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Research Paper

Distribution of Extremely Heat-Resistant *Escherichia coli* in the Beef Production and Processing Continuum

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ABSTRACT

Understanding the dynamics of stress-resistant *Escherichia coli* (*E. coli*) across the meat production and processing continuum is important for tracking sources of such microbes and devising effective modes of control. The Locus of Heat Resistance (LHR) is a ~14–19 Kb genetic element imparting extreme heat resistance (XHR) in Enterobacteriaceae. It has been hypothesized that thermal and antimicrobial interventions applied during meat processing may select for LHR$^+$ *E. coli*. Thus, our goal was to study the prevalence and molecular biology of LHR$^+$ *E. coli* among lots of beef cattle (n = 3) from production through processing. Two hundred thirty-two generic *E. coli* isolated from the same animals through seven stages of the beef processing continuum (cattle in feedyards to packaged strip loins) were examined. LHR$^+$ *E. coli* were rare (0.6%; 1 of 180) among the early stages of the beef continuum (feces and hides at feedlot, feces and hides at harvest, and preevisceration carcasses), whereas the prevalence of LHR$^+$ *E. coli* on final carcasses and strip loins was remarkably higher. Half (14 of 28) of the final carcass *E. coli* possessed the LHR, while 79.2% (19 of 24) of the strip loin *E. coli* did. Eighty-five percent (29 of 34) of the LHR$^+$ *E. coli* presented with the XHR phenotype. The selection or enrichment of LHR$^+$ *E. coli* from harvest steps to the final products appeared unlikely as the LHR$^+$ *E. coli* isolates were effectively controlled by antimicrobial interventions typically used during beef processing. Further, whole-genome sequencing of the isolates suggested LHR$^+$ *E. coli* are persisting in the chilled processing environment and that horizontal LHR transfer among *E. coli* isolates may take place.

The Locus of Heat Resistance (LHR) is a chromosomal or plasmid-borne island of ~14–19 Kb that has been found in several Enterobacteriaceae including *Escherichia coli* (*E. coli)* isolated from meat animals and meat products (Dlusskaya et al., 2011; Guragain et al., 2021; Guragain et al., 2020; Zhang et al., 2020) dairy (Marti et al., 2016), and chlorinated sewage (Zhi et al., 2016). Two major variants of the LHR have been described, both with similar genetic synteny and twelve core genes. LHR1 (~15 kb) and LHR2 (~19 kb) differ mainly in DNA sequences of the ATP-dependent protease ClpK and a few other genes unique to each variant (Guragain et al., 2021). Originally, 15–19 genes of the LHR were grouped into three functional motifs based on their predicted roles in resistance against heat shock, envelope stress, and oxidative stress (Mercer et al., 2015). Recently, the roles of individual LHR genes and motifs in resistance against heat, chlorine, hydrogen peroxide, peroxyacetic acid, and pressure have been dissected using a mutational approach (Li et al., 2020; Wang et al., 2020, 2021). These studies identified that the underlying mechanisms of LHR-mediated stress resistance relied on the prevention of oxidation of cytoplasmic proteins and membrane lipids. Due to these findings, the LHR was retermed as the transmissible locus of stress tolerance (tLST) but will be referred to as the LHR herein, to maintain consistency with our previous reports (Guragain et al., 2022; Gurevich et al., 2013). *E. coli* that possess this genetic element have the potential to resist currently used meat processing interventions like hot water wash (80–85°C), peroxyacetic acid, organic (lactic, citric, acetic) acids, and halogen-based compounds (bromine, acidified sodium chlorite, sodium hypochlorite, and chlorine dioxide) which are intended to ensure beef safety (Kocharunchitt et al., 2020; Koohmaraie et al., 2005; Schmidt et al., 2012).

E. coli are common residents of cattle intestine and can contaminate beef during harvest and processing steps (as reviewed in (Adam and Brulisauer, 2010)). Earlier studies (Guragain et al., 2021; Wang et al., 2020) have shown that the LHR is not common among pathogenic Shiga toxin-producing *E. coli*. However, 0.49% of *E. coli* from acute human gastroenteritis cases, including Shiga toxin (stx_1)-producing isolates (Ma and Chui, 2017; Ma et al., 2020), approximately

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10% of human commensal *E. coli* (Kamal et al., 2021), and 2% of beta-lactam producing nosocomial *E. coli* (Boll et al., 2016) are reported to carry the LHR in their genomes. Given the horizontal transfer capability of the LHR leading to gain of function among pathogenic bacteria (Mercer et al., 2017), the emergence of extreme heat and other stress-tolerant pathogenic *E. coli* may be possible.

Our recent study suggested a higher prevalence of the LHR among *E. coli* isolated from finished meat products compared to *E. coli* isolated from animals and the early stages of meat processing (Guragain et al., 2021). Given the stress tolerance provided by the genes of the LHR, increased prevalence of LHR$^+$ *E. coli* in finished meat products may be a result of enrichment or selection through processing treatments. This could not be determined in our previous study as the isolates examined were from multiple unrelated studies. Therefore, the goal of this study was to confirm increased prevalence of LHR$^+$ *E. coli* in final meat products and understand the dynamics of LHR$^+$ *E. coli* in the beef processing continuum. Further, we characterized the resistance of cattle and beef-borne LHR$^+$ *E. coli* against antimicrobial interventions commonly used in the beef processing industry. Dynamics of the LHR$^+$ *E. coli* in the processing continuum was investigated by using whole-genome sequencing of LHR$^+$ *E. coli* isolates. The phenotypic and genotypic characteristics of LHR$^+$ *E. coli* from this study provide knowledge on the efficacy of current meat processing treatments in controlling LHR$^+$ *E. coli* and point to possible sources of LHR$^+$ *E. coli* in final products.

Methods

Study design and samples

Generic *E. coli* isolates previously recovered during 2013 and archived at the United States Meat Animal Research Center (USMARC) were utilized for the current study (Schmidt et al., 2015). To examine LHR$^+$ and XHR *E. coli* across the beef production continuum, 232 generic *E. coli* isolated from three lots of fed beef cattle along different stages of the beef processing continuum (feces and hides at feedlot, feces and hides at abattoir, preevisceration (hide off, posthead drop) carcasses, final carcasses, and packaged strip loins) were utilized (Schmidt et al., 2015). Multiple hurdle interventions were applied during beef processing. Hide-on exsanguinated carcasses were washed with alkali, followed by a preevisceration carcass wash with organic acid. Final carcasses were treated with hot water wash (80–85°C) followed by organic acid spray as the carcasses entered the spray chill area, where they were sprayed with chlorinated water. Further, as carcasses left the cooler and entered fabrication, a subsequent organic spray was applied. Hides were collected before the hide wash, preevisceration carcasses were sampled before the preevisceration treatment, final carcasses were sampled chilled after the above-mentioned treatments, and the strip loins were collected before any packaging or prepackaging antimicrobial treatments on fabrication lines.

For each stage, a single *E. coli* isolate from each of the 12 samples had been selected. In some cases, the *E. coli* was from directly plated enumeration colonies (generally early stages with greater *E. coli* loads present), while others were from enriched samples plated for prevalence determination (late-stage samples with low *E. coli* loads present). For each stage, 12 isolates were available per lot, except strip loin for which no sample was available for Lot 3, and final carcasses for which only four samples were available for Lot 2 (Table 1). Difco Tryptic Soy Broth (TSB, Beckton Dickinson, USA) grown overnight cultures of each *E. coli* isolate had been archived at −80°C with 17% glycerol as a cryoprotectant. Frozen cultures were revived on Luria Bertani (LB) agar (Beckton Dickinson, Sparks, MD) plates following incubation at 37°C, 15–22 h. A single colony from each plate was utilized for further studies.

Screening of locus of heat resistance

A four-plex PCR assay was used to detect the LHR as previously described (Guragain et al., 2020). Briefly, oligonucleotides designed to target 5', 3', and interior regions of the LHR1 variant (15 kb LHR) were used to differentially amplify four different regions across the LHR. Oligonucleotide primer pairs used were 1266F / 1373R, 4295F / 4505R, 7069F / 7404R, and 14160F / 14699R, where numerals indicate primer location within the LHR. To detect the LHR2 variant (19 kb LHR), *E. coli* isolates were screened for the presence of the marker gene *clpK2* using primers ClpK2F / ClpK2R as previously described (Guragain et al., 2020). All amplicons were resolved on 1.5% agarose gels, electrophoresed in 1X sodium borate buffer (Faster Better Media LLC, USA), and stained with ethidium bromide for visualization. Prevalence was compared using Fisher's exact test.

Screening of extreme heat resistance

XHR *E. coli* were identified in a high throughput 96 well assay format (Guragain et al., 2020; Marti et al., 2016) with modifications. Briefly, each isolated colony was inoculated into LB broth and incubated at 37°C, 100 rpm for 7.5 h. The cultures were diluted 1:16 into fresh LB broth and further incubated at 37°C,100 rpm for 13–14 h. Cell density of each culture was then normalized to OD600 of 0.15 (~1.2 × 10^6 CFU/mL). Nine hundred and fifty microliters of each normalized culture was transferred to a thin-walled plastic test tube for heat treatments. Isolates were exposed to 60°C for 20 min in a hot water bath, then transferred and rapidly cooled in an ice-water bath for 45 s, followed by recovery at room temperature for 1 h. At the end of 1 h, 50 μL of the heat-treated samples was diluted 1:10 in fresh LB broth and incubated overnight at 37°C, 100 rpm for 21 h. At the

Table 1
Prevalence of LHR$^+$ generic *E. coli* by meat processing stages

Sample matrix	Number of *E. coli* isolates			
	Lot#1	Lot#2	Lot#3	Total
Feedlot hide sponge (FH)	12^{A}	12^{M}	12^{X}	36
Feedlot fecal swab (FF)	12^{A}	12^{M}	12^{X}	36
Harvest hide sponge (HH)	12^{A}	$12(1)^{M}$	12^{X}	36(1)
Harvest fecal swab (HF)	12^{A}	12^{M}	12^{X}	36
Preevisceration carcass (PC)	12^{A}	12^{M}	12^{X}	36
Final carcass (FC)	12^{A}	$4(2)^{M}$	$12(12)^{Y}$	$28(14)^{***}$
Strip loin (SL)	$12\ (11)^{B}$	$12(8)^{N}$	NA	$24(19)^{***}$
Total	84(11)	76(11)	72(12)	232(34)

Number in parentheses represents the number of *E. coli* tested positive for LHR. Same superscript letter in each column represents no statistical significance in prevalence ($P \geq 0.05$) at different processing stages. ***, Extremely significant prevalence ($P < 0.0001$).
NA: Sample not available.

end of the incubation period, OD600 of the samples was measured and isolates showing less than 0.5 log reduction in growth compared to the respective untreated samples were considered extremely heat resistant. Previously identified LHR$^+$ XHR strain AW1.7 was included as a positive control in each series of heat treatments. Experiments were conducted in triplicates, with assays for each isolate repeated at least two times using independently grown cultures.

Antimicrobial treatment assay

Treatments with sodium hypochlorite (NaOCl), bromine (BR), lactic acid (LA), peroxyacetic acid (PAA), and hot water (HW) were performed using conditions common in beef processing (50 ppm, 400 ppm, 4%, 200 ppm, and 82°C, respectively) and again with empirically determined conditions (described below) that provided a 1–2 log fold reduction allowing comparison with the control strain AW1.7. The antimicrobial reagents were prepared in sterile deionized water at room temperature: 30 ppm NaOCl, (Sigma-Aldrich, St. Louis, MO), 600 ppm BR (BoviBrom™ 11–28%BR, Albemarle, USA), 4% LA (pH = 2.3, Sigma-Aldrich, St. Louis, MO), and 100 ppm PAA (pH = 3.4, BLITZ organic, FMC Corp., Philadelphia, PA). PAA and BR concentrations were determined by following the manufacturer's recommendation using a PAA kit (Peroxychem, Philadelphia, PA) and bromine photometer II (Hanna Instruments Inc, Smithfield, RI). Antimicrobial susceptibility was tested as described previously (Guragain et al., 2022) with some modifications. Each isolated colony from LB agar was inoculated into 5 mL of LB broth and incubated at 37°C, 150 rpm for 18 h. Cell density of each culture was adjusted to OD600 of 0.2. Then, to 200 μL of the normalized culture, an equal volume of double-strength antimicrobial reagent was added to yield the following final concentrations: 15 ppm NaOCl; 300 ppm BR; 2% LA; and 50 ppm PAA. Cells were exposed to NaOCl for 15 s, BR for 30 s, and LA and PAA for 30 s. In cases of non-treatment controls, the antimicrobial reagent was substituted with an equal volume of sterile deionized water. For NaOCl treatment, the reaction was quenched by adding 12.5 μL of 10% Sodium thiosulfate ($Na_2S_2O_3$, Sigma-Aldrich, St. Louis, MO). For hot water treatment, 200 μL of prewarmed (65°C) sterile water was added to the cultures and incubated in 65°C water bath for 1 min, followed by rapid cooling in an ice-water bath for 45 s. After each antimicrobial treatment (chemical or heat), 100 μL culture were transferred to 900 μL D/E neutralization broth (BD), incubated at room temperature for 1 h, followed by ten-fold serial dilution in normal saline and plated onto LB agar containing 0.0005% 2,3,5 triphenyltetrazolium chloride (Milipore Sigma, St. Louis, MO). Addition of 0.0005% 2,3,5 triphenyltetrazolium chloride facilitated the colony counting without affecting bacterial growth. Viable cells were enumerated by counting colony-forming units (CFUs) and log reduction in CFUs/mL was calculated.

Whole-genome sequencing

Genomic DNA was extracted as described previously (Guragain et al., 2022) from overnight cultures (LB, 37°C) of all LHR$^+$ *E. coli* identified in this study. Briefly, genomic DNA (gDNA) was extracted and purified using Qiamp DNA mini kit (Qiagen, Valencia, CA). Illumina sequencing library was prepared using TruSeq PCR free LP kit (Illumina, San Diego, CA), and paired-end sequencing of 350bp gDNA was performed on a MiSeq platform (v2 chemistry, 300 cycles) (Illumina, San Diego, CA).

Genome assembly, analysis, and annotation

Genomes were assembled, analyzed, and annotated using an in-house genome analysis pipeline as described previously (Guragain et al., 2022). Briefly, adaptor sequences were removed using Trimmomatic version 0.39 (Bolger et al., 2014) and reads were assembled using SPAdes version 3.14.0 (Bankevich et al., 2012). SerotypeFinder version 2018_09_24 (Joensen et al., 2015), MLST version 2019_05_08 (Larsen et al., 2012), ResFinder version 2020_02_06 (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017), and VirulenceFinder version 2.0 (Joensen et al., 2014; Malberg Tetzschner et al., 2020) were respectively used to identify *E. coli* serotypes, multilocus sequence types (MLST), antimicrobial resistance genes, and presence of virulence factors. Phylogroups were determined with Clermon typing 21.03 (Beghain et al., 2018). Sequence quality was further assessed using QUAST (Gurevich et al., 2013), and 30 of the 34 sequences were of good quality and further analyzed.

LHR regions in the newly sequenced genomes were identified as described previously (Guragain et al., 2020). Briefly, high-sensitivity mapping of full-length genome sequences against a reference sequence library was done using Geneious Mapper. Genome regions showing at least 80% overlap identity (>99.9% confidence) with reference LHR sequences upon pairwise alignment over the full length were determined to be LHR. LHR variants were identified primarily based on sequences of *clpK, clsA, dgc,* and *pas* genes. The draft genomes were annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al., 2018; Tatusova et al., 2016). The coordinates of newly identified LHR regions were derived from PGAP annotations and further confirmed with rapid prokaryotic genome annotation software PROKKA1.14.6 (Seemann, 2014).

Phylogenetic analysis

LHR regions and core genomes were used for separate phylogenetic analysis as previously described (Guragain et al., 2022). Briefly, core LHR nucleotide sequences were extracted from 32 individual contigs coming from 22 genomes, each contig harboring the full-length LHR. LHR sequences were mapped against each other and seven reference LHR sequences (GenBank Accession#: *E. coli* AW1.7, CP072541.1; *E. coli* FAM21805, KY646173.1; Chronobacter sakazakii 29544, CP011047.1; *E. coli* 730V1, CP061764.1; *E. coli* 873.10p2, CP061756.1; *E. coli* 873.10p1, CP061755.1; *Salmonella enterica* serovar Senftenberg ATCC 43845 TLPQC1, CP016838.1) by MAFFT aligner (Katoh and Standley, 2013) in Geneious prime V2020.1.2 (Biomatters). The best fit model for maximum parsimony analysis was determined to be GTR+I+G4 using Akaike's information criteria (AIC) by Modeltest-NG v0.1.7 (Darriba et al., 2020; Flouri et al., 2015).

For core genome phylogenetic analysis, the reference genome was identified as *E. coli* strain MEM (GenBank Accession# CP012378.1) by using Patric 3.6.10 (Davis et al., 2020). Prophage sequences within the reference genomes were identified and masked using PHASTER (Arndt et al., 2016; Zhou et al., 2011) and Bedtools, respectively (Quinlan and Hall, 2010). Thirty newly sequenced LHR$^+$ genomes were determined to be good quality by QUAST. Core genomes and the reference genome were aligned using ParSNP version 1.2 (Treangen et al., 2014). The best fit model for maximum parsimony phylogenetic analysis was determined to be GTR+I+G4 using AIC by Modeltest-NG v0.1.7 (Darriba et al., 2020; Flouri et al., 2015).

Maximum likelihood phylogenetic tree was constructed using iQ-tree 1.6.10 (Nguyen et al., 2015) and visualized with iTolv6 (Letunic and Bork, 2021).

Statistical analysis

Antimicrobial treatment assays were repeated at least three times with independently grown cultures. Statistical significance was calculated using unpaired t test. LHR prevalence was expressed as the mean ± standard deviation. Statistical significance for prevalence across production stages for each lot was calculated using Fisher's exact test.

Data availability

Genome sequences have been uploaded to NCBI under BioProject no. PRJNA663878. The GenBank accession numbers for individual PGAP annotated genomes are provided in Supplementary Data File SD1.

Results and discussion

Prevalence of the LHR among E. coli isolated across the beef processing continuum

The LHR was detected in *E. coli* isolated from samples of all three cattle lots (Lot 1 = 13.1%, Lot 2 = 14.5%, Lot 3 = 16.7%). Here, Lots 1 and 2 originated from the same feed yard while Lot 3 originated from a different but nearby feedyard. An earlier study that targeted the LHR in bovine feces cultures found it absent in 41% of cattle that were similar to those sampled here (Central US-fed cattle) (Guragain et al., 2020) with an intact LHR (all four PCR screening bands present) in 18% of the cattle feces. However, attempts to isolate LHR$^+$ *XHR E. coli* from the feces of fed cattle located in the central US yielded only 6 isolates (4.2%). Therefore, care must be taken when assessing and comparing LHR$^+$ *E. coli* prevalence numbers in beef production.

Overall, the LHR1 was detected in 14.7% (34/232) of generic *E. coli* isolated across the beef processing continuum (Table 1). LHR2 variant was detected among 24 *E. coli*, all of which were also LHR1$^+$. Similar LHR prevalence (11.4%) was observed in our earlier study of *E. coli* collections sourced from meat and various animal sources at different processing stages (Guragain et al., 2021). Our results here found no LHR$^+$ *E. coli* among those isolated from feedlot fecal swabs (0/36) and feedlot hide sponges (0/36), harvest fecal swabs (0/36), and preevisceration carcasses (0/36). The only early processing stage *E. coli* that carried an LHR was an isolate from harvest cattle hides (2.8%, 1/36). Low LHR prevalence (4.2%) among *E. coli* from fecal samples of fed beef cattle was also reported earlier (Guragain et al., 2020); hence, few LHR$^+$ *E. coli* appear to be arriving with animals and entering processing plants.

Despite low LHR$^+$ *E. coli* prevalence in early processing stages, in the final stages of processing 50% (14/28) of *E. coli* from final carcass and 79.2% (19/24) of *E. coli* from strip loins possessed an LHR (Table 1). Findings of this work agree with our previous report (Guragain et al., 2021) showing increased prevalence (46.1%) of the LHR in *E. coli* isolated from final pork products, while absent in *E. coli* isolated in earlier pork production and processing stages. All three lots were processed at the same establishment, but Lots 1 and 2 were processed in May and June, while Lot 3 was processed in October (Schmidt et al., 2015). None of the 12 *E. coli* isolated from final carcasses of Lot 1 harbored an LHR. For Lot 2, *E. coli* were detected on only 4 of 74 carcasses sampled. Thus, only four *E. coli* were isolated, of which 2 (50%) were LHR$^+$. For Lot 3, all 12 (100%) *E. coli* isolated from final carcasses were LHR$^+$ (Table 1). Since widely used beef processing interventions (Koohmaraie et al., 2005) have not changed considerably between the time our samples were collected and now, the current prevalence of LHR$^+$ *E. coli* in beef processing is likely similar. Nevertheless, significant lot-to-lot variation can be observed.

Prevalence of the XHR phenotype among E. coli isolated across the beef processing continuum

The LHR is a molecular determinant of extreme heat resistance, but our previous work identified LHR-negative *E. coli* that were XHR. Therefore, we next screened all 232 isolates for the XHR phenotype. Overall, 12.9% (30/232) of the generic *E. coli* from the beef production and processing continuum showed an extreme heat-resistant phenotype (Table 2). This was similar to our earlier report that found a 10.3% prevalence of XHR among *E. coli* in meat animals and meat products (Guragain et al., 2021). The XHR phenotype correlated with LHR$^+$ *E. coli*. LHR was present in 97% (29/30) of the XHR *E. coli*. Five LHR$^+$ *E. coli* failed to show XHR phenotype, and one LHR-negative *E. coli* was XHR. Incongruency of various extents between XHR and LHR has been previously observed by our group (Guragain et al., 2021, 2020). The lack of XHR phenotype in LHR$^+$ *E. coli* highlights the possible need for optimal genetic background for LHR-mediated XHR phenotype. The five LHR$^+$ *E. coli* that failed to show XHR phenotype were spread across the continuum; one feedlot hide, one final carcass and three strip loin isolates, as well as across the three lots (two in Lots 1 and 2 and one in Lot 3). Only one *E. coli* isolate with XHR phenotype that lacked the LHR was detected, and it was from the early production stage of feedlot cattle hide. This contrasts from our earlier study where only 21% of XHR *E. coli* contained an LHR (Guragain et al., 2021). This could be attributed to the impact of different XHR screening approaches that has been noted by others (de Souza Figueiredo et al., 2019).

Antimicrobial susceptibility

A large number of *E. coli* (>50%) isolated from chlorinated wastewater in Alberta, Canada were found to carry the LHR in their genome (Zhi et al., 2016). Further, transformation by an LHR plasmid is reported to increase the resistance of *E. coli* MG1655 to various antimicrobials including chlorine, hydrogen peroxide, and peroxyacetic acid (Wang et al., 2020). Therefore, we assessed the LHR$^+$ isolates from the beef production and processing continuum for their sensitivity to currently used microbial interventions in meat processing. Preliminary screening for resistance against conditions like those used in meat processing facilities (heat, 82°C; PAA, 200ppm; BR, 400 ppm; LA, 4%; 50ppm NaOCl) was initially performed but these over-

Table 2
Prevalence of XHR generic *E. coli* by meat processing stages

Sample matrix	Number of *E. coli* isolates			
	Lot#1	Lot#2	Lot#3	Total
Feedlot hide sponge (FH)	12^{A}	$12(1)^{M}$	12^{X}	36(1)
Feedlot fecal swab (FF)	12^{A}	12^{M}	12^{X}	36
Harvest hide sponge (HH)	12^{A}	12^{M}	12^{X}	36
Harvest fecal swab (HF)	12^{A}	12^{M}	12^{X}	36
Preevisceration carcass (PC)	12^{A}	12^{M}	12^{X}	36
Final carcass (FC)	12^{A}	$4(2)^{MO}$	$12(11)^{Y}$	28(13) ***
Strip loin (SL)	$12\ (9)^{B}$	$12(7)^{NO}$	NA	24(16) ***
Total	84(9)	76(10)	72(11)	232(30)

Number in parentheses represents the number of XHR *E. coli*. Same superscript letter in each column represents no statistical significance in prevalence ($P \geq 0.05$) at different processing stages. ***, Extremely high significant prevalence ($P < 0.0001$).
NA: Sample not available.

whelming concentrations prevented growth of the LHR$^+$ isolates including the reference strain AW1.7 (data not shown). Therefore, treatment conditions were optimized to achieve a 1–2 log fold reduction in cell density of AW1.7 to facilitate the comparison of LHR$^+$ strains with the reference strain. Treatment with 65°C water (1 min), 15ppm NaOCl (15 s), 50 ppm PAA (30 s), 2% LA (30 s), and 300ppm BR (30 s) respectively reduced cell counts of AW1.7 by 1.5 ± 0.5 log,1.7 ± 0.3 log, 1.6 ± 0.3 log, 1.2 ± 0.3, and 1.5 ± 0.6 (Fig 1). A similar reduction in cell density was observed for 28 LHR$^+$ *E. coli* isolated from the beef processing continuum. Among the remaining 8 LHR$^+$ *E. coli* isolates, CE58 from strip loin was less resistant to NaOCl (2.6 ± 0.4 log fold reduction), whereas seven isolates (four final carcasses, three strip loin isolates) showed slight but significantly more resistance to high temperature. Overall, this shows LHR$^+$ *E. coli* isolated from the beef processing continuum possess an antimicrobial resistance profile comparable to AW1.7, and current beef processing interventions should be effective in controlling LHR$^+$ *E. coli*.

Whole-Genome Sequence of LHR+ isolates

Our earlier study (Guragain et al., 2021) clearly suggested the increased prevalence of LHR$^+$ *E. coli* in finished meat products. This increased prevalence in finished meat products may be due to either selection during processing treatments or enrichment during final stages. In the earlier study, however, source could not be tracked for each production stage, as the isolates were from unrelated studies. Therefore, the current study utilizes the advantage of isolates collection from the same animals through the processing stream allowing us to investigate the dynamics of LHR$^+$ *E. coli* in the beef production and processing continuum. Major findings from whole-genome sequence analysis are summarized in supplementary data SD1.

Twelve MLST profiles were predicted for LHR$^+$ *E. coli*. MLST 3105 (26.7%), 399 (13.3%), and 2602, 1316, 607 (10% each) were the most predominant types. The LHR isolates in this study most commonly belonged to serogroup O21:H12 (27%) and phylogroups A (66.7%) and C (8%). A recent study based on large-scale genomic analysis of

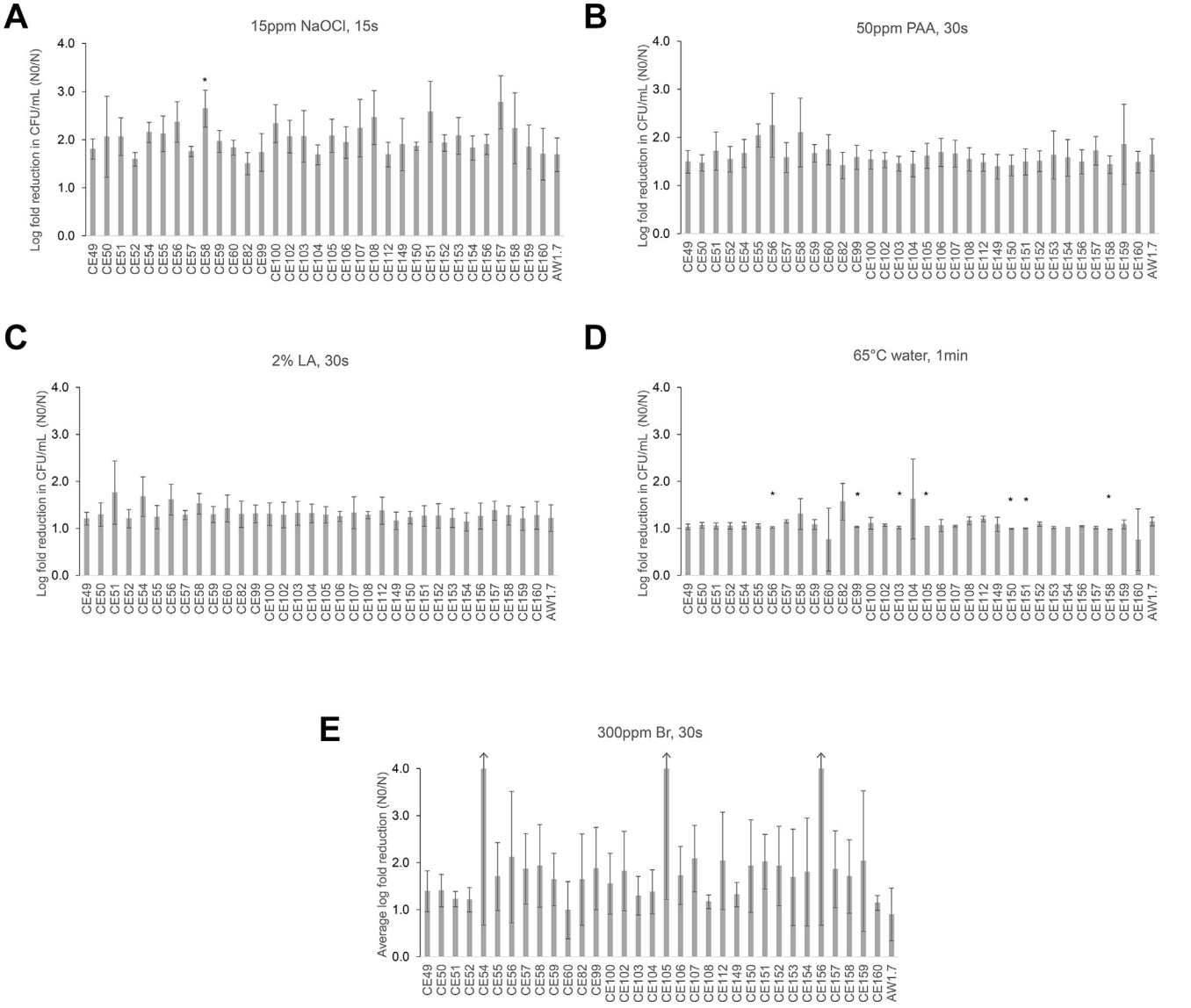

Figure 1. Log fold reduction in colony-forming unit of LHR$^+$ *E. coli* upon treatment with (A) 15 ppm Sodium hypochlorite for 15 s; (B) 50 ppm peroxyacetic acid for 30 s; (C) 2% lactic acid for 30 s; (D) 65°C water for 1 min; and (E) 300 ppm BR for 30 s. Data represent average of three independent biological experiments. ↑, error bars extend to 7.8–7.9 N0/N.

LHR$^+$ *E. coli* also identified that LHR prevalence was highest among phylogroup A, followed by phylogroups C and B (Zhang and Yang, 2022). The antibiotic resistance genes (*mdf(A), tet(A), aadA1, dfrA*) present in these LHR isolates were ones commonly identified in meat-borne *E. coli* (Weinroth et al., 2022). Four LHR isolates (CE112, CE149, CE153, and CE159) showed the presence of *sitABCD* (peroxide resistance). Virulence finder identified the presence of several virulence genes in the LHR isolates. Eleven virulence factors were identified among LHR isolates: long polar fimbriae (*ipfA,*46.7%), colicin (*cba* and *cbm*, 40%), outer membrane protein for complement resistance (*traT*, 40%), and tellurium ion resistance protein (*terC*, 33.3%) being most abundant. Other virulence factors identified were increased serum survival protein *iss*, outer membrane protease *ompT*, iron transport protein *sitA*, heat-resistant agglutinin *hra*, putative type I secretion outer membrane protein *etsC*, outer membrane usher fimbriae *papC*, and EAST-1 heat stable toxin *astA*. This observation, together with previous reports of LHR in human commensal (Kamal et al., 2021) and clinical *E. coli* isolates from human case of acute gastroenteritis (Ma et al., 2020), indicates that the LHR is present in genetic backgrounds equipped with contributory factors associated with colonization, persistence, and evading immune responses.

Phylogenetic analysis

Phylogenetic analysis of core genomes of 30 LHR$^+$ *E. coli* grouped these isolates largely by MLST profile, phylogroups, serogroups, and serotypes. Clustering together of MLST 3104 isolates from Lot 2 samples of final carcass and strip loin suggests the possible transfer from finished carcass to processed whole muscle cuts like strip loin of LHR$^+$ *E. coli*. However, the same was not observed for Lot 1 samples. In most clades, isolates from different processing stages and different lots, but same MLST profile, were grouped together. The only early processing stage isolate, CE82 from harvest hide (Lot 2), grouped together with different MLST isolates from final carcass (Lot 3) and strip loins (Lots 1 and 2).

Thirty-two LHR sequences (14 LHR1 and 18 LHR2) that were present in single full-length contigs were aligned using MAFFT in Geneious prime. These LHR sequences came from 22 LHR$^+$ *E. coli*. Phylogenetic analysis showed our sequenced LHR regions distinctly grouped into two major clusters (Clusters 1 and 2) based on ClpK variants (Fig. 2). Nucleotide sequence identity between cluster 1 and cluster 2 ranged between 61.06 and 79.71%. Fourteen LHR1 nucleotide sequences were grouped into three clades (1A, 1B, and 1C), and interclade nucleotide sequence identity ranged from 98.27 to 99.87%. Eighteen LHR2 sequences formed five major clades (2A, 2B, 2C, 2D, and 2E); interclade nucleotide sequence identity ranged from 71.58 to 99.94%. Within each clade, very high sequence identity was observed (≥99.53% within each LHR1 clade and ≥99.94% within each LHR2 clade). When compared to published LHR sequences, the LHR1 sequences from this study shared greater sequence identity with LHR1 sequences from 730V1 and FAM21805 (97.67–99.51%) compared to LHR1 sequences from 29544, AW1.7, and 873.10p2 (91.36–93.01%). The LHR2 clades 2A and 2E shared greater nucleotide sequence identity (>88.2%) with LHR2 sequences from 873.10p1 and TLPQC1 compared to other LHR2 sequences.

Unique to clade 1A LHR sequences was frameshift mutation in zinc metalloproteases (*ftsH* and *htpX*). On the other hand, LHR1 sequences from isolates CE149 and CE159; CE49 and CE52; CE51, CE54, and CE103; and CE60 and CE82 were identical. However, despite closely related core genomes as well as identical LHR of CE60 and CE82, CE82 failed to show XHR phenotype in this study, suggesting isolate-to-isolate variation in the LHR-mediated phenotype.

Four LHR2 sequences identified in this study differ from other LHR2 sequences regarding their gene composition. Two hybrid LHR2 sequences from CE149 and CE159 (clade 2A) were similar to LHR1 sequences except for the presence of LHR2-specific genes *clpK2* and cardiolipin synthase (*clsA*). As a result, these two LHR sequences shared 85.70–85.93% and 71.58–79.69% identity with LHR1 and LHR2 clades, respectively. LHR2 sequence from CE59 (clade 2B) lacked a gene encoding trypsin-like domain-containing protein, which is otherwise conserved among both LHR variants.

Isolate CE55 (clade 2C) carried the most divergent variant of LHR2 in this study (71.58–74.18% sequence identity with other LHR2). LHR2-specific genes: mechanosensitive channel (*msc*), and two hypothetical proteins were absent in CE55 LHR2. Unique to CE55 LHR2 were transposase genes (*istA* and *istB*) which were positioned immediately upstream of cation proton antiporter, highlighting the possibility of acquiring novel elements in LHR. LHR architectures similar to these four LHR2 sequences were recently reported from analysis of *E. coli* genomes deposited in NCBI where clade 2A architecture was reported to make up 6.3% of LHR variants (Zhou et al., 2011). We found the LHR2 architectures of strains CE59 and CE55 were each among 6.06% and 0.27% of 363 draft genomes with LHR assembled in a single contig. Interestingly, these genomic differences were not reflected in phenotypes investigated in this study. Nevertheless, diversity in LHR indicates continuous LHR evolution, and future investigations into the role of different LHR variants in bacterial fitness and survival will expand understanding of the function of the LHR.

LHR variants and clades do not appear to distribute differentially based on processing stages, phylogroups, serogroups, and serotypes.

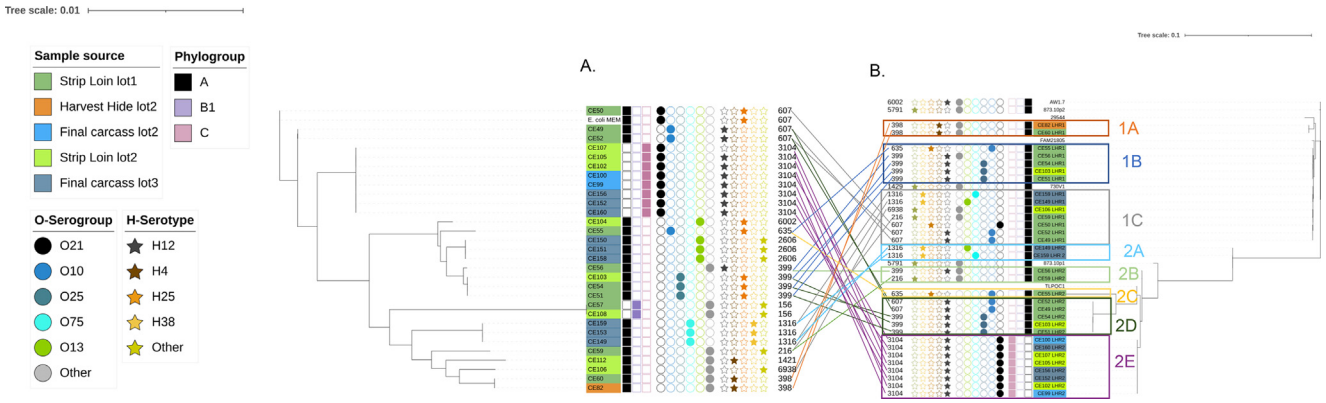

Figure 2. Maximum likelihood phylogenetic tree of (A) 30 core genomes of LHR$^+$ *E. coli* isolated from beef processing continuum and (B) 32 LHR regions from contigs carrying full-length LHR. Phylogenetic trees were constructed using iq-tree and visualized and annotated using iTol6. Numbers on the outermost column represent MLST. Alphanumeric characters outside the boxes represent clade names.

Interestingly, only and all LHR sequences from phylogroup C, MLST 3104 *E. coli* grouped in clade 2E. All of these isolates belong to serotype O21:H12. All of these isolates were grouped under one clade in the core genome analysis. Additionally, when LHR sequences were traced back to the core genome phylogeny, the overlap was observed between only a few core genomes and LHR clades. Combined, this suggests that 1) LHR sequence variations may have preferences for certain host genetic backgrounds, and 2) clonal expansion might be occurring at each stage of processing after introduction. On the other hand, LHR sequences in most of the clades originated from diverse MLST profiles, serogroups, serotypes, and processing stages. This diversity could likely be attributed to the horizontal transfer of LHR among unrelated strains, leading to frequent *de novo* selection resulting in diverse populations.

Our current observations do not support the hypothesis that LHR isolates travel from the initial processing stages to final products, indicating that enrichment or selection of LHR by current meat processing interventions is unlikely. This reinforces previous observations of 1450 *E. coli* isolated from live cattle and meat plant environments between 2002 and 2017 that suggested antimicrobial treatments in meat packing plants do not appear to select for extreme heat-resistant phenotype (Zhang et al., 2020). Nevertheless, our observations here are limited by small number of isolates per processing stage. Larger number of isolates from each processing stage could yield more definitive data on the origin of LHR$^+$ *E. coli* in final beef products. Additionally, other possibilities to be considered are that stress-tolerant LHR$^+$ *E. coli* are introduced during later stages of processing or are protected on process surfaces by resident microbial communities. A recent study reported that when embedded in biofilm, LHR$^+$ *E. coli* are better protected against chlorine, hydrogen peroxide, and peroxyacetic acid than LHR$^-$ strains. This protection was associated with higher biofilm biomass production capacity of LHR$^+$ *E. coli* in both mono- and dual strains biofilms (Xu et al., 2021). Therefore, more research is needed to identify the environmental sources of LHR/XHR *E. coli* in final beef products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfp.2022.100031.

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