorescent signal that is detected from the Vero-d2EGFP cells (Quiñones et al., 2009; Quiñones and Swimley, 2011). Our results indicated low levels of fluorescence, ranging from 5.4 to 19.5%, were observed when the Vero-d2EGFP cells were incubated with culture supernatants from several STEC strains with the serotypes O8:H19, O75:H8, and O146:H8 (Figure 1), recovered from sheep and cattle. Further statistical analysis of the detected EGFP fluorescence indicated that these STEC strains with serotypes O8:H19, O75:H8 and O146:H8 belong to the same k-means cluster, suggesting that the Stx expressed by these non-O157 strains had similar effects on the EGFP fluorescence. Moreover, significantly reduced levels of EGFP fluorescence to approximately 16.7% were also observed after incubation with cell-free culture supernatants from the positive control O157:H7 strain RM2084 (Figure 1).

Intermediate levels of EGFP fluorescence, ranging from 22.9 to 32.4% were observed after incubation with culture supernatants from the ovine STEC strains belonging to serotypes O20:H4, O73:H4, O111:H8, and O146:H21. Interestingly, analysis of the culture supernatants from all O157:H7 strains, recovered from sheep, cattle and chickens in the Culiacan Valley, resulted in significantly higher levels of EGFP fluorescence (Figure 1). The levels of EGFP fluorescence when testing the supernatants from the O157:H7 strains in the Vero-cell based assay ranged from 38.0 to 62.6%. High levels of EGFP fluorescence that also belong to the same k-means cluster group as the O157:H7 strains were also observed when testing culture supernatants from the O157:H7 strains (Figure 1). No reduction of EGFP fluorescence, averaging 98% detected fluorescence, was observed after incubation with culture supernatants from the negative control O157:H4 strain RM8928 strain lacking an stx gene (Figure 1). The fluorescence in the Vero-d2EGFP cells still remained when testing culture supernatants from an stx-negative O157:H7 strain RM4876 (Quiñones et al., 2009) or after incubation with bacterial growth media without toxin added (data not shown).

**DISCUSSION**

In the present study, a genotyping and functional analysis was conducted to further characterize STEC strains, recovered from domestic animals in rural farms in the Culiacan Valley, which is considered one of the most important agricultural regions in Mexico (Amézquita-López et al., 2012). Given that the rural farms were located in communities that may not follow efficient management of animal wastes (Jiménez et al., 2011; Amézquita-López et al., 2012), an understanding of the virulence potential of the...
STEC strains were also positive for
et al., 2008). A previous study showed that these O157 and O111 to be located in the genomic islands OI-122 and OI-71 (Coombes January 2014 | Volume 4 | Article 7|
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room would assist in the development of control measures to prevent the dispersal and transmission of pathogens throughout the environment that could lead to human infections associated with STEC.

The virulence typing analysis revealed that all O157:H7 and O111:H8 STEC strains from domestic animal reservoirs in the Culiacan Valley were positive for several of the nle genes, known to be located in the genomic islands OI-122 and OI-71 (Coombes et al., 2008). A previous study showed that these O157 and O111 STEC strains were also positive for eae (Amézquita-López et al., 2012). Thus, these findings revealed that these STEC strains from the Culiacan Valley harbor the gene signature, eae, ent/espL2, nleA, nleB, nleF, and nleH1-2, which has been proposed to be present in STEC strains with high virulence for humans (Bugarel et al., 2010a). Moreover, EtpD, the pO157 plasmid-encoded type II secretory pathway protein (Burland et al., 1998), was exclusively identified in O157:H7 strains. Finally, the STEC autoagglutinating adhesin (Saa) and subtilase cytotoxin (SubA) were specifically detected in the O8:H19 cattle strains, previously shown to be eae-negative and to display a limited genomic diversity by multilocus variable-number tandem repeat analysis (Amézquita-López et al., 2012). The findings from the present study are in agreement with other reports that documented Saa and SubA to be associated with non-O157 LEE-negative strains (Paton et al., 2001; Jenkins et al., 2003; Kumar et al., 2004; Toma et al., 2004; Zweifel et al., 2004; Kobayashi et al., 2013).

Approximately 79% of the O157 and non-O157 recovered STEC harbored both the iha and sheA genes. Previous studies demonstrated that iha, which codes for the iron-regulated gene A homolog adhesin, has been commonly observed in both LEE-positive as well as LEE-negative strains with different serotypes (Tarr et al., 2000; Schmidt et al., 2001). Moreover, sheA, encoding the cytolysin A or “silent hemolysin” has been shown to be prevalent in certain non-pathogenic E. coli strains and in other enteropathogenic E. coli strains (Del Castillo et al., 1997; Fernández et al., 1998; Ludwig et al., 2004). All O157:H7 strains were positive for katP, a gene mostly identified in STEC strains belonging to seropathotypes associated with HUS (Bugarel et al., 2010b, 2011; Kobayashi et al., 2013). However, the present study also detected katP in O73:NT and O15:NT strains, belonging to serogroups not implicated in causing any human illness (Hussein, 2007).

To examine the relative toxicities of Stx subtypes expressed by the recovered STEC strains, the Vero-d2EGFP fluorescent assay was employed. The assay uses the Vero-d2EGFP cell line, expressing a destabilized variant of EGFP (Quiñones et al., 2009), and measures in mammalian cells the inhibition of protein synthesis by Stx (Quiñones et al., 2009; Quiñones and Swimley, 2011). Given that Vero cells are highly responsive to the effects of Stx (Keusch et al., 1995), the Vero-d2EGFP fluorescent assay is thus a sensitive and quantitative method to examine the potential relative toxicities of STEC strains. The results from the present study demonstrated that STEC strains with serotypes O8:H19, O75:H8, and O146:H8, serotypes previously associated with severe disease in humans (Boerlin et al., 1999; Blanco et al., 2003; Hussein, 2007), displayed a significant reduction in the EGFP signal from producing E. coli O157:H4 strain RM8928 were used as positive and negative controls, respectively. The average ± standard deviation of three independent experiments with duplicate samples for each strain is shown. Bars with the same lowercase letter represent significantly distinct clusters according to the k-means clustering algorithm and the clValid R Software package (Brock et al., 2008; R-Core Team, 2013).
multiple stx subtypes, appear to be more efficient at inhibiting protein synthesis in mammalian cells.

Interestingly, analysis of the Stx activity from the stx2c-positive O157:H7 strains, recovered from chicken, cattle, and sheep in the Culiacan Valley, indicated that the Vero-d2EGFP fluorescence levels were on average threefold higher when compared to the positive control O157:H7 strain RM2084. These findings suggested that the O157:H7 strains from this region in Mexico may have a significantly lower ability to inhibit protein synthesis in mammalian cells. Previous reports have documented that the Stx2a, Stx2c, and Stx2d subtypes have been associated with severe disease symptoms, including HUS and bloody diarrhea, as well as with differential toxicities in mammalian cells (Friedrich et al., 2002; Ethelberg et al., 2004; Persson et al., 2007; Manning et al., 2008; Mütting et al., 2009; Fuller et al., 2011; Quiñones and Swimley, 2011). However, recent evidence has indicated that purified Stx2c appears to have a reduced potency at inhibiting protein synthesis and metabolic activity in mammalian cells and a lower toxicity in mice (Fuller et al., 2011). The findings from the present study have demonstrated that the stx2c-positive O157:H7 strains from this region in Mexico, previously shown to be closely-related by multiple-locus variable-number tandem repeat analysis (Amézquita-López et al., 2012), were less toxic to Vero cells although they possessed other key accessory virulence factors.

Previous reports have documented that the amounts of Stx2 produced may define the severity of disease caused by STEC strains (Zhang et al., 2000; Dean-Nystrom et al., 2003; Muniesa et al., 2004), and the differential expression and induction of Stx2 subtypes appears to contribute to the relative virulence of the STEC strain (Muniesa et al., 2004; Zhang et al., 2005; De Sablet et al., 2008). Therefore, future work is aimed at further characterizing the amounts of Stx produced after induction under different conditions to obtain a more detailed understanding of the pathogenic potential of O157 and non-O157 STEC strains from diverse sampling sites and sampling sources in the agricultural Culiacan Valley region in Mexico.

ACKNOWLEDGMENTS

This work was supported by the USDA-Agricultural Research Service CRIS project number 5325-42000-047-00D and by a Postgraduate Studies Scholarship from The National Council of Science and Technology in Mexico (CONACyT grant #234885) to Bianca A. Amézquita-López. The authors would like to thank Célida Martínez (CIAD in Culiacan, Sinaloa, Mexico) for excellent technical assistance.

REFERENCES

Amézquita-López, B. A., Quiñones, B., Cooley, M. B., León-Félix, J., Castro-Del Campo, N., Mandrell, R. E., et al. (2012). Genotypic analyses of Shiga toxin-producing Escherichia coli O157 and non-O157 recovered from feces of domestic animals on rural farms in Mexico. PLoS ONE 7:e51565. doi: 10.1371/journal.pone.0051565

Bettelheim, K. A. (2007). The non-O157 Shiga-toxigenic (Verocytotoxigenic) Escherichia coli; under-rated pathogens. Crit. Rev. Microbiol. 33, 67–87. doi: 10.1080/10408410601127172

Beutin, L., Krause, G., Zimmermann, S., Kaulfuss, S., and Gleier, K. (2004). Characterization of Shiga toxin-producing Escherichia coli strains isolated from human patients in Germany over a 3-year period. J. Clin. Microbiol. 42, 1099–1108. doi: 10.1128/JCM.42.3.1099-1108.2004

Beutin, L., and Martin, A. (2012). Outbreak of Shiga toxin-producing Escherichia coli (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J. Food Prot. 75, 408–416. doi: 10.4315/0362-028X.JFP-11-452

Bielaszewska, M., Friedrich, A. W., Aldick, T., Schurk-Bulgrin, R., and Karch, H. (2006). Shiga toxin activatable by intestinal mucus in Escherichia coli isolated from humans: predictor for a severe clinical outcome. Clin. Infect. Dis. 43, 1160–1167. doi: 10.1086/508195

Blanco, M., Blanco, J. E., Mora, A., Rey, I., Alonso, J. M., Hermoso, M., et al. (2003). Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing Escherichia coli isolates from healthy sheep in Spain. J. Clin. Microbiol. 41, 1351–1356. doi: 10.1128/JCM.41.4.1351-1356.2003

Boerlin, P., McEwen, S. A., Boerlin-Petzold, E., Wilson, J. B., Johnson, R. P., and Gyles, C. L. (1999). Associations between virulence factors of Shiga toxin-producing Escherichia coli and disease in humans. J. Clin. Microbiol. 37, 497–503.

Bolton, D. J. (2011). Verocytotoxigenic (Shiga toxin-producing) Escherichia coli: virulence factors and pathogenicity in the farm to fork paradigm. Foodborne Pathog. Dis. 8, 357–365. doi: 10.1089/fpd.2010.06099

Brooks, G., Pihur, V., Datta, S., and Datta, S. (2008). iValid: an R package for cluster validation. J. Stat. Softw. 25, 1–22. Available online at: http://www.jstatsoft.org/v25/i04/paper

Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M., Hoekstra, R. M., et al. (2005). Non-O157 Shiga toxin-producing Escherichia coli infections in the United States, 1983-2002. J. Infect. Dis. 192, 1422–1429. doi: 10.1086/465636

Bugarel, M., Beutin, L., and Fach, P. (2010a). Low-density macroarray targeting non-locus of enterocyte effacement effectors (ise genes) and major virulence factors of Shiga toxin-producing Escherichia coli (STEC): a new approach for molecular risk assessment of STEC isolates. Appl. Environ. Microbiol. 76, 203–211. doi: 10.1128/AEM.01921-09

Bugarel, M., Beutin, L., Martin, A., Gill, A., and Fach, P. (2010b). Microarray for the identification of Shiga toxin-producing Escherichia coli (STEC) seropathotypes associated with Hemorrhagic Colitis and Hemolytic Uremic Syndrome in humans. Int. J. Food Microbiol. 142, 318–329. doi: 10.1016/j.ijfoodmicro.2010.07.010

Bugarel, M., Martin, A., Fach, P., and Beutin, L. (2011). Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) Escherichia coli strains: a basis for molecular risk assessment of typical and atypical EPEC strains. BMC Microbiol. 11:42. doi: 10.1186/1471-2180-11-42

Burk, C., Dietrich, R., Acar, G., Moravek, M., Bulte, M., and Mättläuer, E. (2003). Identification and characterization of a new variant of Shiga toxin 1 in Escherichia coli O157:H7 of bovine origin. J. Clin. Microbiol. 41, 2106–2112. doi: 10.1128/JCM.41.5.2106-2112.2003

Burland, V., Shao, Y., Perna, N. T., Plunkett, G., Sofia, H. J., and Blattner, F. R. (1998). The complete DNA sequence and analysis of the large virulence plasmid of Escherichia coli O157:H7. Nucleic Acids Res. 26, 4196–4204. doi: 10.1093/nar/26.18.4196

Cooley, M. B., Jay-Russell, M., Atwill, E. R., Carychao, D., Nguyen, K., Quiñones, B., et al. (2013). Development of a robust method for isolation of Shiga toxin-positive Escherichia coli (STEC) from fecal, plant, soil and water samples from a leafy greens production region in California. PLoS ONE 8:e65716. doi: 10.1371/journal.pone.0065716

Cooley, M., Carychao, D., Crawford-Miksa, L., Jay, M. T., Myers, C., Rose, C., et al. (2007). Incidence and tracking of Escherichia coli O157:H7 in a major produce production region in California. PLoS ONE 2:e1159. doi: 10.1371/journal.pone.0001159

Coombes, B. K., Gilmour, M. W., and Goodman, C. D. (2011). The evolution of virulence in non-O157 Shiga toxin-producing Escherichia coli. Front. Microbiol. 2:90. doi: 10.3389/fmicb.2011.00090

Coombes, B. K., Wickham, M. E., Mascarenhas, M., Gruenheid, S., Finlay, B. B., and Karmali, M. A. (2008). Molecular analysis as an aid to assess the public health risk of non-O157 Shiga toxin-producing Escherichia coli strains. Appl. Environ. Microbiol. 74, 2153–2160. doi: 10.1128/AEM.02566-07

Dean-Nystrom, E. A., Melton-Celsa, A. R., Pohlenz, J. F., Moon, H. W., and O’Brien, A. D. (2003). Comparative pathogenicity of Escherichia coli O157 and...
intimin-negative non-O157 Shiga toxin-producing E. coli strains in neonatal pigs. Infect. Immun. 71, 6526–6533. doi: 10.1128/IAI.71.11.6526-6533.2003

Del Castillo, F. J., Leal, S. C., Moreno, P., and Del Castillo, I. (1997). The _Escherichia coli_ K-12 _sheA_ gene encodes a 34-kDa secreted haemolysin. Mol. Microbiol. 25, 107–115. doi: 10.1046/j.1365-2958.1997.2531813.x

De Sablet, T., Bertin, Y., Vareille, M., Girardeau, J. P., and De Sablet, T. (2014). Tracing Pathogens in the Food Chain, 548–595. doi: 10.1088/9780857090508.4.548

Amézquita-López et al. Virulence profiles in STEC

Kaper, J. B. (1998). The locus of enteroocyte effacement pathogenicity island of Shiga toxin-producing _Escherichia coli_ O157:H7 and other attaching and effacing _E. coli_. Jpn. J. Med. Sci. Biol. 51(Suppl.), S101–S107.

Karmali, M. A. (1989). Infection by verocytotoxin-producing _Escherichia coli_. Clin. Microbiol. Rev. 2, 15–38.

Karmali, M. A. (2009). Host and pathogen determinants of verocytoxigen-producing _Escherichia coli_-associated hemolytic uremic syndrome. Kidney Int. Suppl. 75, S4–S7. doi: 10.1038/ki.2008.608

Karmali, M. A., Gannon, V., and Sargeant, J. M. (2010). Verocytoxigen-producing _Escherichia coli_ (VTEC). Vet. Microbiol. 140, 360–370. doi: 10.1016/j.vetmic.2009.04.011

Karmali, M. A., Steele, B. T., Petric, M., and Lim, C. (1983). Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing _Escherichia coli_ in stools. Lancet 321, 619–620. doi: 10.1016/S0140-6736(83)91795-6

Kerenyi, M., Allison, H. E., Bätäi, L., Sonnevend, Á., Emödy, L., Plaveczyk, N. et al. (2005). Occurrence of _hlyA_ and _sheA_ genes in extraintestinal _Escherichia coli_ strains. _J. Clin. Microbiol._ 43, 2965–2968. doi: 10.1128/JCM.43.6.2965-2968.2005

Keusch, G. T., Iacewicz, M., Acheson, D. W., Donohue-Rolfe, A., Kane, A. V., and McCluer, R. H. (1995). Globotriaosylceramide, Gb3, is an alternative functional receptor for Shiga-like toxin 2e. *Infect. Immun.* 63, 1138–1141.

Kobayashi, N., Lee, K. I., Yamaizaki, A., Saito, S., Furukawa, I., Kono, T., et al. (2013). Virulence gene profiles and population genetic analysis for exploration of pathogenic serogroups of Shiga toxin-producing _Escherichia coli_. _J. Clin. Microbiol._ 51, 4022–4028 doi: 10.1128/JCM.01598-13

Koch, C., Hertwig, S., Lurz, R., Appel, B., and Beutin, L. (2001). Isolation of a lysogenic bacteriophage carrying the _stx2_ gene, which is closely associated with Shiga toxin-producing _Escherichia coli_ strains from sheep and humans. _J. Clin. Microbiol._ 39, 3992–3998. doi: 10.1128/JCM.39.11.3992-3998.2001

Kumar, H. S., Karunasagar, I., Teizou, T., Shima, K., and Yamashiki, S. (2004). Characterisation of Shiga toxin-producing _Escherichia coli_ (STEC) isolated from seafood and beef. _FEMS Microbiol. Lett._ 233, 173–178. doi: 10.1016/j.femsle.2004.02.008

Kyle, J. L., Cummings, C. A., Parker, C. T., Quinones, B., Vatta, P., Newton, E. et al. (2012). _Escherichia coli_ serotype O55:H7 diversity supports parallel acquisition of bacteriophages at Shiga toxin phage insertion sites during evolution of the O157:H7 lineage. _J. Bacteriol._ 194, 1885–1896. doi: 10.1128/JB.00120-12

La Ragione, R. M., Best, A., Woodward, M. J., and Wales, A. D. (2009). _Escherichia coli_ O157:H7 colonization in small domestic ruminants. _FEMS Microbiol. Rev._ 33, 394–410. doi: 10.1111/j.1574-6976.2008.00138.x

Leung, P. H., Peiris, J. S., Ng, W. W., Robins-Browne, R. M., Bettelheim, K. A., and Yam, W. C. (2003). A newly discovered verotoxin variant, VT2g, produced by bovine verocytotoxigenic _Escherichia coli_. _Appl. Environ. Microbiol._ 69, 7549–7553. doi: 10.1128/AEM.69.12.7549-7553.2003

Ludwig, A., Von Rhein, C., Bauer, S., Hüttinger, C., and Goebel, W. (2004). Molecular analysis of cytolyisin A (ClyA) in pathogenic _Escherichia coli_ strains. _J. Bacteriol._ 186, 5311–5320. doi: 10.1128/JB.186.16.5311-5320.2004

Mandrell, R. E. (2011). “Tracing pathogens in fruit and vegetable production chains,” in Tracing Pathogens in the Food Chain, eds S. Brut, P. M. Fratamico, and T. McMeekin (Philadelphia, PA: Woodhead Publishing), 548–595. doi: 10.1533/9780857090508.5.458

Manning, S. D., Motiwala, A. S., Springman, A. C., Qi, W., Lacher, D. W., Ouellette, L. M., et al. (2008). Variation in virulence among clades of _Escherichia coli_ O157:H7 associated with disease outbreaks. _Proc. Natl. Acad. Sci. U.S.A._ 105, 4868–4873. doi: 10.1073/pnas.0710834105

Mathusa, E. C., Chen, Y., Enache, E., and Hontz, L. (2012). Non-O157 Shiga toxin-producing _Escherichia coli_ in foods. _J. Food Prot._ 75, 1721–1736. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/22828483

Melton-Celsa, A., Mohawk, K., Teel, L., and O’Brien, A. (2012). Pathogenesis of Shiga-toxin-producing _Escherichia coli_. _Curr. Top. Microbiol. Immunol._ 357, 67–103. doi: 10.1007/82_2011_176

Muniesa, M., Blanco, J. E., De Simón, M., Serra-Moreno, R., Blanch, A. R., and Jofre, J. (2004). Diversity of _stx2_ converting bacteriophages induced from Shiga-toxin-producing _Escherichia coli_ strains isolated from cattle. _Microbiology_ 150, 2959–2971. doi: 10.1099/mic.0.27188-0
Amézquita-López et al. Virulence profiles in STEC

Muthing, J., Schewepe, C. H., Karch, H., and Friedrich, A. W. (2009). Shiga toxins, glycosphingolipid diversity, and endothelial cell injury. *Thromb. Haemost.* 101, 232–246. doi: 10.1160/TH08-05-0317

Nakao, H., Kimura, K., Murakami, H., Maruyama, T., and Takeda, T. (2002). Subtyping of Shiga toxin 2 variants in human-derived Shiga toxin-producing *Escherichia coli* strains isolated in Japan. *FEMS Immunol. Med. Microbiol.* 34, 289–297. doi: 10.1111/j.1574-695X.2002.tb00636.x

Ogden, I. D., Macrae, M., and Strachan, N. J. (2005). Concentration and prevalence of *Escherichia coli* O157 in sheep faeces at pasture in Scotland. *J. Appl. Microbiol.* 98, 646–651. doi: 10.1111/j.1365-2672.2004.02493.x

Paton, A. W., Srimanote, P., Woodrow, M. C., and Paton, J. C. (2001). Identification and characterization of Shiga toxin 2 from *Escherichia coli* O157:H7 and inhibitors of toxin activity. *Appl. Environ. Microbiol.* 75, 1410–1416. doi: 10.1128/AEM.02230-08

Quíones, B., and Swimey, M. S. (2011). Use of a Vero cell-based fluorescent assay to assess relative toxicities of Shiga toxin 2 subtypes from *Escherichia coli*. *Methods Mol. Biol.* 739, 61–71. doi: 10.1007/978-1-61779-102-4_6

Quíones, B., Swimey, M. S., Narm, K.-E., Patel, R. N., Cooley, M. B., and Mandrell, R. E. (2012). O-antigen and virulence profiling of Shiga toxin-producing *Escherichia coli* by a rapid and cost-effective DNA microarray colorimetric method. *Front. Cell. Inf. Microbiol.* 2:61. doi: 10.3389/fcimb.2012.00061

Scheutz, F., and Strockbine, N. A. (2005). “Genus I. *Escherichia*,” in Bergey’s *Manual of Systematic Bacteriology*, eds G. M. Garrity, D. J. Brenner, N. R. Krieg and J. T. Staley (New York, NY: Springer), 607–624.

Schmidt, H., Zhang, W. L., Henmrich, U., Jelacic, S., Brunder, W., Tarr, P. L., et al. (2001). Identification and characterization of a novel genomic island integrated at selC in locus of enterocyte effacement-negative, Shiga toxin-producing *Escherichia coli*. * Infect. Immun.* 69, 6863–6873. doi: 10.1128/IAI.69.11.6863-6873.2001

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 July 2013; paper pending published: 20 August 2013; accepted: 13 January 2014; published online: 31 January 2014.

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