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Comparison of heating block and water bath methods to determine heat resistance in Shiga-toxin producing *Escherichia coli* with and without the locus of heat resistance

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ABSTRACT

This study found variability in the time required for tubes of media in heating block wells to reach 60 °C, resulting in significant effects on heat resistance measurements. To determine the extent that methodology changed heat resistance measurements, we compared the heat resistance of Shiga-toxin producing *Escherichia coli* (STEC) strains with and without the locus of heat resistance (LHR) using both heating block and water bath methods. A total of 34 strains of STEC were used along with a generic *E. coli* which has been identified as heat-resistant and used as a positive control. The *E. coli* strains were incubated in a water bath and a heating block set at 60 °C to determine come up time to 60 °C (T0) and for 6 additional minutes (T6) to calculate the D_{60} value. After incubation, the colony forming units (CFU) were enumerated and mean log CFU/mL from biological replicates was calculated. To compare reductions from T0 to T6, standard deviations among replicates within heating method and correlation of the D_{60} values generated across methods were determined using Mixed model and Correlation analyses. Our findings indicate that the method chosen to evaluate heat resistance of *E. coli* can dramatically influence results as there was not a significant correlation between D_{60} values for the same isolate determined by water bath and heating block methods. The water bath method generates more reliable and consistent heat resistance data and should be used in future evaluations of heat resistance in *E. coli*. Moreover, PCR screening for the LHR would only be moderately useful for predicting phenotypic heat-resistance of *E. coli*. Considering water bath data only, LHR-positive STEC isolates were either moderately heat-resistant (1 to 5 log reduction) or heat-sensitive (> 5 log reduction). As LHR-negative STEC were also moderately heat-resistant, prediction of phenotypic heat resistance from genotype requires further refinement.

1. Introduction

Foodborne disease due to pathogenic *E. coli* has been often linked to the consumption of undercooked meat (Greig and Ravel, 2009; Thomas et al., 2015; Yeni et al., 2015). Accordingly, heat treatment is often an essential strategy to inactivate pathogenic bacteria and ensure food safety (Woodward et al., 2002; Klaiber et al., 2005; Rajic et al., 2007). Effective application of heat treatment to the control of foodborne pathogens requires accurately estimating the reduction in cell numbers that will be achieved and understanding the factors influencing heat resistance of the pathogen (Li and Ganzle, 2016).

For *E. coli*, heat resistance is not related to the phylogenetic group or serotype (Mercer et al., 2015) and is highly variable (Mercer et al., 2015; Mercer et al., 2017). Under elevated temperatures many biochemical mechanisms may be affected including outer membrane and membrane fluidity, regulation of heat response by heat shock proteins (HSPs), sigma factors σ^E and σ^S, or cross-resistance to acid oxidative or high-pressure stress (Li and Ganzle, 2016). In general, *E. coli* have been considered relatively heat-sensitive, having D-values at 60 °C (D_{60}) ranging from 0.1 to 1 min (Mercer et al., 2015; Li and Ganzle, 2016). However, a heat-resistant strain of *E. coli* with a D_{60}-value exceeding 10 min has been reported (Dlusskaya et al., 2011; Garcia-Hernandez et al., 2015), and this has been attributed to a ~ 15-kb mobile genomic island termed the locus of heat resistance (LHR; Li and Ganzle, 2016; Mercer et al., 2015). In a recent study, the LHR has been found in the genomes of 2% of *E. coli* including food isolates, and pathogens

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harboring the LHR have exhibited extreme resistance to wet heat (Mercer et al., 2015). Accordingly, strains carrying the LHR may potentially resist thermal interventions that are lethal to LHR-negative strains (Dlusskaya et al., 2011).

Currently, studies to determine heat resistance have used a heating block, thermomixer, or PCR thermal cycler (Dlusskaya et al., 2011; Garcia-Hernandez et al., 2015) or a conventional water bath (Liu et al., 2015; Mercer et al., 2015; Mercer et al., 2017; Gill et al., 2019). However, some of our internal studies using two different heating blocks demonstrated variability in the time required for media/bacterial cultures in wells of the heating blocks to reach 60 °C, which would possibly affect heat-resistance measurements. To determine impacts of methodology, in this study we compared the heat resistance of Shiga-toxin producing *E. coli* strains with and without the LHR using both heating block and water bath methods.

2. Material and methods

2.1. Bacterial strains and culture

A total of 35 isolates of *E. coli* were used in this study, 34 strains were from the culture collection of Alberta Agriculture and Forestry and were isolated from cattle or their environment from 2002 to 2017 (Table 1). The highly-heat resistant *E. coli* strain AW1.7 (Aslam et al., 2004) was used as a positive control. All strains were PCR screened for the LHR (Mercer et al., 2015) and virulence factors *stx1*, *stx2 eae* and *ehxA* (Conrad et al., 2014). Isolates positive for the LHR ($n = 17$) were matched with isolates lacking the LHR collected at a similar date with similar virulence factors.

From a glycerol working stock, a loop of suspension was streaked on a MacConkey plate (Difco™, USA) and incubated aerobically for 18 h at 35 °C (CO_2 Incubator, Thermo Scientific, Canada). A single colony was then sub-cultured in 15 mL Luria Bertani broth (LB, Difco™, USA) at 35 °C with shaking at 100 rpm (VWR Mini-Shaker, New Brunswick Scientific, Canada). All experiments were performed with overnight cultures in the stationary phase of growth. Two replicates per heating treatment were evaluated from each overnight culture. Optical density (OD_{600} nm) was determined on 3.0 mL of the overnight culture using a spectrophotometer (Thermo Fischer Scientific, Canada) to estimate initial total cell concentrations (Conrad et al., 2014).

2.2. Determining come up time

For the water bath method, a 28-l water bath (model FSGPD28, Fisher Scientific®, Canada) with forced water circulation and interior dimensions of 11.7 × 19.7 × 7.99 cm (L x W x H) was used. Duplicate 2 mL-microcentrifuge tubes (Eppendorf, Canada) each containing 1.5 mL of blank LB were included. The time required for media to reach 60 °C (come up time, T0) was measured by a long needle probe attached to a thermometer (Thermapen® Mk4, Thermoworks, USA) stabbed into the medium through the lids of the tubes. A total of 14 microtubes were incubated at any one time in the water bath. The water bath and heating blocks were pre-set to 60 °C prior to each test. For the heating block method, the come up time for each individual well of two 24-well heating blocks (Thermomixer® R, Eppendorf Canada and ThermoMixer® C, Eppendorf Canada) was determined using triplicate tubes of LB media for each well. Subsequent studies were conducted using the Eppendorf Thermomixer® R, dimensions: (25 L × 22 W x 12H

Table 1
E. coli isolates and their response in LB broth to heat treatment in water bath and heating block.

Isolate	Description	Virulence	LHR	Reduction (Log CFU/ml) WB	HB	D_{60} (min) WB	HB	Heat resistance classification WB	HB
1	AW1.7	n.d.	+	0.72	1.87	8.30	4.40	High	Moderate
25	O26:H9	*stx1*	+	1.39	3.19	4.30	1.88	Moderate	Moderate
23	O26:H9	*ehxA, eae, stx2*	+	1.52	3.78	3.95	1.59	Moderate	Moderate
20	O26:H9	*ehxA, eae, stx2*	+	1.66	2.94	3.61	2.04	Moderate	Moderate
21	O26:H9	*ehxA, eae, stx1*	+	1.79	3.50	3.34	1.71	Moderate	Moderate
11	O45:H34	*ehxA, eae, stx2, stx1*	+	1.85	3.96	3.25	1.52	Moderate	Moderate
26	O26:H9	*ehxA, eae, stx1*	+	2.00	2.96	3.00	2.03	Moderate	Moderate
8	O26:H11	*ehxA, eae, stx1*	−	2.65	1.34	2.30	7.10	Moderate	High
2	O157:H12	n.d.	+	2.58	4.13	2.30	1.45	moderate	Moderate
30	O157	n.d.	+	2.58	4.00	2.30	1.50	moderate	Moderate
7	O157:H12	n.d.	+	2.68	1.51	2.24	3.97	Moderate	Moderate
34	O157	*ehxA, eae, stx2*	+	2.89	0.94	2.08	6.41	Moderate	High
17	O157:H12	*ehxA, eae,*	+	2.94	4.30	2.04	1.40	Moderate	Moderate
27	O157	*eae*	+	2.97	1.99	2.02	3.02	Moderate	Moderate
15	O157:H12	*n.d*	+	3.13	3.33	1.92	1.80	Moderate	Moderate
14	O145:NM	*ehxA, eae, stx2, stx1*	+	3.29	1.91	1.83	3.14	Moderate	Moderate
33	O157	*ehxA, eae, stx1*	+	3.43	1.00	1.75	5.98	Moderate	Moderate
13	O145:NM	*ehxA, eae*	−	3.64	3.69	1.65	1.63	Moderate	Moderate
9	O26:H46	*ehxA, eae*	+	4.33	4.43	1.38	1.36	Moderate	Moderate
28	O26	*eae, stx2*	−	4.91	7.35	1.22	0.82	Moderate	Sensitive
18	O26	*ehxA, stx2*	−	5.13	0.60	1.17	9.92	Sensitive	High
16	O157:NM	*stx2*	−	5.18	Indeterminate	1.16	Indeterminate	Sensitive	Indeterminate
29	O157	*stx2*	−	5.26	6.93	1.14	0.87	Sensitive	Sensitive
32	O157	eae	−	5.47	Indeterminate	1.10	Indeterminate	Sensitive	Indeterminate
3	O103:NM	*stx2*	−	5.84	5.14	1.03	1.17	Sensitive	Sensitive
5	O157:H7	*stx1*	−	5.86	Indeterminate	1.02	Indeterminate	Sensitive	Indeterminate
6	O157:H12	n.d.	−	6.05	1.81	0.99	3.31	Sensitive	Moderate
12	O157:NM	*stx1*	−	6.21	1.34	0.97	8.4	Sensitive	High
22	O26	*ehxA, eae, stx2*	−	6.45	2.10	0.93	2.86	Sensitive	Moderate
31	O157	n.d.	−	6.49	1.06	0.92	5.69	Sensitive	Moderate
10	O45:H4	n.d.	−	6.75	Indeterminate	0.89	Indeterminate	Sensitive	Indeterminate
19	O26:NM	*ehxA, eae, stx1*	−	6.80	2.26	0.88	2.65	Sensitive	Moderate
24	O26:NM	stx1	−	7.28	2.29	0.82	2.62	Sensitive	Moderate
4	O103:H2	stx1	+	7.74	4.95	0.78	1.21	Sensitive	Moderate
35	O157	*ehxA, eae, stx1*	−	8.03	3.64	0.75	1.65	Sensitive	Moderate

Note: n.d, none detected; '+', presence; '-', absence. WB, water bath, HB, heating block. Heat resistance, > than 5 log (CFU/mL) reduction after 6 min at 60 °C = heat-sensitive, 1 to 5 log (CFU/mL) reduction = moderately heat-resistant, < 1 log (CFU/mL) = highly heat-resistant.

cm).

2.3. Heat treatments

For each overnight culture of *E. coli*, with OD_{600} nm value approximately 0.5, 1.5 mL was dispensed into eight 2.0 mL micro tubes. Four tubes were incubated simultaneously, with two in the water bath and two in heating block as described above to determine the come up time to 60 °C (T0). The remaining four tubes were also split between water bath and heating block and incubated for 6 min plus the come up times (T6). After incubation, tubes were immediately placed in an ice bath and allowed to cool completely, approximately 30 min. Serial dilutions (10^{-1} to 10^{-8}) for each incubated culture were then prepared in 1500 µL 0.1% peptone water and 1 mL of each dilution was incubated on Petrifilm (3M™ Petrifilm™ Aerobic count) for 18–24 h at 35 °C. Dilutions and original tubes were kept at 4 °C overnight, and if additional dilutions were needed the following day.

2.4. Colony enumeration and data analysis

After incubation, the colony forming units (CFU) on the Petrifilm agar plates with 30–300 CFU were enumerated per manufacturer's instructions and mean log CFU/mL from independent duplicates was calculated. The log reduction in bacterial populations from T0 to T6 and D_{60} values were calculated from the linear slope of the death curve (Dlusskaya et al., 2011; Liu et al., 2015; Garcia-Hernandez et al., 2015). Strains were classified into phenotypic groups based on their survival after heating, using the following criteria for stratification. Strains with a reduction in cell counts of > 5 log (CFU/mL) after 6 min at 60 °C were classified as heat-sensitive. Those demonstrating a reduction in cell counts of 1 to 5 log (CFU/mL) were classified as moderately heat-resistant while strains with reductions < 1 log ($CFUmL^{-1}$) were designated as highly heat-resistant according to Mercer et al. (2015). To compare all reductions, standard deviations among duplicates within a heating method and correlation of the D_{60} values generated across methods were determined using mixed model and correlation analyses in SAS (version 9.4, Cary, NC, USA). A significance level of 0.05 was used for all analyses.

3. Results

3.1. Come up times

The come up times for media in microtubes in the water bath ranged between 3.4 and 3.6 min. In contrast, twelve and nine wells of Thermomixer® C and Thermomixer® R, respectively, either failed to heat the media in tubes placed in these wells up to 60 °C within 30 min or showed a large variability between repeated measurements, i.e. the standard deviation among triplicates was larger than 1 min (Fig. 1). The come up time for media in microtubes in the remaining wells ranged from 4.3 to 8.3 min and from 4.3 to 18.9 min for heating blocks R and C, respectively. For subsequent studies, the Eppendorf Thermomixer R was chosen. Wells failing to reach 60 °C when the temperature was set to 60 °C or showing > 1 min standard deviation among the duplicates were excluded from further use (Fig. 1).

3.2. Heat resistance measured by water bath

After heat treatment at 60 °C for 6 min, the reductions in cell counts ranged from 0.72 to 8.03 log CFU/mL and D_{60} ranged from 0.75 to 8.30 min (Table 1). The median and mean of cell reduction was 3.21 and 3.81 CFU/mL, respectively (Fig. 2). Among duplicates, the standard deviation at T0 and T6 was 9.09 and 4.93, CFU/mL respectively (Table 2). The positive control *E. coli* AW1.7 was the only isolate with high thermal-resistance. Other LHR-positive isolates were either classified as moderately heat-resistant (n = 16) or heat-sensitive (n = 1). Most LHR- negative isolates were generally heat-sensitive except for three isolates which were moderately heat-resistant. According to the results from the water bath method, heat resistance (D_{60}) of *E. coli* was increased ($P < .05$) by the presence of the LHR.

3.3. Heat resistance measured by thermomixer® R

The seven wells (green zone in Fig. 1B) in Thermomixer R with relatively lower come up times were used for the heat resistance tests. The reductions after thermal treatment at 60 °C for 6 min ranged from 0.60 to 7.35 $\log_{10}$ CFU/mL (Table 1). The median cell reduction was 2.42 $\log_{10}$ CFU/mL and averaged 2.82 $\log_{10}$ CFU/mL (Fig. 2), approximately 1 $\log_{10}$ CFU/mL lower than that from the water bath method ($P < .05$). There was no difference in standard deviation among duplicates at T0 using the water bath or heating block methods, but the standard deviation among duplicates at T6 was higher ($P < .05$) using the heating block as compared to the water bath (Table 2). Using the heating block, D_{60} values ranged from 0.82 to 9.92 min (Table 1). In contrast to the water bath method, the positive control *E. coli* AW1.7 showed only moderate thermal-resistance and three strains without the LHR were highly thermal-resistant. For four isolates, it was not possible to determine D_{60} because there was no reduction in cell numbers after 6 min at 60 °C. Only one isolate with the LHR had high thermal-resistance. All 17 other strains with LHR were classified as moderately thermal-resistant and seven strains lacking the LHR were also moderately thermal-resistant. The D_{60} rank for heat resistance among isolates using both the water bath and heating block methods is shown in Table 1. Using the heating block method, presence of the LHR did not affect D_{60} values ($P > .05$).

4. Discussion

4.1. Heating block vs water bath for determining bacterial heat resistance

Other heating block systems have been designed to measure thermal inactivation of bacteria in liquid, semi-solid and solid foods (Chung et al., 2008; Jin et al., 2008; Yuk et al., 2009; Kou et al., 2016). These heating blocks are also made of aluminum and were developed to provide uniform heating, good control of heating rates and short come up times to accurately determine bacterial heat resistance in foods. In contrast to thermomixers where there are multiple wells, these other heating block systems (Chung et al., 2008; Jin et al., 2008; Yuk et al., 2009; Kou et al., 2016) have a few cells designed to put samples directly in contact with aluminum, thus increasing thermal conductivity. The thermomixers evaluated in the present study were not designed to determine bacterial heat resistance and were functioning within

A) Thermomixer® C

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

B) Thermomixer® R

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

Fig. 1. Come up times to 60 °C for wells in two heating blocks when heating 1.5 mL Luria Bertani broth in 2 mL Eppendorf tubes.
Legend: RED wells either failing to heat media to 60 °C within 30 min or showing a > 1-min standard deviation (SD) among the replicates. GREEN: wells with mean come up time < 5 min) and a SD < 1 min. WHITE, wells mean come up time > 5 min and a SD < 1 min.

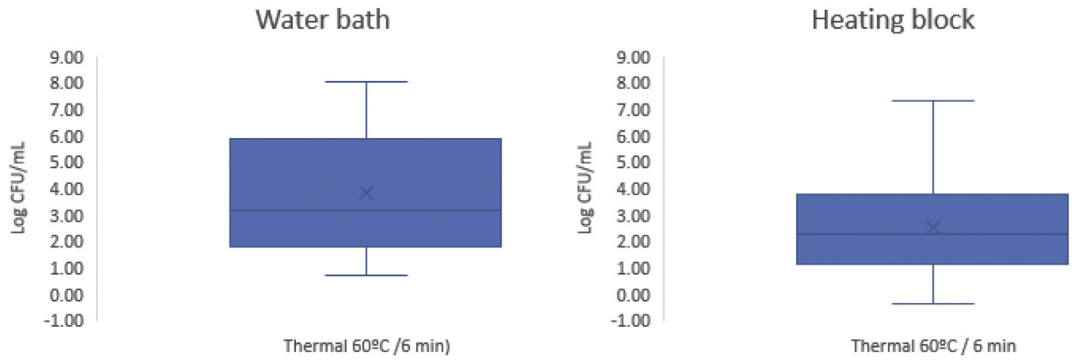

Fig. 2. Log Reductions of 35 strains of *E. coli* after treatment at 60 °C for 6 min in water bath and heating block. Boxes indicate ranges of results for log reduction, line inside of box indicate median, "x" marks indicate average and whiskers indicate the lowest and highest data points.

Table 2
Reduction (log CFU/mL) of *E. coli* after heat at 60 °C for 6 min treatment using two different methods.

Method	Strains	Reduction[1]	Median[2]	CV[3]	STDEV T0[4]	STDEV T6[5]	D_{60}[6]
Water bath	35	0.75–8.30	3.21 A	58 A	9.09 ± 8.74A	4.93 ± 5.57A	2.49 ± 1.96A
Heating block	35	0.82–9.92	2.42 B	116 B	14.23 ± 12.9A	33.87 ± 38.4B	4.06 ± 6.05B

[AB]Means within a column with different letters differ, ($P < .05$).
[1] Cell count Log (cfu/mL) after 60 °C / 6 min.
[2] Median values.
[3] Coefficient of variation.
[4] Standard deviation average "Time 0" Log (cfu/mL) after reaching a temperature of 60 °C.
[5] Standard deviation average "Time 6" Log (cfu/mL) after 60 °C for 6 min.
[6] D_{60} values average.

manufacturer's specifications. According to Thermo Fisher Scientific (2007), Eppendorf Thermomixer® R has a within well temperature accuracy ± 2.0 °C at temperatures > 45 °C and a heating rate of approximately 5 °C/min in empty wells, which would be reduced in tubes containing media.

Comparing the two heating methods, the heating block had a higher come up time (5.2 min) than water bath (3.5 min). According to Buchner et al. (2012), devices used to determine heat resistance of bacteria need a short come up time to avoid thermal adaptation of bacteria and isothermal conditions for uniform sample temperature distributions. With the heating block, the standard deviation among duplicates at T6 was approximately 10 x higher than for the water bath, ($P < .05$) as well as having a longer come up time, which would have resulted in slower heating during the incubation period. Slow heating rates have often resulted in enhanced heat resistance of bacteria as determined by higher D-values at the same target temperatures due to a prolonged time in sub-lethal temperatures (Chung et al., 2007; Chung et al., 2008; Yuk et al., 2009). These differences in bacterial heat resistance are likely the result of acclimation and physiological adjustment during the slower heating, and most probably caused by the production of heat shock protein (Wiegand et al., 2009; Urban-Chmiel et al., 2013; Kou et al., 2016).

Generally, more than two time points are used to calculate D_{60} values, which was not possible in the present study after splitting the overnight culture between the two heat treatments. D_{60} values were estimated only to demonstrate the magnitude of changes possible in this measurement from minor changes in heating methodology. It was not possible to estimate D_{60} value for all isolates in the heating block, because 4 isolates actually had increased cell numbers after 6 min at 60 °C (Table 1). This likely occurred due to sub-lethal temperature in the wells used for those isolates. Even though wells in the heating block with slower heating and increased variation among duplicates were not used, this situation could not be controlled. In contrast, using the water bath method it was possible to estimate D_{60} values for all isolates and the water bath also showed less variability among duplicates than the heating block (Fig. 2, Table 1, and Table 2). These results are most likely related to the stability and uniformity of the temperature of the water bath, with manufacturer's specifications of ± 0.2 °C, for temperatures up to 70 °C (Thermo Scientific, 2015).

The Pearson correlation coefficient (r) between water bath and heating block D_{60} values was – 0.07, revealing that there was no significant relationship between results of the two methods. As our findings indicate that the water bath method generates more consistent data than did the heating block, the water bath method should be used in future *E. coli* heat-resistance studies to avoid inflation of D_{60} values and overstating the risk to public health.

4.2. Impacts of the LHR

The strains with LHR did not differ in D_{60} when evaluated by heating block and water bath ($P = .068$), but for strains without the LHR D_{60} values were higher in the heating block than in water bath ($P < .001$). The heating block results disagree with previous studies (Mercer et al., 2017; Mercer et al., 2015; Dlusskaya et al., 2011) which demonstrated that the presence of the LHR led to increased heat resistance. With the longer come up time of the heating block, highest heat resistance was due to unknown mechanisms, as all isolates with high heat resistance were LHR-negative. Multiple mechanisms are known to be responsible for heat resistance in *E. coli* (Mercer et al., 2015; Li and Ganzle, 2016) and it was only with the rapid and consistent heating of the water bath method that the superior heat resistance of LHR-positive isolates was revealed.

Considering water bath results, most *E. coli* harboring the LHR had moderate heat resistance and one LHR-positive isolate was heat-sensitive (Table 1). These findings demonstrate that the presence of the LHR in *E. coli* does not guarantee that strains will display a phenotype of high heat-resistance. Other studies have associated the presence of the LHR with high heat-resistance in *E. coli* (Mercer et al., 2017; Mercer et al., 2015; Dlusskaya et al., 2011), but much of this work has focused on isolate AW1.7 which also showed high heat-resistance in the present

study.

Previous studies of *E. coli* AW1.7 have demonstrated that the LHR encodes small heat shock proteins (Orf2 and Orf7), proteins with unknown function (Orf8 and Orf9), heat shock proteases (Orf3, Orf15 and Orf 16), thioredoxin (Orf12), and a sodium/hydrogen antiporter (Orf13) which may contribute to turnover of misfolded or aggregated proteins, the osmotic stress response and mitigate oxidative stress (Mercer et al., 2015; Li and Ganzle, 2016). These LHR-encoded effects are species specific, and high heat-resistance in *E. coli* necessitates heat shock proteins acting in concert with other biochemical functions (Li and Ganzle, 2016). Accordingly, the results of the present study indicate that individual *E. coli* strains carrying the LHR can display phenotypes ranging from high heat-resistance (AW1.7), to sensitive.

Considering water bath results only, LHR-positive strains generally exhibited moderate heat resistance, similar to that of some other strains lacking the LHR (Table 1). Accordingly, mechanisms other than the LHR provided phenotypic heat resistance equal to that of the LHR, with the resistance of AW1.7 atypical. The mechanisms used by these non-LHR carrying isolates are unknown, but will be investigated in a subsequent study.

5. Conclusions

The method chosen to evaluate heat resistance of *E. coli* can dramatically influence results. We did not find a significant correlation between D_{60} values for the same isolate determined by water bath and heating block methods. We also found that the water bath method generates more reliable and consistent heat-resistance data and should be used in future evaluations of heat resistance in *E. coli*. Based on the range in heat resistance exhibited by isolates carrying the LHR in the present study, PCR screening for the LHR would be of limited value in predicting thet *E. coli* strains displaying a highly heat-resistant phenotype. Additional assays are required to identify LHR-positive and LHR-negative isolates with high phenotypic heat resistance.

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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