Heat-induced injury in *Listeria monocytogenes*

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SUMMARY

Heating of *Listeria monocytogenes* (Scott A strain) in potassium phosphate buffer (0.1 M, pH 7.2) at 52°C for 1 h led to injury, with the heat-injured cells failing to produce colonies on agar medium containing 5% NaCl. The detection of injury was based on the use of differential media: plating on tryptose phosphate broth + 2% agar and 1% sodium pyruvate (TPBA + P) and on tryptose phosphate broth + 2% agar and 5% NaCl (TPBA + S). Only non-injured *Listeria* formed colonies on TPBA + S whereas both heat-injured and non-injured cells formed colonies on TPBA + P. The bacterial count on TPBA + P minus that on TPBA + S represents the extent of heat injury. A large number of selective agars were tested and compared to TPBA + P for their ability to support repair and colony formation of heat-injured *L. monocytogenes*. Media containing 0.025% phenylethanol, 0.0012–0.0025% acriflavin, 0.1–0.2% potassium tellurite, 0.001% polymyxin B sulfate, 5% NaCl or a combination of these ingredients were detrimental to the recovery of heat-injured *L. monocytogenes*. Media currently in use for *L. monocytogenes* are not satisfactory for the recovery of injured cells.

INTRODUCTION

Concern about foodborne listeriosis, particularly from dairy products, has been steadily increasing. The principle source of *Listeria monocytogenes* in dairy foods is still unclear. *Listeria* are killed at pasteurization temperatures [6,10,11]; therefore, the presence of *L. monocytogenes* in dairy products should only be due to improper pasteurization or post-processing contamination. While post-processing contamination is the more likely source of *Listeria* [10], improper pasteurization with concomitant generation of injured cells cannot be ruled out.

Microorganisms present in food may encounter sublethal stresses due to food processing conditions and are physiologically injured but not killed. On non-selective agar media, injured cells can repair the damage induced by the stress and grow; however, on selective agars containing bile salts, NaCl, antibiotics, etc., injured cells undergo additional stresses and fail to repair the initial damage [22].

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Thus, if a particular foodborne pathogen has been injured, the food microbiologist may either fail to detect it or will underreport its numbers, particularly if selective agars are used directly.

While there has been a substantial amount of recent research pertaining to the heat resistance of *L. monocytogenes* only one report has appeared which demonstrates the susceptibility of *L. monocytogenes* to heat injury. Beuchat et al. [4] observed that two strains of *L. monocytogenes* when heated at 52°C in cabbage juice formed fewer colonies (4–5 log decrease) on tryptic soy agar (TSA) + 4% NaCl than they did on TSA lacking added salt. There is currently a need for sound quantitative data on the incidence of *L. monocytogenes* in food products, particularly those that receive some limited degree of thermal processing. Isolation techniques that can effectively enumerate the population of *L. monocytogenes* including cells that may be injured as a result of sublethal stress are not available. Therefore, the objectives of the current study were to determine the incidence of injured cells after thermal stress and assess quantitatively the ability of various selective media to detect thermally injured *L. monocytogenes*.

METHODS AND MATERIALS

L. monocytogenes, Scott A strain, was maintained in brain heart infusion broth (BHI; Difco) stored at 5°C. A flask (1 liter Erlenmeyer) containing 100 ml BHI with added glucose (final glucose concentration, 0.5% w/v) was inoculated with *L. monocytogenes* and incubated on a rotary shaker (200 rpm) at 37°C for 20 h. Cells were harvested by centrifugation, washed twice with sterile distilled water and resuspended in 5 ml sterile distilled water.

To determine heat injury, one or more wide-mouth screw cap 160 ml dilution bottles containing 20 ml sterile potassium phosphate buffer (pH 7.2; 0.1 M) were equilibrated to 52°C and then 1 ml of washed *L. monocytogenes* was added to each bottle. At zero time, and at regular intervals during the heating cycle, 0.1 ml of culture was removed from each bottle and placed into 9.9 ml sterile 0.1% (w/v) Bacto peptone water and successive dilutions were prepared. Using a spiral plater (Spiral Systems Instruments, Inc., Bethesda, MD), appropriate dilutions were plated onto tryptose phosphate broth + 2% (w/v) agar (TPBA) + 1% (w/v) sodium pyruvate (TPBA + P) and onto TPBA + 5% (w/v) added NaCl (TPBA + S). Other media were tested similarly. Plates were incubated at 37°C and were counted after 3 days.

Bacteriological media and protein hydrolysates were obtained from Difco Laboratories, except for Lab-Lemco powder, which was obtained from Oxoid U.S.A. All Difco media were prepared by using preformulated preparations, except for McBride, Modified McBride, and Phenylethanol agars, which were prepared from individual ingredients according to Difco specifications. Chemicals utilized in the study were obtained from Sigma Chemical Co., except for glucose, potassium thiocyanate, and lactic acid obtained from J.T. Baker Chemical Co., lithium chloride from Merck & Co., and moxalactam from E. Lilly Co.

RESULTS AND DISCUSSION

The number of injured cells was determined by using a differential plating system consisting of TPBA + P and TPBA + S. Both injured and non-

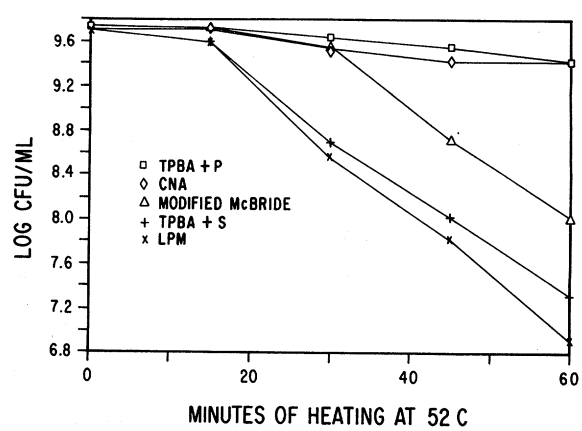

Fig. 1. Colony formation by heat-induced (52°C) injured cells of *L. monocytogenes* on various media.

injured *L. monocytogenes* formed colonies on TPBA + P, but since injured cells were unable to repair heat-induced damage in the presence of 5% salt, only non-injured cells formed colonies on TPBA + S. Thus, the difference in bacterial count between TPBA + P and TPBA + S gave the number of injured cells. Injured bacteria typically have increased sensitivity to hydrogen peroxide and the addition of exogenous H_2O_2 decomposers such as catalase or sodium pyruvate has been recommended to maximize repair and colony formation of injured cells [5,19]. Therefore, TPBA + P was routinely incorporated into every experiment to ensure maximum recovery of heat-injured *L. monocytogenes* and to serve as the positive control; similarly, TPBA + S served as the negative control.

Examples of responses typical of those obtained when *L. monocytogenes* heated at 52°C was plated on various media are shown in Fig. 1. Columbia CNA agar was similar to TPBA + P in its ability to support repair and colony formation of heat-injured cells. Modified McBride agar was less able to support colony formation of injured cells than TPBA + P but was not as inhibitory as TPBA + S, i.e., it supported the repair and growth of some but not all of the thermally injured cells. LPM (LiCl-Phenylethanol-Moxalactam) agar was more inhibitory than TPBA + S (Fig. 1).

In order to compare the various media for their ability to support colony formation of heat-injured *L. monocytogenes*, a 'recovery inhibition coefficient' (RIC) was developed. All experimental data were graphed as in Fig. 1 and the area under each curve was determined. The RIC was then calculated by determining the differential between the curve for the medium being assessed and that of the TPBA + P control. Accordingly, the RIC for the TPBA + P controls which detected both injured and non-injured cells was 0.0, while the value for the TPBA + S control which recovered only non-injured cells was −53.6. The RIC values for the various media evaluated are presented in Table 1.

Examination of the RIC values identified general trends concerning compounds that prevent repair and subsequent colony formation by heat-injured *L. monocytogenes*. Media containing phenylethanol (0.25%), acriflavin (0.0012–0.0025%), oxgall (4.0%), potassium tellurite (0.1–0.2%), polymyxin B sulfate (0.01%; approximately 1600 IU/100 ml), and sodium thiosulfate (0.008%) appear to inhibit repair and colony formation of heat-injured cells. It should be emphasized that growth of unheated, i.e., non-injured *L. monocytogenes* on the media listed in Table 1 was comparable to that which occurs on TPBA + P.

TPBA (containing 0.2% glucose) without pyruvate was slightly less effective (RIC = −6.8) than TPBA + P in recovering heat-injured *L. monocytogenes* (Table 1). However, TPBA lacking both glucose and pyruvate was considerably less (RIC = −14.8) able to support colony formation of injured cells (Table 1).

Brilliant Green agar, EMB agar, GN broth, and MacConkey agar, which are used to isolate gram-negative enteric bacteria, did not support the growth of non-injured (nor injured) *L. monocytogenes* (Table 2). The inhibitory compounds in these isolation media are probably the various dyes and/or bile salts. Thioglycollate medium which contains thioglycollic acid and KF Streptococcus agar which contains azide also did not support growth of injured or non-injured cells. The Selective Enrichment Procedure (SEP) for *Listeria* developed by Doyle and Schoeni [12] inhibited growth of *L. monocytogenes* due to the presence of toxic levels of polymyxin and acriflavin (Table 2).

Addition of 1–3% sodium pyruvate to modified McBride medium did not improve recovery of heat-injured *L. monocytogenes*. Sodium lactate was reported to improve the isolation of heat-injured *Salmonella typhimurium* [7] but not *S. senftenberg* [21]. The addition of lactate to modified McBride medium did not improve colony formation by heat-injured *Listeria*.

The results shown in Table 1 indicated that most media used currently for the enrichment and isolation of *Listeria* from clinical and food samples contain inhibitory substances that prevent the repair and subsequent colony formation by stress-injured cells. The presence of phenylethanol (McBride and Modified McBride medium, LPM agar, FDA Enrichment broth), acriflavin (Listeria

Table 1

Ability of various media to support repair and growth of heat-injured (52°C) *L. monocytogenes* (Scott A)

Media	Suspected inhibitor(s)	RIC[a]	Ref.
Tryptose phosphate broth (Difco) + 2% agar (TPBA) with cycloheximide	0.02% cycloheximide	+3.8	this paper
Brucella agar (Difco)	–	+2.6	3
GBNA Listeria medium	0.005% nalidixic acid	+1.1	18
TPBA + P (1.0% Na pyruvate)	–	0.0	this paper
TPBA with thallium acetate and nalidixic acid	0.02% thallium acetate 0.005% nalidixic acid	−1.2	16
TPBA with ferric citrate and esculin	–	−2.1	11
Pseudomonas F agar (Difco)	–	−2.6	2
Columbia CNA agar (Difco)	0.001% colistin sulfate 0.0015% nalidixic acid	−3.4	3
TPBA with K thiocyanate	3.75% thiocyanate	−4.1	this paper
VJ agar (Difco) minus K tellurite	0.5% LiCl 1.0% glycine	−5.8	3
Jay's Listeria medium (CNP agar)	0.3% 1,3-cyclohexanedione 0.1% phenylethanol 0.0045% nalidixic acid	−6.4	13
TPBA with nalidixic acid	0.01% nalidixic acid	−6.7	this paper
TPBA	–	−6.8	this paper
TPBA with moxalactam	0.002% moxalactam	−8.1	this paper
Phenylethanol agar (Difco)	0.25% phenylethanol	−13.3	3
TPBA minus glucose	–	−14.8	this paper
Listeria Enrichment broth + 2% agar	0.004% nalidixic acid 0.0012% acriflavin HCl	−18.4	9
Modified McBride medium	0.05% LiCl 0.25% phenylethanol 1.0% glycine anhydride 0.02% cycloheximide	−20.2	17
Modified McBride medium minus glycine anhydride	0.05% LiCl 0.25% phenylethanol 0.02% cycloheximide	−23.7	17
Bile Esculin agar (Difco)	4.0% oxgall	−25.0	3
VJ agar (Difco) with K tellurite	0.02% tellurite 0.5% LiCl 1.0% glycine	−28.3	3
Trypan Blue Enrichment medium	0.008% trypan blue 0.005% nalidixic acid 5.0% NaCl	−30.1	8
APT agar (Difco)	0.02% sorbitan monooleate	−34.4	3

Table 1 contd.

Media	Suspected inhibitor(s)	RIC[a]	Ref.
Baird Parker agar + egg yolk tellurite enrichment (Difco)	0.5% LiCl 1.2% glycine 0.01% K tellurite	−35.4	3
McBride medium	0.25% phenylethanol 0.05% LiCl 1.0% glycine	−43.0	20
TPBA with K thiocyanate, nalidixic acid and acriflavin HCl	3.75% K thiocyanate 0.01% nalidixic acid 0.0025% acriflavin HCl	−45.2	14
Pseudomonas P agar (Difco)	?	−45.4	2
LPM agar	0.25% phenylethanol 1.0% glycine anhydride 0.5% LiCl 0.002% moxalactam	−62.9	15
FDA Enrichment broth + 2% agar	0.0015% acriflavin HCl 0.004% nalidixic acid 0.005% cycloheximide	−64.4	12
TPBA with acriflavin HCl	0.0025% acriflavin HCl	−139.7	this paper
TPBA with polymyxin	0.01% polymyxin B sulfate	−155.0	this paper
Peptone Iron agar (Difco)	0.008% Na thiosulfate	−188.9	1

[a] Values represent averages of 2–4 independent determinations.

Table 2

Media that supported little or no growth[a] of non-injured *L. monocytogenes* (Scott A)

Media	Suspected inhibitor(s)	Ref.
Brilliant Green agar (Difco)	0.00125% brilliant green	3
EMB agar (Difco)	0.04% eosin Y 0.0065% methylene blue	3
GN broth, Hajna (Difco) + 2% agar	0.05% Na desoxycholate	3
MacConkey agar (Difco)	0.15% bile salts No. 3 0.0001% crystal violet	3 3
Thioglycollate medium minus glucose or indicator (Difco)	0.03 ml thioglycollic acid	3
KF Streptococcus agar (Difco)	0.04% azide	3
SEP + 2% agar	1600 IU polymyxin B 0.001% acriflavin HCl 0.004% naladixic acid	12

[a] When the initial count of *L. monocytogenes* was approximately 5×10^9 cells/ml, plating a 10^2 dilution on these media gave <5 colonies per plate (when approximately 5×10^7 would be expected).

Enrichment broth), NaCl (Trypan Blue Enrichment medium), and polymyxin-acriflavin (SEP of Doyle and Schoeni) were found to be detrimental to the recovery of heat-injured *L. monocytogenes*. Thus, most media in use as selective media for *L. monocytogenes* do not appear to be suitable for the recovery of heat-injured cells. Before quantitative surveys can be done to determine the incidence of *Listeria* in foods and food products receiving a thermal treatment, a medium must be developed that is selective and also permits the recovery of injured cells.

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