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Short communication

Validation of high-resolution melting assays for the detection of virulent strains of *Escherichia coli* O26 and O111 in beef and pork enrichment broths

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ABSTRACT

Extensive validation of diagnostic assays using widely collected surveillance samples is critical for developing pathogen detection assays. The detection of potentially virulent *E. coli* strains is critical to the red meat industry. We previously developed two high-resolution melting (HRM) assays for detecting potentially virulent and avirulent *E. coli* O26 and O111 strains. Assays were validated using enriched beef (n = 36) and pork (n = 36) samples collected as part of a U.S. federal regulatory surveillance program. Data from this study showed more than 90% sensitivity and specificity for both the HRM assays, demonstrating suitability for the red meat industry and regulatory agencies.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens of great concern. In the United States, *E. coli* O157:H7 and six non-O157 STEC serogroups (i.e., O26, O45, O103, O111, O121, and O145) are considered adulterants in non-intact beef. The Centers for Disease Control and Prevention (CDC) National outbreak reporting system (NORS) for the year 2018 reported a total of 1228 illnesses, 293 hospitalization, and nine deaths, which were associated with STEC strains. Further, in 2019, the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) initiated seven recalls due to the presence of STEC, which resulted in the recall of 298,500 pounds of food (USDA, FSIS, 2019). These recalled foods were comprised of raw ground beef, raw beef products, non-intact beef, and meat and poultry salad (USDA, FSIS, 2019).

The United States gross income from red meat sales (i.e., cattle, calves, hogs, and pigs) in 2019 was totaled at $88.7 billion (USDA, 2020). The presence of STEC strains in raw non-intact beef products has been extensively documented in the past. However, only limited studies have been published on the presence of STEC in finished pork products (Bardasi et al., 2017; Scott et al., 2020). The food industry, especially the red meat industry, is constantly threatened by outbreaks associated with STEC serogroups. Contamination by STEC and recalls such as these can significantly impact the United States gross income from red meat sales (i.e., cattle, calves, hogs, and pigs).

The virulent strains of STEC serogroups possess the Shiga toxin gene (*stx*) and the adherence factor intimin (*eae*) gene. These two genes are considered essential for causing severe human infection. Human infection by a virulent STEC strain may result in acute illness, bloody diarrhea, hemorrhagic uremic syndrome, and renal failure (Majowicz et al., 2014). *E. coli* strains lacking these genes can be considered avirulent as far as causing bloody diarrhea and severe complications of the enterohemorrhagic disease. *E. coli* belonging to the seven adulterant STEC serogroups are comprised of both virulent and avirulent strains. That is, many isolates are positive for the serogroup. However, they lack the *eae* and *stx* virulence genes. Various virulent (or potentially virulent) and avirulent strains of *E. coli* serogroups are an integral part of cattle microbiota. These serogroups may contaminate the carcasses during animal slaughter and carcass processing (Brichta-Harhay et al., 2008; Nastasijevic et al., 2020). Real-time PCR-based 5′-nuclease assays are commonly used to test STEC serogroups present in food samples (Singh et al., 2019; Singh & Mustapha, 2015). The USDA, FSIS describes the detection of STEC serogroups by initially screening for the presence of *stx* and *eae* genes. Samples testing positive for these two genes are then screened for the presence of *E. coli* O157:H7 and six non-O157 STEC serogroups using three sets of multiplex 5′-nuclease assays (USDA FSIS, 2020). A major limitation of this official method and other commercially available 5′-nuclease assays for STEC testing is that they may result in the diversion of meat products to thermal lethality steps due to the presence of avirulent strains of *E. coli* serogroups (i.e., false-positive test

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result). According to a USDA, FSIS Office of Public Health Science 2018 report, the false positive rates of potential screening were 93% for beef manufacturing trimming, 81% for ground beef, 100% for bench trim, and 94% for other components (USDA, FSIS, 2020b). This high false-positive rate results in an annual loss of approximately $47 million for the beef industry (USDA, FSIS, 2020b).

This study aimed to validate our previously standardized two high-resolution melting (HRM) assays. These two assays targeted SNPs on the serogroup-specific *fnl*1 and *wbd*k genes for O26 and O111, respectively (Singh et al., 2020). The HRM assays can identify the presence of specific SNP associated with potentially virulent or avirulent strains. During HRM analysis, each gene variant generates a unique melt profile facilitating discrimination of isolates into potentially virulent or avirulent groups. Spiked food sample studies are often performed in controlled conditions, which is different from surveillance samples. Therefore, this study aimed to perform robust validation of our assays using DNA isolated from beef and pork enrichments obtained from the U.S. federal regulatory surveillance program (USFRSP).

2. Methods

2.1. Samples

DNA from 12 pure culture *E. coli* O26 strains (O26-2, O26-1, O26-3, O26–16.2, O26–699.1, O26 766.1, O26–859.3, O26–946.1, O26-DEC10B, O26-97-3250, O26-MT#10, and O26-TB352A) and 14 pure culture *E. coli* O111 strains (O111-hSTEC 08, O111-C4-462-2_7095, O111-F-A 790.1, O111 739.3, O111-7075, O111-F6627, O111-0201 9611, O111-3007-85 O111-LF1, O111-LF2, O111-LF3, O111-LF4, O111-LF5 and O111-LF6) were used (Table 1). The assay was validated using selected naturally contaminated beef (n = 36) and pork (n = 36) enrichment samples, which were obtained from official testing laboratories (Supplementary Tables 1 and 2), which were processed according to the USDA FSIS MLG Chapter 5c. A portion of the initial enrichment broth was stripped of identity, mixed with glycerol (final concentration 17%) and frozen at −20 °C, then shipped to the Meat Safety and Quality Research Unit laboratory for testing. The frozen broths were thawed, processed for STEC isolation according to FSIS MLG Chp 5c, and a separate portion was used for bacterial DNA isolation using the Bacterial Genomic DNA Isolation Kit (Norgen, Biotek Corp, Ontario, Canada) following the manufacturer's recommendations. Samples that tested positive by the iQ-Check STEC VirX and SerO PCR Detection Kits (Bio-Rad Laboratories, Hercules, CA) were processed for the culture isolation of the specific serogroup of STEC identified following the method described in the USDA FSIS MLG Chapter 5c. Obtained isolates were characterized for the presence of virulence genes as described previously (Paton & Paton, 1998). DNA isolated from the enrichments was shipped to Florida State University, Food Microbiology laboratory to validate HRM assays. The DNA samples were quantified using a Nanodrop One Spectrophotometer (Thermo Fisher, DE, USA). Samples were diluted to 10 ng/μl and used for the real-time PCR HRM assays.

Table 1
List of pure culture strains used as a control for validation of HRM assays.

Strains	*stx*	*eae*
E. coli O26-2	1	1
E. coli O26-1	1	1
E. coli O26-3	1	1
E. coli O26–16.2	0	0
E. coli O26–699.1	0	1
E. coli O26–766.1	0	0
E. coli O26–859.3	0	0
E. coli O26–946.1	0	0
E. coli O26-DEC10B	1	1
E. coli O26-97-3250	1	1
E. coli O26-MT#10	1	1
E. coli O26-TB352A	1	1
E. coli O111-hSTEC 08	0	1
E. coli O111-C4-462-2_7095	1	1
E. coli O111-F-A 790.1	0	1
E. coli O111 739.3	0	0
E. coli O111-7075	1	0
E. coli O111-F6627	1	1
E. coli O111-0201 9611	1	1
E. coli O111-3007-85	1	1
E. coli O111-LF1	0	0
E. coli O111-LF2	0	0
E. coli O111-LF3	0	0
E. coli O111-LF4	0	0
E. coli O111-LF5	0	0
E. coli O111-LF6	0	0

2.2. Oligonucleotides

The oligonucleotides for this study were commercially synthesized and purchased from Integrated DNA Technologies (IDT, IA, USA). The *E. coli* O26 strains in the samples were detected by amplifying a fragment of serogroup specific *fnl*1 gene using O26-32F: 5′-GTG GCA CTG GTT CTT TTG GT-3′ and O26-118R: 5′-TTT CAT CCC TGC TAA ATA TTC G-3' (Singh et al., 2020). Whereas the *E. coli* O111 strains were detected by amplifying the *wbd*K gene using O111-634F: 5′-CTT CGA GCT CAT GGT TGG AC-3′ and O111-717R: 5′-CGA CTC TTC GAA AAT ATC ATC A-3′ primer-pair (Singh et al., 2020).

2.3. High-resolution real-time PCR assay

The PCR assays were performed on the LightCycler LC96 system (Roche Diagnostics, IN, USA) using 2 × LightCycler® 480 High Resolution melting master (Roche Diagnostics, IN, USA). The PCR assays were performed as previously described (Singh et al., 2020). Each 10 μl PCR reaction consisted of 20 ng of DNA, 2.5 mM $MgCl_2$, and 0.5 μM of each primer. The PCR amplifications were performed using one initial denaturation step (95 °C for 10 min), followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 62 °C for 30 s. At the end of the amplification cycle, a high-resolution melt step was added. The amplification and high-resolution melt data were collected in Channel 1 (470/514 nm) of the LightCycler LC96 system. The HRM data for O26 amplicons were analyzed with a pre-melt and post-melt regions of 73.3–74.3 °C and 78.7–79.7 °C, respectively. Whereas the HRM data of O111 amplicons were analyzed with a pre-melt and post-melt regions of 71.5–72.5 °C and 78.2–79.2 °C, respectively. Samples testing negative by the assay were removed from the HRM analysis.

2.4. Sequencing

The DNA samples testing positive by only the O26 and O111 HRM assays were confirmed by Sanger sequencing. The PCR amplicons generated using the above-mentioned primers pairs were purified using sodium acetate and ethanol precipitation (Sambrook & Russell, 2006). Each purified amplicon was diluted to a 10 ng/μl concentration. Samples were sequenced using 3.2 μM of forward primer at Florida State University molecular cloning facility (Tallahassee, FL, USA). Obtained sequence chromatograms were edited using Chromas Lite v2.1.1 (Technelysium, Brisbane, Australia). Obtained sequence data was used for serogroup confirmation after BLAST analysis.

2.5. Data analysis

The sensitivity, specificity, false-positive (FP) rate, false negative (FN) rate, positive predictive value (PPV), negative predictive value (NPV), and test accuracy were calculated as previously described (Bosilevac et al., 2019). Assays showing sensitivity, specificity, positive predictive value, negative predictive, and test accuracy value greater

than 90% were considered suitable for diagnostic applications (USDA, FSIS, 2010).

3. Results

Out of 72 enriched beef and pork samples tested in this study using our O26 HRM assay, a total of 16 were positive for the presence of O26. Twelve of these 16 samples correlated with the USDA FSIS MLG Bio-Rad SerO assay, while four were positive only by the HRM assay (Supplementary Tables 1 and 2). The four samples testing only by the O26 HRM assay and had a melting peak associated with potentially avirulent STEC-O26 strains, and these strains were further confirmed by Sanger sequencing. Therefore, these four samples were classified as true positive. The standardized O26 HRM assay for the serogroup identification showed sensitivity (100.0%), specificity (100.0%), FP rate (0%), FN rate (0.0%), PPV (100%), NPV (100.0%) and test accuracy (98.8%). Based on HRM analysis, all 16 samples from beef and pork enrichments that tested positive by the O26 HRM assay grouped with the avirulent control strains (Fig. 1 A and B). Further, investigation of these samples showed they could not be culture-confirmed to possess an adulterant SETC-O26 strain.

The O111 HRM assay was previously validated using DNA from a limited set of *E. coli* O111 strains. Therefore, we expanded that validation here to a total of 14 pure culture *E. coli* O111 strains. Five were the previously characterized strains used as positive controls, and six were recently isolated O111 strains lacking *stx* and *eae* genes (O111-LF1, O111-LF2, O111-LF3, O111-LF4, O111-LF5, and O111-LF6). During the HRM analysis, the six new avirulent strains and 7075 strain (*stx* positive and *eae* negative) clustered with other previously identified avirulent *E. coli* O111 strains. In contrast, the three STEC-O111 strains (i.e., F6627, 0201 9611, and 3007-85) clustered with potentially virulent strains.

Among 72 beef and pork enrichments, only 12 pork broths tested positive for the presence of O111 serogroup (Supplementary Tables 1 and 2). However, only four broths were identified as positive for *E. coli* O111 by the MLG method. Due to this, the O111 HRM assay, when compared with results generated using the MLG Chp 5c method, iQ-Check STEC SerO PCR Detection Kit (Bio-Rad Laboratories, Hercules, CA), showed a false-positive rate of 10.5%. All samples generating false-positive results (n = 8) using the HRM assay had a specific melt peak of 76.2 °C, and after HRM analysis, the eight samples grouped with melt profiles of avirulent strains. The amplicons generated by these disagreeing samples (n = 8) were further verified by Sanger sequencing. Sequencing data confirmed the amplicons were indeed the expected *E. coli* O111 sequence and had not been identified by the official method (i.e., iQ-Check STEC SerO PCR Detection assay). Therefore, the eight samples were considered as true positive for further analysis. As such, the O111 HRM assay showed sensitivity (100%), specificity (100%), FP rate (0%), FN rate (0%), PPV (100%), NPV (100.0%) and test accuracy (100%).

During HRM analysis, the 12 O111 positive pork enrichments and O111 control samples grouped into four distinct groups. The potentially virulent O111 strain C4-462-2_7095 (*stx* positive and *eae* positive) grouped slightly apart (Tm: 75.6 °C) from other potentially virulent strains that possessed *stx* and *eae*. Whereas strains LF-2 and LF-3 formed a slightly distinct group compared (Tm: 76.8 °C) to other avirulent strains (Tm: 76.5) (Fig. 1 C and D). Culture confirmation of all 12 broth samples showed a lack of an adulterant O111 strain.

4. Discussion

In addition to the culture-based method for pathogen isolation, diagnostic laboratories worldwide are moving towards culture-independent diagnostic tests (CIDTs) for pathogen detection (CDC, 2019). Real-time PCR-based detection methods are recommended and commonly used for STEC detection. Multiple real-time PCR chemistries (e.g., intercalating dye, dual-labeled probe) are primarily used for the real-time detection of amplified products. Currently, USDA, FSIS method (i.e., MLG5) uses a combination of 9 primer pairs and nine dual-labeled probes to detect seven STEC serogroups. One of the major limitations of the FSIS official method (i.e., MLG5c) and other commercially available assays for detecting non-O157 STEC is their inability to distinguish avirulent from virulent strains in a single reaction. These standard and commercially available methods commonly rely on *stx* and *eae* gene multiplex assay for the screen of potentially positive broths. Multiple *E. coli* serogroups, *Hafnia alvei*, *Citrobacter freundii, Shigella,* and temperate phages are known to possess *stx* genes (Margot et al., 2013; Quirós et al., 2015).

Additionally, the intimin gene has been reported among *Campylobacter*, *Citrobacter*, *Escherichia,* and *Shigella* genera (Gassama et al., 2001; Hyma et al., 2005). Therefore, screening based on the *stx* and *eae* gene results in higher potentially positive broths (Bosilevac & Koohmaraie, 2012). Between 2014 and 2020, the USDA FSIS tested 18,339 beef samples and among them identified 1008 as potentially positive following STEC screening (stx + eae + O-group+). Out of the 1008 positive samples, STEC was confirmed only in 114 samples (Dr. J. Emilio Esteban, USDA, FSIS, personal communication, 2020). The difference of 894 samples (or 89%) that could not be confirmed was most likely due to the detection of interfering non-pathogenic strains of top 6 STEC serogroups by the commercial assays. Further, when these sorts of broths are identified by beef producers as part of their process verification testing, the samples with avirulent strains result in product diversion and economic losses. In cases where a processor does not manufacture frozen products, the time required for culture confirmation is critical. Further, a screen positive product still cannot be released into commerce due to the difficulties of proving the screen result was the product of a mixed culture rather than a true positive.

In this study, a total of four and eight enrichments tested positive for

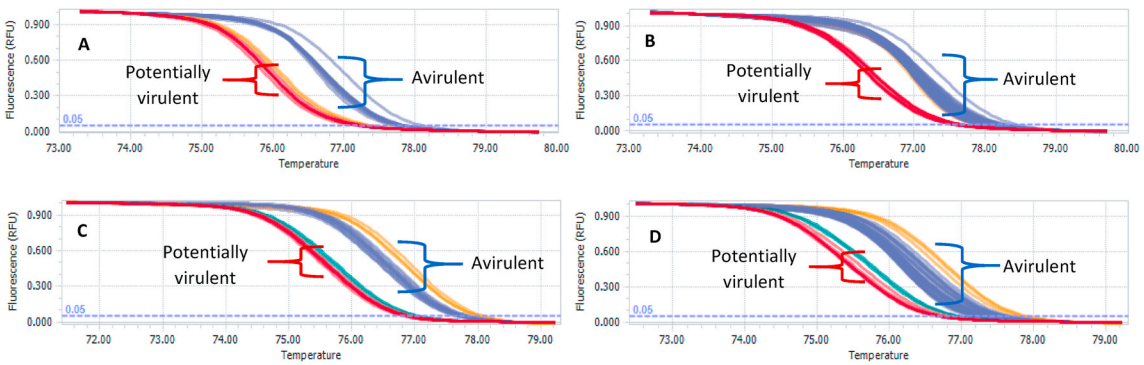

Fig. 1. Validation of *E. coli* O26 HRM assay using enriched beef samples (A) and enriched pork samples (B). Validation of *E. coli* O111 HRM assay using enriched beef samples (C) and enriched pork samples (D).

the presence of O26 and O111, respectively, only by the developed HRM assays (Supplementary Tables 1 and 2), which were further confirmed by Sanger sequencing of the amplicons. The observed variation between the developed HRM assays and the iQ-Check STEC SerO PCR Detection assay can be due to variations in primer binding site used for developing an assay, primer amplification efficiency, assay chemistry, or reaction conditions. Commercially available STEC serogroup detection assays rely on three to four target multiplex PCR reactions, which can result in competition in the PCR reaction resulting in lower PCR amplification efficiency. On the contrary, the HRM is always performed in a singleplex format, avoiding any reaction competition.

The O26 HRM assay gave mixed results for three pork enrichment broths. First, a false negative result, upon retesting by the O26 HRM assay, gave a positive result with a high Cq value (Cq = 33–35). The DNA concentration of these three samples was quantified and retested to overrule the chances of any handling errors. We speculate that these three samples may be contaminated with low levels of O26, causing variation between repeated assays.

The pork enrichments showed a high positive rate for the *E. coli* O26 and O111 strains. Similar results were reported for 465 not-ready-to-eat and 97 ready-to-eat pork products collected from the retail market and processing plants located in Italy. These samples were tested using ISO TS 13136:2012 method (Bardasi et al., 2017). Results from the study showed the presence of *E. coli* O26 and O111 in 76.2% (n = 32/42) and 19% (n = 8/42) of not-ready-to-eat samples, respectively (Bardasi et al., 2017). Despite the high PCR detection rate of STEC serogroups in pork samples collected from Italy, culture-based methods failed to isolate any STEC strain belong to the top seven STEC serogroups (Bardasi et al., 2017). Similar findings were reported by (Scott et al., 2020), which tested 1395 pork samples for STEC. Among these, 309 samples tested positive for the *stx* and *eae* genes and finely resulted in the isolation of only two adulterant STEC strains. These results demonstrate the high prevalence of potentially avirulent or non-viable STEC strains in the pork samples, which can interfere with testing and cause product diversion and loss of product value.

The O26 and O111 HRM assays validated using samples collected as part of a U.S. federal regulatory surveillance program provided sensitivity, specificity, positive predictive value, negative predictive, and test accuracy value greater than 90%, which is recommended by the FSIS Guidance for test kit manufacturers (USDA, FSIS, 2010). Therefore, the two HRM assays can be considered suitable for diagnostic applications. The HRM assays that specifically detect the potentially virulent strains of STEC serogroups can substantially reduce the number and costs associated with the laborious multi-day process of culture confirmation of samples initially testing positive by molecular assays.

5. Conclusion

In conclusion, this study further validated our previously published STEC-O26 and STEC-O111 HRM assays using seventy-two enriched beef (n = 36) and pork (n = 36) broths. The O26 and O111 HRM assays are low-cost methods that distinguish between adulterant potentially virulent strains and avirulent background strains that interfere with current methods. Their use in standard practice will reduce the amounts of product diverted to lethality steps and increase the accuracy of regulatory monitoring for these adulterant STEC serogroups.

Supplementary Table 1
Data for beef enrichments.

Sl. No.	*stx*	*eae*	O26 USFRSP	O26 HRM	O111 USFRSP	O111 HRM
1	1	1	0	0	0	0
2	1	1	0	0	0	0
3	1	1	0	0	0	0
4	1	1	0	0	0	0

(*continued on next column*)

Supplementary Table 1 (*continued*)

Sl. No.	*stx*	*eae*	O26 USFRSP	O26 HRM	O111 USFRSP	O111 HRM
5	1	0	0	0	0	0
6	1	0	0	0	0	0
7	1	0	0	0	0	0
8	1	0	0	0	0	0
9	1	0	0	0	0	0
10	0	1	0	0	0	0
11	1	0	0	0	0	0
12	1	0	0	0	0	0
13	1	1	0	0	0	0
14	1	1	0	0	0	0
15	1	0	0	0	0	0
16	1	0	0	0	0	0
17	1	0	0	0	0	0
18	1	0	0	0	0	0
19	0	1	0	0	0	0
20	1	1	1	1	0	0
21	1	1	0	0	0	0
22	1	0	0	0	0	0
23	1	0	0	0	0	0
24	0	1	0	0	0	0
25	1	0	0	0	0	0
26	0	1	0	0	0	0
27	1	1	0	0	0	0
28	0	1	0	0	0	0
29	1	1	0	0	0	0
30	1	1	0	0	0	0
31	0	1	0	0	0	0
32	0	1	0	0	0	0
33	0	1	0	0	0	0
34	0	1	0	0	0	0
35	1	1	0	0	0	0
36	1	1	0	0	0	0

DNA samples at the FSIS laboratory were tested for the presence of *stx* and *eae* genes using Biorad VirX. Samples for the presence of STEC serogroups were tested using the BioRad sero O screening assay and O26 and O111 HRM assays standardized by (Singh et al., 2020). The O26 and O111 USFRSP represent the data test results provided by the USDA. The O26 and O111 HRM represent the results from the assay developed in our study. In the table, "0" represents negative, and "1" represents positive.

Supplementary Table 2
Data for pork enrichments.

Sl. No.	stx	eae	O26 USFRSP	O26 HRM	O111 USFRSP	O111 HRM
1	1	1	0	0	0	0
2	1	1	1	1	0	1
3	1	1	0	0	0	0
4	1	1	0	0	1	1
5	1	1	1	0	0	0
6	1	1	1	1	0	1
7	1	1	0	0	0	0
8	1	1	1	1	0	0
9	1	1	1	1	0	0
10	1	1	1	0	0	0
11	1	1	0	1	0	1
12	1	1	0	1	0	0
13	1	1	0	0	0	1
14	1	1	1	1	0	1
15	1	1	1	1	0	0
16	1	1	0	0	0	0
17	1	1	0	0	0	1
18	1	1	0	0	1	1
19	1	1	0	0	0	0
20	1	1	0	1	0	0
21	1	1	1	1	0	0
22	1	1	0	0	0	0
23	1	1	1	1	0	0
24	1	1	0	0	0	0
25	1	1	0	0	0	0
26	1	1	1	1	0	1
27	1	1	0	0	0	0
28	1	1	0	0	0	0
29	1	1	0	0	0	0
30	1	1	1	0	0	0
31	1	1	0	0	0	0

(*continued on next page*)

Supplementary Table 2 (*continued*)

Sl. No.	stx	eae	O26 USFRSP	O26 HRM	O111 USFRSP	O111 HRM
32	1	1	0	1	0	1
33	1	1	0	0	0	0
34	1	1	1	1	1	1
35	1	1	1	1	1	1
36	1	1	0	0	0	0

DNA samples at the FSIS laboratory were tested for the presence of *stx* and *eae* genes using Biorad VirX. Samples for the presence of STEC serogroups were tested using the BioRad sero O screening assay and O26 and O111 HRM assays standardized by (Singh et al., 2020). The O26 and O111 USFRSP represent the data test results provided by the USDA. The O26 and O111 HRM represent the results from the assay developed in our study. In the table, "0″ represents negative, and "1″ represents positive.

CRediT authorship contribution statement

Frank J. Velez: Investigation. **Joseph M. Bosilevac:** Resources, Writing – review & editing. **Prashant Singh:** Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Supervision, Project administration.

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