



# Are Antimicrobial Interventions Associated with Heat-Resistant *Escherichia coli* on Meat?

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**ABSTRACT** Decontamination practices, which often involve thermal treatments, are routinely performed in beef packing plants and have generally improved the safety of meat in North America. We investigated whether *Escherichia coli* in the beef production chain is becoming more heat resistant due to those treatments. Cattle isolates ( $n = 750$ ) included seven serogroups (O157, O103, O111, O121, O145, O26, and O45) which were collected between 2002 and 2017. Beef plant isolates ( $n = 700$ ) from carcasses, fabrication equipment, and beef products were included. Heat resistance was determined in Luria-Bertani broth at 60°C and by PCR screening for the locus of heat resistance (LHR). The decimal reduction for *E. coli* at 60°C ( $D_{60^\circ\text{C}}$  values) ranged from 0 to 7.54 min, with 97.2% of the values being <2 min. The prevalence of *E. coli* with  $D_{60^\circ\text{C}}$  values of >2 min was not significantly different ( $P > 0.05$ ) among cattle and meat plant isolates. *E. coli* from equipment before sanitation (median, 1.03 min) was more heat resistant than that after sanitation (median, 0.9 min). No significant difference in  $D_{60^\circ\text{C}}$  values was observed among *E. coli* isolates from different years, from carcasses before and after antimicrobial interventions, or from before and during carcass chilling. Of all isolates, 1.97% harbored LHR, and the LHR-positive isolates had greater median  $D_{60^\circ\text{C}}$  values than the LHR-negative isolates (3.25 versus 0.96 min). No increase in heat resistance in *E. coli* was observed along the beef production chain or with time.

**IMPORTANCE** The implementation of multiple hurdles in the beef production chain has resulted in substantial improvement in the microbial safety of beef in Canada. In this study, we characterized a large number of *Escherichia coli* isolates ( $n = 1,450$ ) from various sources/stages of beef processing to determine whether the commonly used antimicrobial interventions would give rise to heat-resistant *E. coli* on meat, which in turn may require alternatives to the current control of pathogens and/or modifications to the current cooking recommendations for meat. The findings show that the degree and rate of heat resistance in *E. coli* did not increase along the production chain or with time. This furthers our understanding of man-made ecological niches that are required for the development of heat resistance in *E. coli*.

**KEYWORDS**  $D$  value, *Escherichia coli*, antimicrobial interventions, beef packing plant, cattle, heat resistance, locus of heat resistance

*Escherichia coli* is a Gram-negative, facultative anaerobe that inhabits the gut of warm-blooded animals. Most strains of *E. coli* are nonpathogenic, but some strains are pathogenic to humans and animals (1). Clinical signs of intestinal diseases caused by pathogenic *E. coli* range from self-limiting diarrhea to life-threatening hemolytic uremic syndrome (HUS), the latter of which is most often caused by Shiga toxin-producing *E. coli* (STEC) strains. Cattle are natural reservoirs of STEC, but they lack vascular receptors for Shiga toxins (2). The STEC which has caused the most cases of human disease is *E. coli* O157:H7, which can result in up to 10% of infected people

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developing severe and potentially fatal HUS (3). In North America, more than 50% of STEC infections are caused by *E. coli* O157:H7/NM, and the rest, primarily by serogroups O103, O111, O121, O145, O26, and O45 (4, 5). Together, they are commonly referred to as the “Top 7” serogroups.

Hides of cattle are often heavily contaminated with *E. coli*, which may be transferred to previously bacterium-free carcasses or the environment of beef processing plants (6, 7). To control the contamination of beef carcasses with *E. coli*, hazard analysis critical control point (HACCP) programs have been required in federally inspected meat plants in North America since the 1990s, and antimicrobial interventions are often incorporated at various stages of meat processing (6–10). Commonly used antimicrobial interventions may include washing hides on carcasses with a solution of 1.5% sodium hydroxide at 55°C (11, 12), washing skinned carcasses with organic acids such as lactic, acetic, and peroxyacetic acids, and pasteurization of carcass sides with hot water (85°C) or steam (>90°C) (10). To prevent bacterial growth, dressed carcasses are chilled to a low temperature before further processing (7, 8). Beef processing equipment surfaces are cleaned and sanitized daily, if not more often (13, 14). These decontamination practices can reduce the number of bacteria, including *E. coli*, on carcasses and beef products to a very low number, as evidenced by recent studies carried out in commercial beef packing plants (11, 15–18), and in turn, led to the decline of human disease incidence associated with *E. coli* O157:H7/NM in Canada (18). However, the use of these physical and chemical treatments may also lead to development of bacterial resistance.

*E. coli* is considered to be a species sensitive to heat, with the decimal reduction at 60°C ( $D_{60^\circ\text{C}}$  values; i.e., the time required to inactivate 90% of a bacterial population at 60°C) being mostly less than 2 min (19, 20). However, in recent years, a remarkably heat-resistant *E. coli* strain (AW1.7), originally isolated from beef carcasses at a Canadian beef packing plant, has been reported (21), followed by isolation of heat-resistant *E. coli* strains from other sources with  $D_{60^\circ\text{C}}$  values of >10 min (22–24). Thus, it would be important to look into whether the benefits of antimicrobial interventions, which often include thermal treatments at various temperatures, currently implemented at beef packing plants are compromised by potentially giving rise to heat-resistant *E. coli*. The extreme heat resistance in *E. coli* strains is attributable to a genomic island, the locus of heat resistance (LHR), which contains genes encoding heat shock proteins and genes related to membrane and envelope integrity and oxidative stress (25–27).

Therefore, the present study characterized the relative heat resistance in *E. coli* isolates recovered from cattle over time and from various sources in meat plants. All isolates were screened for the presence of LHR, with the objectives to (i) better understand the flow of potentially heat-resistant *E. coli* in the beef production chain and (ii) determine whether antimicrobial interventions currently in use in beef processing plants select for heat-resistant *E. coli*. Considering the large strain variability in the bacterial response to stress, a population-based approach involving 1,450 isolates was taken in this study.

## RESULTS

**Overall heat resistance.** Heat resistance ( $D_{60^\circ\text{C}}$  [min]) was determined for a total of 1,450 isolates comprising both generic ( $n = 700$ ) and the Top 7 ( $n = 750$ ) *E. coli* (Tables 1 and 2). The  $D_{60^\circ\text{C}}$  values did not follow normal distribution ( $P < 0.05$ ; Fig. 1A); hence, nonparametric analysis was performed for pairwise comparison, and median values instead of means were reported. The  $D_{60^\circ\text{C}}$  values ranged from 0 to 7.54 min, with the median value being 0.96 min. The great majority of isolates (97.2%, 1,410) had  $D_{60^\circ\text{C}}$  values of <2 min. For the *E. coli* isolates with  $D_{60^\circ\text{C}}$  values of >2 min, there was no significant difference ( $P > 0.05$ ) in their distribution among cattle (24/750) and meat plant (16/700) isolates (Tables 1 and 2).

The LHR was found in 28 (1.97%) isolates (see Table S1 in the supplemental material). No significant difference ( $P > 0.05$ ) in LHR prevalence was found between cattle (Top 7; 17/750) and meat plant (generic; 11/700) *E. coli*. Among Top 7 *E. coli*, the LHR was found in O157 (8 isolates), O26 (6 isolates), O103 (1 isolate), O145 (1 isolate),

**TABLE 1** *Escherichia coli* isolates from cattle<sup>b</sup>

Serogroup <sup>a</sup>	No. of strains isolated in yr:											Total
	2002	2003	2004	2005	2006	2007	2011	2013	2014	2015	2017	
O103	0	0	0	0	0	0	0	40	86	25	6 (2)	157 (2)
O111	0	0	0	0	0	0	0	3	2	7	0	12
O121	0	0	0	0	0	0	0	41 (3)	33 (1)	28	1	103 (4)
O145	0	0	0	0	0	0	0	1	16 (1)	18	0	35 (1)
O157	14	28 (1)	10	9	6	57	67	11 (2)	19 (1)	12 (1)	6 (1)	239 (6)
O26	0	0	0	0	0	0	0	42 (1)	37 (6)	21 (3)	0	100 (10)
O45	0	0	0	0	0	0	0	34	40 (1)	28	2	104 (1)
Total	14	28 (1)	10	9	6	57	67	172 (6)	233 (10)	139 (4)	15 (3)	750 (24)

<sup>a</sup>The cattle *E. coli* isolates were recovered from feces or hides of cattle in feedlots and transportation trucks to slaughter plants (1–5; Reuter et al., unpublished data).

<sup>b</sup>Numbers in parentheses indicate *E. coli* isolates with  $D_{60^\circ\text{C}}$  values of >2 min.

and O45 (1 isolate). LHR-positive generic *E. coli* isolates were from carcasses after hide-on wash (3 isolates), carcasses before chilling (6 isolates), and beef products (2 isolates).

$D_{60^\circ\text{C}}$  values for the LHR-positive *E. coli* isolates (median, 3.25 min; range, 0.78 to 7.54 min) were significantly larger ( $P < 0.05$ ) than those for the LHR-negative isolates (median, 0.96 min; range, 0 to 3.93 min) (Fig. 1B). The  $D_{60^\circ\text{C}}$  values within the respective LHR-positive and -negative *E. coli* groups varied widely (Fig. 1C and D). Among the LHR-positive *E. coli* group (Fig. 1D; Table S1), four generic *E. coli* isolates, which were recovered from carcasses before chilling, showed  $D_{60^\circ\text{C}}$  values of  $\geq 6$  min, comparable to the positive-control *E. coli* AW1.7 ( $6.16 \pm 2$  min), while all the other LHR-positive *E. coli* isolates had  $D_{60^\circ\text{C}}$  values of less than 6 min, with the lower limit being 0.78 min. Among the LHR-negative *E. coli* group, five of the Top 7 isolates, comprising O26 (1 isolate), O103 (1 isolate), and O157 (3 isolates), were all extremely sensitive to heat and were killed during the come-up time; the  $D_{60^\circ\text{C}}$  values for these isolates were arbitrarily assigned as 0 min. In contrast, two O103 isolates and one O26 isolate that were LHR negative had  $D_{60^\circ\text{C}}$  values of 3.93, 3.50, and 3.57 min (Table S2), and these values were numerically higher than the median  $D_{60^\circ\text{C}}$  value for the LHR-positive isolates, 3.25 min.

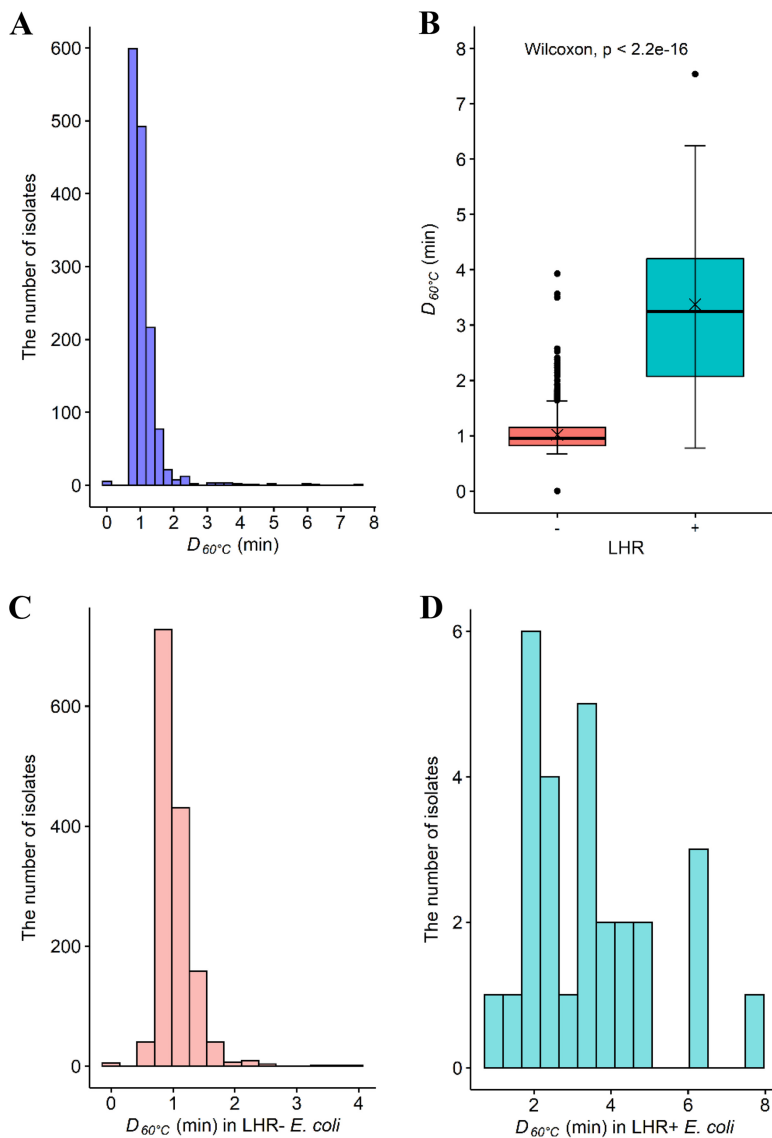
**Effect of antimicrobial interventions on heat resistance in *E. coli*.** Various antimicrobial interventions are generally in use in meat processing plants in North America (7, 8). This study compared the heat resistance in *E. coli* recovered from hide-on carcasses before any antimicrobial treatment was applied (before hide-on wash) and from carcasses along the dressing line where multiple antimicrobial interventions were applied (after hide-on wash), from dressed carcasses before and during carcass chilling from a beef plant where no antimicrobial interventions were used during the dressing process, and from beef processing equipment surfaces before and after sanitation to investigate whether these interventions select for heat-resistant *E. coli* (Table 2). The median  $D_{60^\circ\text{C}}$  value for the isolates recovered from beef processing equipment before sanitation (1.03 min) was larger ( $P < 0.05$ ) than that for isolates recovered after sanitation (0.9 min; Fig. 2). No significant difference ( $P > 0.05$ ) in heat resistance was found

**TABLE 2** *Escherichia coli* isolates from beef packing plants

Beef processing plant	Isolation source	Stage of process	No. of isolates <sup>b</sup>	Reference(s)
B	Hide-on carcasses	Dressing/before hide-on wash	100 (1)	11
	Hide-on/dressed carcasses	Dressing/after hide-on wash	100 (3)	
A	Dressed carcasses	Before chilling	100 (6)	54, 55
		During chilling	100	
A	Fabrication equipment surface	Before sanitation	100 (4)	9
		After sanitation	100	
A	Beef products <sup>a</sup>	Fabrication	100 (2)	16

<sup>a</sup>Isolates were recovered from samples collected from beef cuts and trimmings during routine production.

<sup>b</sup>Numbers in parentheses indicate *E. coli* isolates with  $D_{60^\circ\text{C}}$  values of >2 min.

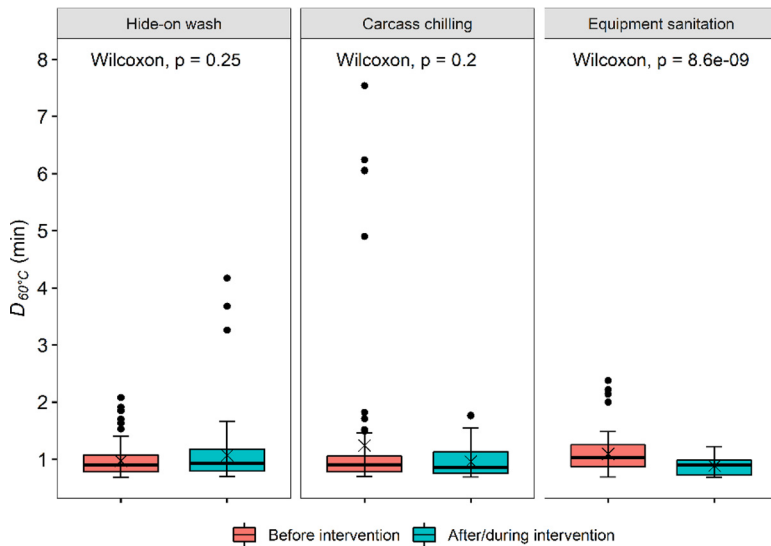


**FIG 1**  $D_{60^{\circ}\text{C}}$  values (min) for *Escherichia coli* isolates ( $n = 1,450$ ) from various sources. (A, C, and D) The distribution of  $D_{60^{\circ}\text{C}}$  in all *E. coli* isolates (A), in LHR-negative ( $-$ ,  $n = 1,422$ ) isolates (C), and in LHR-positive ( $+$ ,  $n = 28$ ) isolates (D). (B) The comparison of  $D_{60^{\circ}\text{C}}$  between the LHR-positive ( $+$ , green shading) and -negative ( $-$ , orange shading) *E. coli*. Central horizontal lines and "x" marks indicate medians and means, respectively; whiskers indicate the lowest data point within 1.5 interquartile range (IQR) of the first quartile and the highest data point within 1.5 IQR of the third quartile. Filled circles indicate data points below or above the 1.5 IQR of the first or third quartile, respectively.

between *E. coli* isolates from carcasses before and after hide-on wash or from carcasses before and during chilling (Fig. 2).

**Year of isolation and heat resistance in *E. coli*.** The Top 7 *E. coli* isolates in this study were recovered over 15 years, from 2002 to 2017 (Table 1). To investigate whether the heat resistance in *E. coli* changed over time, the correlation between  $D_{60^{\circ}\text{C}}$  values and years of recovery was determined using Spearman's method. The serogroup O111 had fewer than 10 isolates from individual years and, as such, was excluded from the comparison to avoid bias in the statistical analysis. No significant correlation ( $P > 0.05$ ) between  $D_{60^{\circ}\text{C}}$  values for each of the other six serogroups by year was noted (Fig. 3).

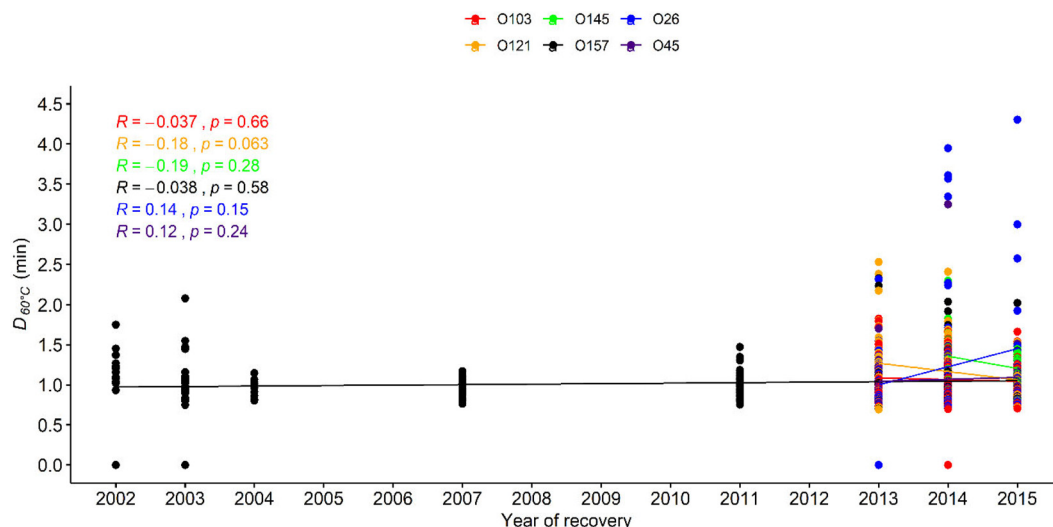
**Heat resistance in generic versus the Top 7 *E. coli*.** To investigate whether the heat resistance was different in the Top 7 and generic *E. coli*, the two groups of isolates



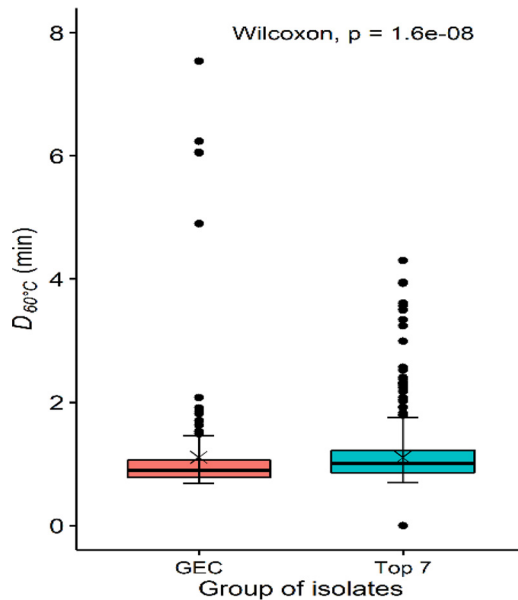
**FIG 2**  $D_{60^{\circ}\text{C}}$  values (min) for *Escherichia coli* isolates ( $n = 600$ ) recovered from beef packing plants. From left to right are *E. coli* from carcasses before and after the hide-on wash intervention, *E. coli* from dressed carcasses before and during carcass chilling, and *E. coli* from fabrication equipment before and after the daily routine sanitation. Central horizontal lines and “x” marks indicate medians and means, respectively; whiskers indicate the lowest data point within 1.5 IQR of the first quartile and the highest data point within 1.5 IQR of the third quartile. Filled circles indicate data points below or above the 1.5 IQR of the first or third quartile, respectively. Each group included 100 *E. coli* isolates.

from the common source, cattle, were involved. The Top 7 group comprised 750 STEC isolates from cattle, and the generic *E. coli* group comprised 200 isolates from carcasses that did not experience any antimicrobial treatment, 100 from hide-on carcasses before hide-on wash from plant B, and 100 from dressed carcasses before carcass chilling from plant A. The Top 7 isolates had a slightly higher ( $P < 0.05$ ) median  $D_{60^{\circ}\text{C}}$  value than the generic *E. coli* isolates (1.0 versus 0.9 min; Fig. 4).

Among the Top 7 *E. coli*, O145 isolates were the most heat resistant, with a median  $D_{60^{\circ}\text{C}}$  value of 1.2 min, followed by O121, O111, O103, O45, O157, and O26 with  $D_{60^{\circ}\text{C}}$  values of 1.11, 1.0, 1.0, 1.0, 0.99, and 0.87 min, respectively (Fig. 5).



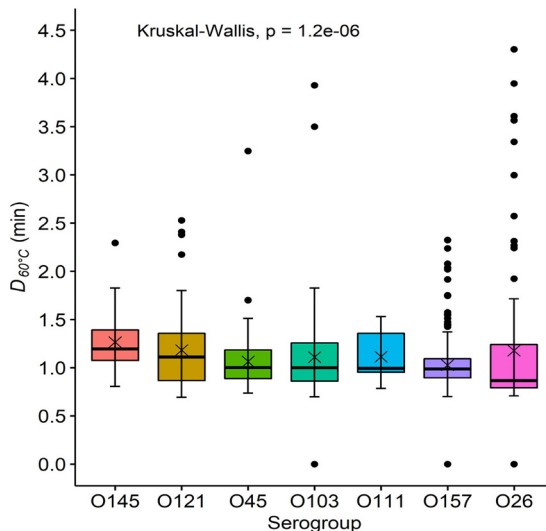
**FIG 3**  $D_{60^{\circ}\text{C}}$  values (min) for six *Escherichia coli* serogroups (O157, O26, O45, O103, O121, and O145) recovered from different years. The data points show the  $D_{60^{\circ}\text{C}}$  values for *E. coli* of the six serogroups, and each line shows the trend of the  $D$  value change for each group along the years of isolation. As the  $D$  value of each serogroup did not follow normal distribution, the correlation between heat resistance in each serogroup and recovery year was examined using Spearman's method. The Spearman's rank correlation coefficients ( $R$ ) and  $P$  values are shown in the figure.



**FIG 4**  $D_{60^{\circ}\text{C}}$  values (min) for generic (GEC,  $n = 200$ ) and the Top 7 ( $n = 750$ ) *E. coli* isolates. Central horizontal lines and "x" marks indicate medians and means, respectively; whiskers indicate the lowest data point within 1.5 IQR of the first quartile and the highest data point within 1.5 IQR of the third quartile. Filled circles indicate data points below or above the 1.5 IQR of the first or third quartile, respectively.

#### Heat resistance in *E. coli* recovered from different seasons and sample types.

This study also explored the potential difference in heat resistance in generic *E. coli* from different sample sources and different sampling seasons. No significant difference ( $P > 0.05$ ) was noted between *E. coli* from hide-on carcasses, dressed carcasses, equipment surfaces, and beef products or between different seasons in which they were recovered (Fig. S1 and S2).



**FIG 5**  $D_{60^{\circ}\text{C}}$  values (min) for the Top 7 *Escherichia coli* isolates. Central horizontal lines and "x" marks indicate medians and means, respectively; whiskers indicate the lowest data point within 1.5 IQR of the first quartile and the highest data point within 1.5 IQR of the third quartile. Filled circles indicate data points below or above the 1.5 IQR of the first or third quartile, respectively. The Top 7 *E. coli* included serogroups O103 ( $n = 157$ ), O111 ( $n = 12$ ), O121 ( $n = 103$ ), O145 ( $n = 35$ ), O157 ( $n = 239$ ), O26 ( $n = 100$ ), and O45 ( $n = 104$ ). The serogroups in the figure are ordered according to the level of the median  $D_{60^{\circ}\text{C}}$  value in each group.

## DISCUSSION

This study compared the relative heat resistance of 1,450 *E. coli* isolates from live cattle and meat plant environments to better understand whether antimicrobial interventions commonly employed at commercial beef packing plants in North America would enrich for heat-resistant *E. coli*. Muscle tissues of healthy animals are largely free of bacteria. Most bacteria found on beef carcasses have been transferred to the meat from the hide during hide removal operations (6, 28, 29). Therefore, STEC from cattle and generic *E. coli* on carcasses can all be regarded as being of cattle origin. The finding that the median  $D_{60^{\circ}\text{C}}$  values for STEC isolates from cattle and generic *E. coli* from carcasses prior to antimicrobial treatments only differed slightly (1.0 versus 0.9 min; Fig. 4) further confirms the similarity of these populations of *E. coli*. Due to the large number of isolates used in the study, a fixed incubation time, i.e., 6 min, was used for determination of  $D$  values for most of the isolates. This may have led to overestimation of  $D$  values for some isolates that were completely inactivated by this incubation time.

Overall, LHR-positive *E. coli* showed higher  $D_{60^{\circ}\text{C}}$  values than LHR-negative *E. coli*, which is in agreement with previous reports that LHR increases heat resistance of *E. coli* (25, 30). However, of the 28 LHR-positive *E. coli* isolates, 5 had  $D_{60^{\circ}\text{C}}$  values of <2 min, with one being as low as 0.78 min. LHR-positive isolates with relatively low heat resistance were also reported in three recent studies (23, 31, 32). The LHR is also present in other *Enterobacteriaceae* species, including *Salmonella enterica*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*, and it provides genus-specific protection from heat treatment (25, 30). In addition, two copies of LHR have been found in a few strains of *E. coli* from raw milk cheese, with one in the chromosome and the other in a plasmid, and the strains with the extra copy had higher heat resistance (33, 34). Interestingly, a number (1.2%, 17/1,422) of LHR-negative isolates had  $D_{60^{\circ}\text{C}}$  values of >2 min, with 3.93 min being the largest value, which is comparable to the median value (3.25 min) for LHR-positive *E. coli* isolates. Taken together, these findings suggest that factors in addition to the LHR are required to confer extreme heat resistance to *E. coli* and that more than the simple presence or absence of the LHR dictates the extent of heat resistance in *E. coli*.

The majority (97.2%) of the 1,450 *E. coli* isolates had  $D_{60^{\circ}\text{C}}$  values of <2 min, which is consistent with published accounts (19, 20, 35–39). The  $D_{60^{\circ}\text{C}}$  values ranged from 0 min (5 isolates from cattle were killed during the come-up time) to 7.54 min (an isolate from a carcass that did not encounter any antimicrobial treatments before chilling), suggesting large strain variation in heat resistance for naturally occurring *E. coli*. Isolates that had  $D_{60^{\circ}\text{C}}$  values of >2 min were similarly distributed among cattle and meat plant *E. coli* sources. In addition, there was no difference observed in  $D_{60^{\circ}\text{C}}$  values among *E. coli* from before or after antimicrobial interventions (before hide-on wash versus after hide-on wash and during the dressing process, before hide-on wash and beef products), suggesting that the proportion of heat-resistant *E. coli* found on carcasses and meat largely reflects the naturally occurring heat resistance in *E. coli* of cattle origin. The prevalence of LHR in *E. coli* recovered from the beef production chain (1.97%) is similar to that in the published genomes (2%) of *E. coli* (25). In contrast, a much larger fraction of LHR-positive *E. coli* was found in raw milk cheese (36.3%, 93/256) and treated municipal wastewater (59%, 41/70) (24, 34, 40). The former was attributed to the commercial practice of thermization of milk at 57 to 68°C for at least 15 s. However, information on the prevalence level of heat-resistant *E. coli* in raw milk cheese before heat treatment was unavailable. The much-increased prevalence of heat-resistant *E. coli* in treated wastewater, compared to 5% in untreated wastewater, was likely from coselection of chlorine and heat resistance by the wastewater treatment practice (24, 26, 27). Bacteria in general can develop resistance responses when exposed to stress conditions, and in the case of heat stress, “heat shock response” will be elicited when exposed to sublethal temperatures (41). Several studies have demonstrated that a higher temperature, especially with a step-wise increase (up to 50°C), and extended incubation time (up to 2 to 3 h) in rich medium that can support the

development of a heat response can aid thermotolerance acquisition (42–44). The carcass *E. coli* populations before and after hide-on wash were collected from a plant where the following multiple antimicrobial interventions were used: washing hide-on carcasses with 1.5% NaOH at 55°C, spraying skinned carcasses with 5% lactic acid, and pasteurization of carcass sides for 12 s with steam at a temperature of >90°C (11, 15, 45). Considering the short duration of heat exposure of carcasses (a few seconds) and environments with limited nutrients (equipment surfaces), the lack of increased heat resistance level in the *E. coli* populations exposed to multiple antimicrobial interventions is not surprising. It is also possible that the after hide-on wash *E. coli* population survived the treatments not owing to their higher tolerance of the treatments but owing to evading the treatments through the topostructural properties of carcass surfaces. Interestingly, the range of  $D_{60^{\circ}\text{C}}$  values and the prevalence of LHR-positive isolates were much more reduced after than before air chilling even though the median values did not differ significantly. This corroborates the finding that the *E. coli* population after sanitation was more heat sensitive than the population before sanitation in that the latter population had a large fraction of *E. coli* isolates that were adapted to the low-temperature environment of the fabrication floor of a beef processing plant through persistence (9, 46). Whether chilling would help combat heat-resistant *E. coli* warrants further investigation.

The levels of heat resistance of the Top 7 *E. coli* isolates collected in different years were comparable, suggesting that they are not naturally evolving to be more heat resistant. Of the seven serogroups, O26 (0.87 min) and O145 (1.20 min) had the smallest and largest median  $D_{60^{\circ}\text{C}}$  values, respectively. A similar observation was made in a study conducted by Liu et al. (35), where O26 ( $n = 10$ ) had smaller  $D_{60^{\circ}\text{C}}$  values in phosphate-buffered saline (PBS) than serogroups O111 ( $n = 10$ ), O121 ( $n = 10$ ), and O145 ( $n = 10$ ), though the latter groups did not differ. A study conducted by Enache et al. (37) also showed that O26 ( $n = 3$ ) and O157 ( $n = 3$ ) were the least and the most heat resistant, respectively, among the Top 7 in apple juice at 60°C. Median log reductions of O157 ( $n = 50$ ) and non-O157 STEC ( $n = 63$ ) at 60°C did not differ, but the median reduction of O145 ( $n = 10$ ) was less than that of O111 ( $n = 10$ ) by 1.4 log units (47). These findings seem to suggest that the O group may play a role in heat resistance. On the other hand, low variability in heat resistance between closely related *E. coli* O157 strains has been reported (48). It is possible that the disparities between studies could also be attributed to the narrow phylogenetic lineage for some serogroups or the limited number of strains being compared. However, the Top 7 *E. coli* in the present study had diverse origins, and 100 or more isolates were evaluated for all serogroups except for O111 and O145.

In conclusion, most *E. coli* strains from cattle and beef packing plant environments are heat sensitive, and the commonly used antimicrobial interventions in meat packing plants do not seem to select for heat-resistant *E. coli*. The likelihood of a cold environment such as the fabrication facility for meat to be a reservoir for heat-resistant *E. coli* seems low.

## MATERIALS AND METHODS

**Bacterial isolates and culture conditions.** *Escherichia coli* isolates ( $n = 1,450$ ), comprising 750 Top 7 and 700 generic *E. coli* isolates, were used in this study (Tables 1 and 2). These isolates were from the culture collections of Alberta Agriculture and Forestry and of the Lacombe Research and Development Centre of Agriculture and Agri-Food Canada, respectively. The Top 7 *E. coli* isolates were recovered between 2002 and 2017 from feces or hides of cattle in feedlots or transportation trucks going to slaughter plants in Alberta (Table 1) (49–53; Tim Reuter, Shaun Cook, and Lisa Tymensen, unpublished data). Studies including non-O157 *E. coli* used methods described by Stanford et al. (49) for isolation and confirmation as STEC. Earlier, O157:H7-only studies used methods described by Stephens et al. (52) for isolation and STEC confirmation. The cattle *E. coli* did not experience antimicrobial interventions, and all were classified as STEC at initial isolation. Generic *E. coli* isolates were obtained between 2013 and 2015 from two federally inspected beef processing plants (A and B) in Alberta (Table 2) (9, 11, 16, 54, 55). At plant A, no decontaminating treatment was used other than carcass trimming and cold-water wash, and dressed carcasses were air chilled (16, 54). Sanitation of fabrication equipment occurred at the end of each fabrication day, which primarily included physical removal of soil, prerinsing with pressurized water at 40 to 50°C, spraying with a chlorine-based alkaline foaming agent, and sanitization with a quaternary



ammonium compound sanitizer (9). During the period of strain isolation, plant B employed the following decontamination interventions: hide-on carcass wash with 1.5% sodium hydroxide at 55°C, spraying skinned carcasses with 5% lactic acid, and pasteurization of carcass sides at the end of the dressing process with steam at >90°C (11, 15). Isolates obtained from plant A after air chilling and after routine sanitation and from plant B after hide-on wash were regarded as isolates after antimicrobial interventions.

All bacterial stock and working cultures were maintained at –80°C and 4°C, respectively, as described previously (56). The species identity of all *E. coli* isolates was verified via PCR using the primers URL-301, 5'-TGTTACGTCCTGTAGAAAGCCC-3', and URL-432, 5'-AAAACCTGCCTGGCAGCAATT-3', as described previously (57). Each *E. coli* isolate was streaked on MacConkey agar (Oxoid) and incubated at 35°C for 24 ± 2 h and was then subcultured in Luria-Bertani (LB; BD Difco, Fisher Scientific, Canada) at 35°C with shaking at 80 to 100 rpm for 16 h. The LB overnight cultures were used for heat resistance determination.

**Phenotypic characterization of heat resistance.** Heat inactivation experiments were carried out in a water bath (Isotemp 228; Fisher Scientific, Nepean, ON, Canada) with an SC100 heated immersion circulator (Fisher Scientific) measuring 13.2 by 5.4 by 7.8 in. (height [H] by width [W] by diameter [D]) to ensure even water temperature distribution. The come-up time, i.e., the time required to reach the target temperature, was determined by measuring the time it took for 1.5 ml of LB ( $n = 40$ ) to reach 60°C using a type K thermocouple probe connected to a portable EasyLog USB temperature logger (Thermoworks, Lindon, UT). The come-up time was also verified twice on the day of heat treatment. A portion of 1.5 ml (~9 log CFU/ml) of each overnight culture was added to a 2-ml microcentrifuge tube (Eppendorf, Canada), with 4 tubes included for each culture. Samples were heated in a 60°C water bath for the duration of the come-up time ( $2.51 \pm 0.004$  min), at which point two tubes for each culture were immediately removed from the water bath (T0), and then the remaining two tubes were removed after another 6 min (T6). Upon removal from the water bath, the tubes were immediately placed in an ice water bath for rapid cooling. The cultures were then serially diluted in 0.1% (wt/vol) peptone water, and 1 ml of appropriate dilutions or the original undiluted bacterial suspensions was plated onto Petrifilm aerobic count plates (AC; 3M Corp., St. Paul, MN, USA) following the manufacturer's instructions. The plates were incubated at 35°C for 18 to 24 h, after which the CFU were enumerated, with a preference for plates bearing 20 to 200 CFU. *E. coli* AW1.7, the benchmark heat-resistant *E. coli*, was included as a positive control.

Bacterial counts were transformed to log values. Mean log transformed values (log CFU/ml) were determined from duplicate technical replicates, and log reduction was calculated ( $\log \text{CFU/ml}_{T_0}$  to  $\log \text{CFU/ml}_{T_6}$ ). For heat-treated samples from which no bacteria were recovered at the detection limit of 1 CFU/ml, a value of –0.5 was arbitrarily assigned for calculation. For the isolates with a log reduction of >2, the  $D$  value at 60°C ( $D_{60^\circ\text{C}}$ , min) was calculated by dividing the incubation time (6 min) by the log reduction. To obtain more accurate  $D_{60^\circ\text{C}}$  values for the isolates with relatively smaller log reductions ( $\leq 2$ ), a longer incubation time was used. Each culture was incubated in a 60°C water bath as before, but for up to 30 min, with duplicate tubes withdrawn at 5-min intervals. Surviving populations were determined as described before. For those isolates, log counts were plotted against incubation time, and the regression of the plot was used to calculate  $D_{60^\circ\text{C}}$  for each isolate (58).

**Screening for LHR.** DNA of each overnight culture was extracted using a water boiling method described by Yang et al. (16). All isolates were screened for LHR using a PCR method (25, 59) with modifications (31). DNA of *E. coli* AW1.7 was used as a positive control, and sterile distilled water was used as the negative control. An *E. coli* isolate was considered to be LHR positive if all three open reading frames (ORFs)/fragments were amplified and negative otherwise.

**Data analysis.** All statistical analyses in this study were performed in R version 3.6.0. The  $D_{60^\circ\text{C}}$  values were grouped into sets according to isolation source, LHR characteristics, and serogroups and year of isolation for the Top 7. Most data sets did not follow normal distribution, as determined by the Shapiro-Wilk test. As such, nonparametric analyses, namely, Wilcoxon rank sum test and Kruskal-Wallis test, were performed to compare two and more sets of data, respectively. Dunn's test was used for pairwise comparison following the Kruskal-Wallis test. To determine whether the heat resistance of cattle isolates changed over time, potential correlation between the heat resistance of each serogroup and the year of recovery was examined using Spearman's method. A contingency table containing the composition of *E. coli* with  $D_{60^\circ\text{C}}$  values of >2 min was analyzed using Fisher's exact test. The difference/effect in each analysis was regarded as significant if the  $P$  value was <0.05.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.01 MB.

**SUPPLEMENTAL FILE 2**, PDF file, 0.3 MB.

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