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Phenotypic and genomic comparison of three human outbreak and one cattle-associated Shiga toxin-producing Escherichia coli O157:H7

Nathan Peroutka-Bigus, Daniel Nielsen, Julian Trachsel, Kathy Mou, Vijay Sharma, Indira Kudva, and Crystal Loving

Corresponding Author(s): Crystal Loving, USDA-ARS-National Animal Disease Center

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Editor: Luxin Wang

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1128/spectrum.04140-23

Re: Spectrum04140-23 (Phenotypic and genomic comparison of human outbreak and cattle-associated Shiga toxin-producing Escherichia coli O157:H7)

Dear Dr. Crystal L Loving:

Thank you for submitting your work to Spectrum. The reviewers have shared their comments and I hope that you can address these comments in your revision. Please return the manuscript within 60 days; if you cannot complete the modification within this time period, please contact me. If you do not wish to modify the manuscript and prefer to submit it to another journal, notify me immediately so that the manuscript may be formally withdrawn from consideration by Spectrum.

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• Upload point-by-point responses to the issues raised by the reviewers in a file named "Response to Reviewers," NOT IN YOUR COVER LETTER
• Upload a compare copy of the manuscript (without figures) as a "Marked-Up Manuscript" file
• Upload a clean .DOC/.DOCX version of the revised manuscript and remove the previous version
• Each figure must be uploaded as a separate, editable, high-resolution file (TIFF or EPS preferred), and any multipanel figures must be assembled into one file
• Any supplemental material intended for posting by ASM should be uploaded separate from the main manuscript; you can combine all supplemental material into one file (preferred) or split it into a maximum of 10 files, with all associated legends included

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Thank you for submitting your paper to Spectrum.

Sincerely,
Luxin Wang
Editor
Microbiology Spectrum

Reviewer #1 (Comments for the Author):

This manuscript reports a large genotypic and phenotypic comparison of four E. coli O157:H7 strains that used diverse in-vitro, in-silico and in-vivo experiments. The central non-null hypothesis of this study was that epidemiological strains were phenotypically and genomically different than strains isolated from cattle. While the list of multiple comparisons is rather unique, the novelty of this research is limited because there have been multiple previously published analyses that included many more strains from clinical and cattle origin, and most of those cases the results seem to be similar, genotypically there may be some differences, but phenotypically there were not many.
The main issue with this manuscript is the unbalanced experimental design to address that core hypothesis. The use of only three clinical strains and one cattle isolate for testing the above hypothesis has extremely low power to be able to find significant

differences between the two origin types or lineages. In addition to this inherent limitation, an additional confounding factor is that two of the clinical strains are LSPA6 lineage I and the reminder clinical and bovine strains are LSPA6 lineage I/II. Did you consider including at least two bovine strains belonging to this lineage instead of a clinical and a cattle isolate?
Title - modify title to reflect the limited scope of this manuscript: "Phenotypic and genomic comparison of three human outbreak strains and one cattle-associated strain of Shiga-toxin-producing Escherichia coli O157:H7"
Abstract - modify broadly. As it stands it provides excessive background information (9 out of 19 lines) and very few results. For the number of results that this paper is reporting, abstract is not even a balanced summary of them. Please include quantitative results.
Supplementary figures and tables - it is not clear what criteria was used to decide when a table or figure was placed in the supplemental material. Several supplemental figures and tables were cited more than once in the text that they should be considered part of the main document. With 3 tables and 7 figures plus 4 supplemental tables and 4 supplemental figures that may be needed in the main text, it is an unusually large manuscript. I wonder if it should be split into two parts instead.
L76 - replace "food" with "beef"
L125-129 - delete this information as it is already in M&M. Delete Table 1 as most of the information is in the M&M text or can be incorporated there.
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L278-293 - these results seem quite odd. They may be an artifact of the particular method use for biofilm growth. I would suggest eliminating it from the paper entirely as they bring little information to answer the core hypothesis.
L472 and Table 1 - clarify if the strains were isolated from spinach and lettuce or if they were clinical isolates in outbreaks that involved spinach and lettuce. Also, what other criteria was used to select FRIK 1989 among multiple other cattle isolates that could have been used here? Delete Table 1.
L547 - insert a space in 10g and 50ml
Reference section - needs extensive editing to make sure that all reference titles and journal names conform with Spectrum's format.
Fig. S1 - only one plot has noticeable error bars, please revisit. Make the plot symbols more easily distinguishable among each other, either larger or use different choices.

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It is not clear why (O157) is included in the first line of the abstract. And O157 should not be used as an abbreviation. The name E. coli O157:H7 should be written.

The abstract is missing data.

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Line 67 includes data from 2010-2017, the authors should include here more recent information?

Line 104 is in humans?

In lines 186-189 what are the levels at which the cattle are shedding?

Are the differences in the Caco-2 and RSE cell culture assays expected?

The qRT-PCR section should be clarified and broken into separate paragraphs.

In the discussion section it would be great if the authors could speculate on super shedders based on their findings. This goes along with the information in line 366.

The tables are useful.

In Figure 2 AULC should be defined.

Reviewer #3 (Comments for the Author):

The manuscript "Phenotypic and genomic comparison of human outbreak and cattle-associated Shiga 2 toxin-producing Escherichia coli O157:H7" was reviewed. In general, the protocols employed and data analysis seem well executed. The manuscript is very well written and should be published after some corrections.

I have comments to address in the manuscript:

1. In the methods section, whenever you use the words "as previously described" (example: lines 549 and 550), you should instead say "as described elsewhere" and add the reference of the paper.

2. In some portions of the methods, it will be beneficial to add a brief description about "why" that experiment was conducted so the manuscript's experiments won't look disconnected. For example, for the cattle in vitro experiment, I don't see a strong connection with other experiments; explaining and justifying will help the reader connect the human-related experiment with animal experiments.

3. Regarding the in vivo assays studying O157 colonization in cattle, a decline in bacterial concentration was observed over time. After day 14, no O157 was detected. Considering the days that animals take for rumination, one could argue that no colonization was evident, as seen by the loss of the organism over time. These results should be addressed better in the discussion and connected with the overall research.

4. Line 540 indicates an inoculation of 6x109. Was this per ml or per 10 ml? Please clarify in the manuscript.

5. Did you test animals for the presence of E. coli O157 in feces before infecting them?

6. Please discuss the need for the growth curves and the obtained results in the manuscript. Make sure you explain the significance of this assay for your research. Why were the growth curves needed?

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2. In some portions of the methods, it will be beneficial to add a brief description about “why” that experiment was conducted so the manuscript’s experiments won’t look disconnected. For example, for the cattle in vitro experiment, I don’t see a strong connection with other experiments; explaining and justifying will help the reader connect the human-related experiment with animal experiments.

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Subject: Spectrum04140-23 Decision Letter

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Thank you for submitting your paper to Spectrum.

Sincerely,
Luxin Wang
Editor
Microbiology Spectrum

Reviewer #1 (Comments for the Author):

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The main issue with this manuscript is the unbalanced experimental design to address that core hypothesis. The use of only three clinical strains and one cattle isolate for testing the above hypothesis has extremely low power to be able to find significant differences between the two origin types or lineages. In addition to this inherent limitation, an additional confounding factor is that two of the clinical strains are LSPA6 lineage I and the reminder clinical and bovine strains are LSPA6 lineage I/II. Did you consider including at least two bovine strains belonging to this lineage instead of a clinical and a cattle isolate?

Response: It was difficult to narrow down the analysis to the four isolates selected. We respect the comment and agree, it is difficult to make sweeping generalizations from the data. However, we started with what we could, and think a major finding of the report is the actual lack of major

differences even between the isolates we selected, particularly as it relates to shedding from cattle, yet we see significant differences in other *in vitro* phenotypes (Cell adherence, biofilm, toxin production, gene expression) between these isolates. We've tried to capture these similarities and differences throughout the manuscript.

1. *Title - modify title to reflect the limited scope of this manuscript: "Phenotypic and genomic comparison of three human outbreak strains and one cattle-associated strain of Shiga-toxin-producing Escherichia coli O157:H7"*

Response: We agree and thank the reviewer for the comment and we have amended the title to describe the study more accurately "Phenotypic and genomic comparison of three human outbreak and one cattle-associated Shiga toxin-producing *Escherichia coli* O157:H7".

2. Abstract - modify broadly. As it stands it provides excessive background information (9 out of 19 lines) and very few results. For the number of results that this paper is reporting, abstract is not even a balanced summary of them. Please include quantitative results.

Response: Background information was removed and a better description of the manuscripts finding was included in the abstract.

3. Supplementary figures and tables - it is not clear what criteria was used to decide when a table or figure was placed in the supplemental material. Several supplemental figures and tables were cited more than once in the text that they should be considered part of the main document. With 3 tables and 7 figures plus 4 supplemental tables and 4 supplemental figures that may be needed in the main text, it is an unusually large manuscript. I wonder if it should be split into two parts instead.

Response: The criteria used to decide if a table or figure was included in the main text or supplemental had to do if the figure/table dealt directly with a prominent phenotype. Supplementary figures/table were those that added additional insight into these phenotypes or provided background data that may be desirable for readers. We agree that some supplemental figures are better suited to be in the main text, and we have taken two supplemental figures (Growth curve and Gene presence/absence heat map) and incorporated them into figure one. We agree the manuscript is large, but as a whole it is a thorough detailing of these *E. coli* O157:H7 isolates and splitting the manuscript up would lose some of the connection between experimental results.

4. L76 - replace "food" with "beef"

Response: Thank you catching this, "beef" would be the correct terminology. This has been changed in the manuscript.

5. L125-129 - delete this information as it is already in M&M. Delete Table 1 as most of the information is in the M&M text or can be incorporated there.

Response: We appreciate the reviewers note of showing similar information in two ways. We have elected to leave the information because it presents the isolates and background on the isolates early in paper and does not necessitate the readers having to jump ahead to the methods to find the information. We find tables like Table 1 are somewhat common and can be useful in reading the paper.

6. L131 - Fig. S1 should be Fig. 1 because a subsection is dedicated to it.

Response: We agree. Fig. S1 is now part of Fig. 1. The subsection originally describing it has been incorporated into the Comparative genomics subsection which is now referred to Comparative genomics and isolate characterization. The original lines are now found at lines 168-170.

7. L141-144 - this supports the idea that the selection of the non-clinical strain was not well justified.

Response: The following statement was added to better explain why FRIK1989 was used (lines 159-167) "It should be noted that this comparison was intended to be between *E. coli* O157:H7 isolates that possess the full spectrum of virulence genes, so it was not unexpected that FRIK1989 phylogeny was similar to clinical isolates. Inclusion of FRIK1989 in the comparison was intended to highlight differences or similarities between known human outbreak isolates and an isolate taken from cattle with no known association with clinical illness in humans. In addition, FRIK1989 was found to possess a larger accessory genome than the other isolates, with FRIK1989 having 419 accessory genes, while EDL933, TW14588, and RM6067W have 251, 353, and 364 accessory genes respectively."

8. *L278-293 - these results seem quite odd. They may be an artifact of the particular method use for biofilm growth. I would suggest eliminating it from the paper entirely as they bring little information to answer the core hypothesis.*

Response: Both crystal violet and resazurin are commonly used methods for measuring biofilms, including with *E. coli* O157:H7. References have been added in the Materials and Methods section to support this, lines 839. It is also not uncommon for there to be significant differences in biofilm production across *E. coli* O157:H7 isolates, including intrastrain differences depending on the status of genes such as *rpoS*. Within the discussion at lines 463-464 the following statement has been added to support the differences measured, "The stark difference in biofilm production between the isolates is expected as biofilm production in *E. coli* O157:H7 is

strain specific (52).” As biofilm formation could be an important phenotype of O157, we elected to include it as a measure.

9. L472 and Table 1 - clarify if the strains were isolated from spinach and lettuce or if they were clinical isolates in outbreaks that involved spinach and lettuce. Also, what other criteria was used to select FRIK 1989 among multiple other cattle isolates that could have been used here? Delete Table 1.

Response: Table one indicates the source of all isolates used. TW14588 was isolated from lettuce that was associated with the 2006 Taco John outbreak. RM6067W was isolated from spinach that was associated with an outbreak in 2006. The description of these isolates in the table has been updated with the header “Associated Environmental Source” in place of “Source”,. FRIK1989 was chosen because it appeared to be genetically interesting as its lineage placement was initially ambiguous and it possesses a large accessory genome in relation to the other three isolates (EDL933 has 251 accessory genes, RM6067W has 364 accessory genes, TW14588 has 353 accessory genes, while FRIK1989 has 419 accessory genes). However, it also encodes O157 virulence genes as an STEC. It is difficult to select a single strain that has the potential to cause disease (based on genes) but to date was only associated with cattle. We also had already received FRIK1989 into our collection to begin studies in a timely manner. See comment #7 for additional text added to the manuscript to justify using FRIK1989. See comment #5 regarding Table 1.

10. L547 - insert a space in 10g and 50ml

Response: Thank you for catching this. Manuscript has been corrected.

11. Reference section - needs extensive editing to make sure that all reference titles and journal names conform with Spectrum's format.

Response: Thank you for catching this. The reference section has been edited.

12. Fig. S1 - only one plot has noticeable error bars, please revisit. Make the plot symbols more easily distinguishable among each other, either larger or use different choices.

Response: We thank the reviewer for catching this. Some of the error bars were hidden behind the plot symbols. To make the error bars visible and to make the different isolates more distinguishable the plot was changed from having symbols denote the different isolates to using a color scheme with thicker lines and removing the plot symbols. This figure is now incorporated into main figure 1.

Reviewer #2 (Comments for the Author):

1. It is not clear why (O157) is included in the first line of the abstract. And O157 should not be used as an abbreviation. The name E. coli O157:H7 should be written.

Response: We have made the suggested change.

2. The abstract is missing data.

Response: The abstract was rewritten to include a better description of the manuscript's findings.

3. The statement in lines 51-53 is not true. There are many papers that clearly demonstrate the genes in E. coli O157:H7 and their phenotypic actions in humans. If the authors mean in cattle this should be clarified.

Response: We thank the reviewer for asking for this piece of information for improved clarity. Indeed, it was in reference to cattle. The text has been updated to clarify this point with "The relationship between *E. coli* O157:H7 genome and phenotype as it relates to colonization in cattle is unclear, and improved understanding could lead to additional strategies to limit *E. coli* O157:H7 in the food chain.", lines 52-55.

4. Line 67 includes data from 2010-2017, the authors should include here more recent information?

Response: The section was updated with data from the CDCs National Outbreak Reporting System; the data is now current to 2021, which is the most recent year posted in the database. (National Outbreak Reporting System (NORS) Dashboard | CDC). And the manuscript was changed accordingly, lines 67-72 now read "Thirty-two percent of confirmed *E. coli* O157 serotype outbreaks during the years 2009–2021 were attributed to foodborne illness, with beef (31%), vegetable row crops (25%), dairy (10%), and fruit (4%) being the primary food sources (2). Illnesses associated with *E. coli* O157 serotype have resulted in 76 multistate outbreaks in the U.S. from the years 2009-2021, and in 5,004 illnesses and 30 deaths (2)."

5. Line 104 is in humans?

Response: Yes, this is in reference to illness in humans. The text has been updated to reflect this. It now reads "The *hlyA* gene, along with *eae* and *stx2,* was present in the majority of *E. coli* O157:H7 HUS cases in humans.", lines 103-104.

6. In lines 186-189 what are the levels at which the cattle are shedding?

Response: The text (lines 196-202) has been updated to reflect the CFUs/g in feces for the cattle that were still shedding. "By the end of the 14-day monitoring period not all calves were negative for *E. coli* O157:H7, with one of seven calves in EDL933 group (600 CFUs/g feces), two of eight calves in TW14588 group (both by enrichment culture only), and two of eight calves

in FRIK1989 group (2400 CFUs/g feces and by enrichment culture only) and three of seven calves in RM6067W group (1150 CFUs/g feces and the other two by enrichment culture only) still shedding."

7. Are the differences in the Caco-2 and RSE cell culture assays expected?

Response: Genes (*eae*) associated with adherence in other tissue culture cell lines are not known to impact adherence to RSE cells (reference #60) so the differences in adherence between Caco-2 and RSE cells were not surprising and could reflect the different nature of adherence between bovine and human cells. This was noted in the manuscript (lines 441-443) as "A clear association between the two models in similarity of adherence is lacking, but these results may underline how different *E. coli* O157:H7 genetic backgrounds influence cattle vs human colonization."

8. The qRT-PCR section should be clarified and broken into separate paragraphs.

Response: The following statement was added to clarify with the reader why AULC was done "…the AULC analysis was incorporated to capture overall trends in gene expression as growth phase had a large effect on expression across the isolates.", lines 258-259. The single long paragraph was broken up into three sections, one introducing the section and then separate paragraphs for toxin related genes and adherence related genes.

9. In the discussion section it would be great if the authors could speculate on super shedders based on their findings. This goes along with the information in line 366.

Response: The super shedder notation can be somewhat controversial in the field, and we try to limit speculation and instead just report the results so inferences can be made by the reader. The following paragraph has been added to the discussion, lines 411-424: "While shedding was greater than 10^4 CFUs/g feces at some time points, which is the amount indicative of a super-shedder (61, 62), shedding at $>10^4$ CFUs/g feces was transient and not detected in all cattle within a group. Supper-shedding is thought to play a prominent role in environmental and cattle carcass contamination with *E. coli* O157 (63, 64). RM6067W was the only isolate to have all inoculated cattle shed at $>10^4$ CFUs/g feces (i.e., super-shedder levels), but this level of shedding was again transient and for three cattle was only noted at a single sampling timepoint. Given the definition of super-shedder ($>10^4$ CFUs/g feces) all four *E. coli* O157:H7 isolates resulted in the transient presence of supper-shedding cattle. The role of biofilm sloughing off in the intestines is suggested to play a role in supper-shedding (62), yet here no significant difference in shedding observed between the poorest biofilm producer RM6067W and the strongest biofilm producer EDL933. The relationship between biofilm formation and sloughing in cattle and the observed *in vitro* biofilm results is unclear, but our data suggest there is little relationship between the two phenotypes."

10. The tables are useful.

Response: Thank you.

11. In Figure 2 AULC should be defined.

Response: AULC is defined in the figure legend “Jersey calves were orally inoculated with 6 x 10^9 CFUs of indicated E. coli O157:H7 isolates (EDL933, TW14588, FRIK1989, or RM6067W) and feces and recto-anal junction mucosa swabs (RAMS) were collected on days 1, 2, 3, 4, 5, 7, 9, 11, and 14 to enumerate O157 levels as indicated in materials and methods. Area Under the Log Curve (AULC) was performed (trapezoid Area Under the Curve model; RStudio version 2021.09.2)”

Reviewer #3 (Comments for the Author):

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I have comments to address in the manuscript:

1. In the methods section, whenever you use the words "as previously described" (example: lines 549 and 550), you should instead say "as described elsewhere" and add the reference of the paper.

Response: The text has been updated.

2. In some portions of the methods, it will be beneficial to add a brief description about "why" that experiment was conducted so the manuscript's experiments won't look disconnected. For example, for the cattle in vitro experiment, I don't see a strong connection with other experiments; explaining and justifying will help the reader connect the human-related experiment with animal experiments.

Response: Additional text was added to the method sections to clarify why particular experiments were performed and how they relate/add to the experimental methodology. For example, current lines 785-787 state “Gene expression of several shared genes related to adherence and toxin production were assessed to allow association with relevant phenotypes and if differences in expression associated with gene polymorphisms.”

3. Regarding the in vivo assays studying O157 colonization in cattle, a decline in bacterial concentration was observed over time. After day 14, no O157 was detected. Considering the days that animals take for rumination, one could argue that no colonization was evident, as seen by the

loss of the organism over time. These results should be addressed better in the discussion and connected with the overall research.

Response: We thank the reviewer for their comment. The following text (lines 398-410) has been added to the discussion to address this " Sporadic *E. coli* O157:H7 shedding has been observed in longitudinal cattle herd studies (57). The short plateau and subsequent rapid decline in recovered CFUs from the feces and RAJ, along with the absence of recovered CFUs in the later sampling timepoints, suggests that the *E. coli* O157:H7 isolates chosen colonized at low levels as many of the positive cattle at later timepoints had *E. coli* O157:H7 detectable only through enrichment cultivation. Similar colonization trends are observed in other experimentally infected cattle studies (55, 56, 58), suggesting that *E. coli* O157:H7 colonize cattle at low CFU burdens or frequent transient colonization is sufficient to maintain *E. coli* O157:H7 in cattle herds. Studies report the absence of *E. coli* O157:H7 recovery from feces one day and then the presence of *E. coli* O157:H7 from feces the next day from the same animal (55, 58), which was observed in several cattle from this study. Given that O157 readily attaches to bovine intestinal cells (59, 60) some amount of colonization is anticipated, and it's unclear how a small amount of shedding relates to contamination at processing."

4. Line 540 indicates an inoculation of 6x109. Was this per ml or per 10 ml? Please clarify in the manuscript.

Response: This was per 10 ml. The text has been updated to clarify this (lines 666-668) with "Calves were inoculated orally with 10 mL of the prepared inoculum, which contained approximately 6 x 10^9 total CFUs of each respective isolate."

5. Did you test animals for the presence of E. coli O157 in feces before infecting them?

Response: Yes, animals were prescreened. *E. coli* was recovered but was not O157 based on agglutination testing. The following text has been added to the methods (lines 659-661) "The calves were pre-screened for the presence of *E. coli* O157:H7 and were found to be negative based on O157 agglutination testing". Additionally, the PATS profiles of recovered *E. coli* O157:H7 isolates from the test animals matched that of the inoculating isolates. This was stated in the results in lines 190-192 "The PATS profile of isolates recovered matched that of the respective inoculum administered to the animals, thereby suggesting the lack of pre-existing *E. coli* O157:H7".

6. Please discuss the need for the growth curves and the obtained results in the manuscript. Make sure you explain the significance of this assay for your research. Why were the growth curves needed?

Response: The following text was added to the discussion (lines 377-384) "While the growth curves of the four isolates was unremarkable when grown in LB, when grown in low glucose DMEM the EDL933 isolates OD measurements indicated a slower rate of growth and lower

overall cell density once stationary phase was reached. We attribute these lower OD measurements to EDL933's unique propensity to flocculate when grown in low glucose DMEM, which was not witnessed in the other three isolates. This flocculating phenotype of EDL933 is likely driven by its propensity to produce curli fimbriae (54) and would have implications in adherence and biofilm formation." Additional text was added to the methods section to explain why growth curves were needed (lines 560-563) "Growth curves of the isolates were determined using a Bioscreen microplate reader (Growth Curves USA, Piscataway, NJ) in order to discern differences in growth rate which could indicate differences in substrate utilization or varied growth characteristics."

Re: Spectrum04140-23R1 (Phenotypic and genomic comparison of three human outbreak and one cattle-associated Shiga toxin-producing Escherichia coli O157:H7)

Dear Dr. Crystal L Loving:

I am pleased to inform you that your manuscript has been accepted, and I am forwarding it to the ASM production staff for publication. Your paper will first be checked to make sure all elements meet the technical requirements. ASM staff will contact you if anything needs to be revised before copyediting and production can begin. Otherwise, you will be notified when your proofs are ready to be viewed.

One reviewer did provide some additional comments, please make sure you address these in your final manuscript.

Data Availability: ASM policy requires that data be available to the public upon online posting of the article, so please verify all links to sequence records, if present, and make sure that each number retrieves the full record of the data. If a new accession number is not linked or a link is broken, provide production staff with the correct URL for the record. If the accession numbers for new data are not publicly accessible before the expected online posting of the article, publication may be delayed; please contact ASM production staff immediately with the expected release date.

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The ASM Journals program strives for constant improvement in our submission and publication process. Please tell us how we can improve your experience by taking this quick Author Survey.

Thank you for submitting your paper to Spectrum.

Sincerely,
Luxin Wang
Editor
Microbiology Spectrum

Reviewer #1 (Comments for the Author):

For the most part the authors were able to address most issues. However, the abstract still needs some work, some results were incorporated, but they still lack any quantitative data. For example, if there in any claim of increased expression, what were the actual fold-differences? If there was no difference in biofilm formation or cattle colonization, what were the parameters used to determine these. There were no differences in biofilm formation and cattle colonization. because of your small sample number, you cannot conclude anything that they were not related. Also, last statement, please delete "that caused the incidence of hemolytic uremic syndrome" because it seems to imply that the other strains used in this study caused also HUS. The differences in vero toxicity and stx2 expression should also be reported in the abstract.

Reviewer #2 (Comments for the Author):

The authors have responded thoughtfully to each comment and made significant changes in the manuscript to reflect the complexity of the analysis, but also to refine the information that is provided and at the center of the manuscript. Many specific points have been clarified that improve the content and impact of the manuscript.

It is important that scientists compare these phenotypic and genomic aspects of isolates, especially as surveillance and longitudinal studies continue to identify the presence of STEC and other pathogens in the environment.

The cattle work in this paper is quite unique. As is the comparison if Caco and Vero cells for attachment and toxin production.

The way that the figures are arranged and described in the text has improved in this manuscript revision.