

Monitoring the microbial contamination of beef carcass tissue with a rapid chromogenic *Limulus* amoebocyte lysate endpoint assay

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G.R. SIRAGUSA, D.H. KANG AND C.N. CUTTER. 2000. A chromogenic *Limulus* amoebocyte lysate (LAL) endpoint assay was found to be an accurate and rapid means of gauging levels of beef carcass microbial contamination within 10 min. The assay demonstrated a high correlation with the total mesophilic bacterial and coliform surface populations from inoculated beef carcass surface tissues. This assay was tested on a set of actual beef carcass surface samples ($n = 121$) demonstrating the utility of the chromogenic LAL test as a means of monitoring carcass microbial contamination in a near real-time fashion. Classifying the chromogenic LAL results into four contamination groups was found to be a sound means of utilizing the resultant chromogenic LAL data for detecting carcasses with high levels of microbial contamination. For beef carcass testing, this assay can be used with no instrumentation other than the required 37°C incubator and, as an option, a microplate reader.

INTRODUCTION

Converting live animals into meat inevitably results in some amount of faecal contamination. Faecal contamination, whether from the intestinal tract, hooves or hide of the animal, is widely held as the main source of enteric pathogenic bacteria, including salmonellae and enteropathogenic *Escherichia coli*, associated with raw meats.

A method for monitoring carcass microbial and faecal contamination levels should indicate to processors the level of hygiene of their process. Relying on microbial culture is limited in efficacy because of two major disadvantages: (1) the inherent variability and inadequate representation of carcass sampling, and (2) lengthiness of assay time. Growth-based microbial assays include standard plate cul-

ture methods, impedometric techniques, dye reduction assays, chromogenic/fluorogenic tests, cultural enrichment tests coupled to enzyme immunoassays or polymerase chain reaction- (PCR) based tests. All of the aforementioned require some minimal growth period of usually no less than 8–16 h. Clearly, for purposes of monitoring faecal contamination on carcasses, such time frames render these tests of limited usefulness as process monitors. Ideally, real-time monitoring of a carcass for faecal contamination would allow that carcass to either be shunted off for a second round of decontamination or be tagged for decontamination downstream prior to entering the chiller. To date, real-time assays for faecal contamination are not commercially available; therefore, relying on faecal indicators that require just minutes in assay time offers perhaps the best alternative for process monitoring of meat animal conversion operations.

Near real-time assays that have been described for carcasses have relied on newly developed modifications of microbial ATP bioluminescence procedures. Such assays (including collecting the carcass sample) require from 5 min (Siragusa *et al.* 1995) to 15 min (Bautista *et al.* 1995), and provide estimates of the levels of total microbial contamination on animal and poultry carcasses. Microbial ATP assays, while rapid and accurate, are predicated on

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the measurable and relatively constant per-cell ATP content. Microbial ATP levels are very sensitive to most processing environmental conditions and microbial stressors including heating, chemical acidification and chilling.

Congruent with the rationale behind microbial ATP testing of carcass samples, high levels of carcass microbial contamination are most likely derived from faecal contamination whether the source of the faeces is contaminated hair, hide or hooves or the gastrointestinal tract itself (Siragusa *et al.* 1998). Therefore, since faecal contamination is dominated by Gram-negative bacteria from the intestinal tract, testing for lipopolysaccharide (LPS) is a logical gauge of faecal contamination on the otherwise clean carcass surface.

The most widely used means to test for LPS is the *Limulus* amoebocyte lysate (LAL) test. This assay, based on the initial research of Levin and Bang (Levin *et al.* 1964) is widely used for testing of medical devices and parenteral solutions. Previously, Jay (1977, 1981) and Jay *et al.* (1979) had utilized the *Limulus* amoebocyte lysate (LAL) test for testing ground beef. This original work demonstrated a high correlation between the LAL result and the microbial quality of the product. These early LAL assays were read by scoring for an endpoint gelling reaction in a tube and required approximately 1–2 h of assay time. Newer modifications of the LAL test are chromogenic and quantitative, therefore offering not only greater precision (Svensson and Hahn-Hagerdal 1987), but also considerably shorter assay times of approximately 10 min. In this report we present data on the use of a chromogenic *Limulus* amoebocyte lysate assay for detecting LPS contributed from the cell walls of Gram-negative bacteria in carcass samples as a means to monitor beef carcass microbial loads.

MATERIALS AND METHODS

Modified chromogenic LAL assay

The QCL-1000 Quantitative Chromogenic LAL test (BioWhittaker, Inc, Walkersville, MD, USA; Catalogue no. 50–647 U) was modified in order to reduce reagent volumes. LPS-positive controls provided by the manufacturer (purified from *E. coli* O111:B4) were used to assess consistent reactivity of each newly rehydrated LAL reagent ranging from 1.0 down to 0.1 EU (endotoxin units) per millilitre. Carcass sample swabs (described below) are placed in a 13 × 125 mm screw capped pyrogen-free test tube containing 5 ml of pretested pyrogen-free water (Milli-Q water system, Millipore, Bedford, MA, USA) and vortexed for 15 s at the highest setting. This sample is then serially diluted threefold in a plastic 96-well microtitre plate containing appropriate amounts of pyrogen-free water. Ten-microlitre aliquots of the diluted samples are

transferred to a fresh microtitre plate followed by 10 µl of the LAL assay reagent. Following incubation at 37 °C for 10 min, 20 µl of the LAL chromogenic reagent is added per well then incubated an additional 6 min at 37 °C. The reaction is stopped by adding 20 µl of stop solution (25% v/v glacial acetic acid in distilled water). The absorbance at 405 nm was measured using a Model 340-I BioTek Instruments microplate reader (BioTek Instruments, Inc., Model 340-I, Winooski, VT, USA). The endpoint was determined as the last well with an absorbance of greater than twice the pyrogen-free water control. The LAL value was defined as the log₁₀ of the reciprocal of the endpoint dilution of the last well yielding a positive reaction.

Bacterial enumeration

Samples were diluted in buffered peptone water (Difco). One millilitre of each diluted sample was plated on duplicate Petrifilm Aerobic Count plates and Petrifilm *E. coli* count plates (3M, Minneapolis, MN, USA) and incubated at 37 °C for 24 h, then counted according to manufacturer's instructions. All counts were normalized to square centimetre areas and transformed into the log₁₀ value.

Experiment one: correlation of the chromogenic LAL test with microbial plate counts

This protocol was designed to obtain a range of microbial, as well as detectable pyrogen levels on actual beef carcass surface tissues. Beef carcass surface tissue sections (cutaneous trunci with intact superficial fascia) were inoculated with bovine faecal suspensions. Faecal inocula were made as follows: on each day of an experiment, faeces were obtained from three cows fed a corn-silage ration containing no antibiotics, 50 g of each faeces sample (total of 150 g) was mixed together with 150 ml of sterile distilled water. This slurry was mixed using a metal spatula for 2 min, then passed through three layers of cotton cheesecloth (Kendall Co., Chicago, IL, USA), the suspension was serially diluted in pyrogen-free water. One-millilitre aliquots of each dilution were spread over the surface of tissue sections and allowed to incubate at room temperature for 15 min prior to sampling. Samples were obtained by swabbing a 100-cm² area with a pyrogen-free cotton-tipped swab (Puritan brand, Hardwood Products Co., Guilford, ME, USA) premoistened with pyrogen-free water, and returned to a screw capped tube containing 5 ml of pyrogen-free water. Samples were vortexed vigorously for 15 s then subjected to the chromogenic LAL and bacterial plate count assays as described.

Experiment two: testing uninoculated carcass surface samples with the chromogenic LAL test and microbial plate counts

Beef carcass surface samples were obtained from the abattoir of the Roman L. Hruska U.S. Meat Animal Research Center (MARC) and from a mid-western located medium-sized cow/bull processing plant. Samples were tested according to methods described above for the modified chromogenic LAL test and microbial plate counts.

Statistical analysis

Enumerated bacterial populations were normalized to an area basis (cfu cm^{-2}) and transformed to \log_{10} values. Correlation coefficients and linear regression trend lines were calculated and plotted using Microsoft Excel 97 or GraphPad InStat Ver. 3.00 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Preliminary results using lean and adipose beef carcass surface tissues (BCT) inoculated with diluted faecal suspensions indicated no differences in LAL test response (data not shown); therefore samples were obtained from BCT samples that were varying in lean or adipose fascia covering. A highly significant linear relationship ($P < 0.001$) was observed between microbial populations (total mesophilic and coliforms) and the modified LAL test results (Fig. 1a,b). Response scatter plots indicated a higher degree of association between LAL values and the total mesophilic aerobic surface count (total) than between the LAL value

and the coliform count, $r_{\text{total}}^2 = 0.90$ vs $r_{\text{coliform}}^2 = 0.73$, respectively. Considering the contribution of LPS from all Gram-negative faecal bacteria to the LAL value, this is not surprising since only a portion of Gram-negative faecal bacteria associated with faecal contamination will grow on the *E. coli*/coliform Petrifilm plates and of those that do grow, not all will ferment lactose. Therefore we chose to use the aerobic mesophilic bacterial plate count as a comparator to the LAL value based on its previously reported utility as a gauge of overall carcass hygiene and an indicator of biotype 1 *E. coli* incidence on beef carcasses (Siragusa *et al.* 1998). Chromogenic LAL test response values appeared to be linear at levels less than $\log_{10} 1.5 \text{ cfu cm}^{-2}$ in the case of both total mesophilic and coliform counts, a level of sensitivity which is more sensitive than that reported previously ($> \log_{10} 2.5 \text{ cfu cm}^{-2}$ for the rapid microbial ATP animal carcass test; Siragusa *et al.* 1995). The rapid microbial ATP test, while indeed quicker (5 min vs approximately 16 min), was less sensitive (response threshold of $> \log_{10} 2.5 \text{ cfu cm}^{-2}$), depending on the adenylate energy charge of the bacterial cells comprising the entire population of the sample. The LAL test detects a preformed cellular component (LPS) which, although variable and capable of being shed, is not in a state of constant flux as is ATP. While it is possible that unbound LPS contributes to the LAL value, our hypothesis was limited to the question of the LAL value and its correlation with a measurable index of hygiene, the plate count.

The LAL offers several advantages as an in-plant monitor of carcass microbial load. One distinct advantage is the highly readable end-point result based on colour. Although results were detected using a microwell plate reader, a visual determination of the endpoint titre could easily be

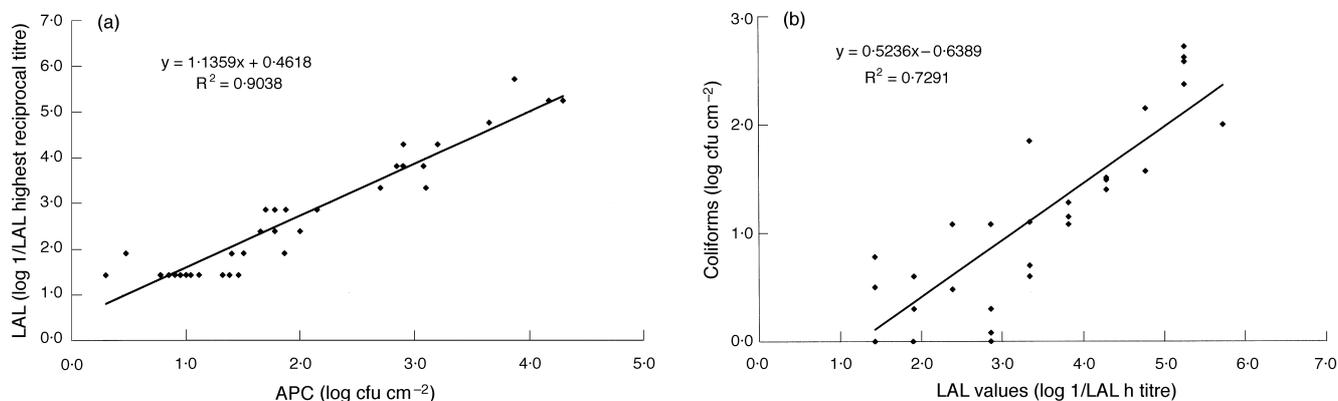


Fig. 1 Scatter plot of chromogenic LAL test values (\log_{10} of the reciprocal endpoint dilution) and relationship to inoculated beef carcass surface (a) microbial counts (\log_{10} mesophilic aerobic bacterial count per unit surface area), and (b) presumptive coliform bacterial counts (\log_{10} mesophilic aerobic bacterial count per unit surface area)

made. Since the analyte is not affected by carcass temperature, the chromogenic LAL test should be adaptable to testing chilled carcasses. In no case was the highest titre beyond eight dilution wells, so it is possible that at least eight carcasses could be tested in a single 96-well microtitre plate. In practice it is possible that more than eight samples per microtitre plate could be tested depending on the average contamination levels in a particular plant and whether the carcasses were tested before or after antimicrobial interventions. In some instances within this study, levels of contamination were such that as many as 24 carcass samples could be adequately diluted and tested per microtitre well plate. Clearly the time frame of this test, while much more rapid than culture methods, is still not ideal for process monitoring. Shortening the chromogenic LAL incubation time while perhaps possible, was not an objective of the current research.

Actual carcass samples were obtained and tested with the chromogenic LAL test. Typically 94–99% of beef carcass surfaces harbour a microbial load of $< \log_{10} 4.0 \text{ cfu cm}^{-2}$ mesophilic APC (Anonymous 1994; Siragusa *et al.* 1998). The 121 carcass samples analysed in the current research fell into that same range. As presented in Fig. 2 the carcass surface test data from the set of uninoculated carcasses. The predicted values were calculated from the regression line equation from the data set composing Fig. 1(a). It can be seen that at actual microbial load levels of less than about $\log_{10} 1.5 \text{ cfu cm}^{-2}$, the response is no longer linear, as would be predicted by the LAL sensitivity. Such LAL results were only obtained from carcasses with low to minimal levels of microbial contamination.

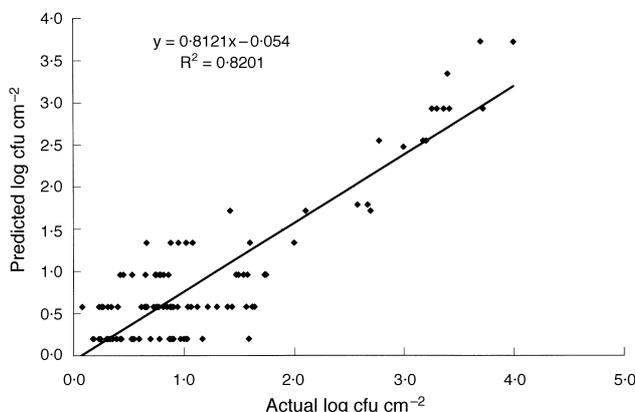


Fig. 2 Beef carcass surface counts predicted from regression line calculated from data presented in Fig. 1(a) and their relationship to the actual microbial counts of those samples

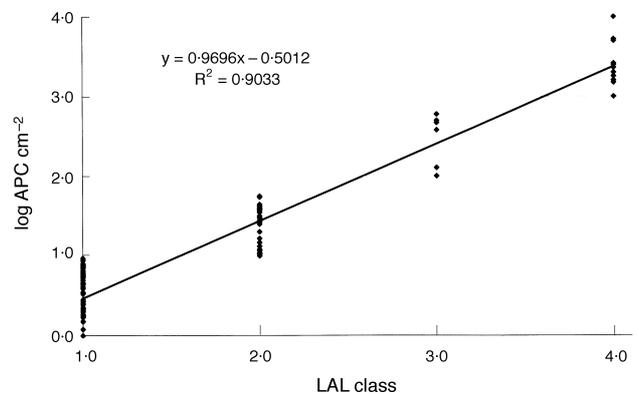


Fig. 3 Classification of LAL values into categories and dispersal of counts within each category. Classification example criteria defined in text

Previously this lab has demonstrated the relationship between the carcass microbial load and the frequency of isolating biotype 1 *E. coli* (Siragusa *et al.* 1998). Again, the importance of this relationship in monitoring for faecal contamination cannot be overstated. For processing plants to gain the most from data such as that provided by the chromogenic LAL test, the user must rely on the ability of the test to categorize carcasses into broad classes based on contamination levels. Figure 3 is an example of how this might be achieved. The LAL test result from the carcasses tested was classified into one of four different LAL categories (class 1: 0–1; class 2: > 1–2; class 3: > 2–3; and class 4: 3–4 LAL value) and plotted against the corresponding total mesophilic microbial load. The association between predefined arbitrary LAL value classes and microbial load classes (based on total mesophilic aerobic bacteria per square centimetre of carcass surface samples reflective of those reported by the USDA-FSIS (Anon. 1994) was found to be highly significant ($\chi^2 = 42.3$, $P < 0.001$). The agreement between the categorical LAL value result and the microbial load provides a useful means to gauge or monitor processing and antimicrobial intervention effectiveness without actually determining the culture-based microbial plate count. Such data are only useful as a semi-quantitative process monitor and are in no way meant to be a substitute for those instances where accurate quantitative microbial counts are required. In practice, a processor would develop test curves relating microbial levels (by culture) to the rapid chromogenic LAL test response of the normal process over several weeks of operation and differing incoming animal conditions. Next, these data could be applied to establishing acceptable limits of microbial loads following various points within the process, such as hide

removal, evisceration or final antimicrobial intervention. The information obtained would likely be specific to an individual plant.

The advent of a chromogenic peptide substrate for pyrogen testing offers both rapidity and sensitivity to the LAL test, key features if it is to be used in a processing environment. Early reports based on first generation LAL tests required lengthy incubation times and the presence of between 10^5 and 10^6 cfu of Gram-negative bacteria per millilitre of sample for the threshold of sensitivity to be reached (Jay 1977). Differing from the current data set, Misawa *et al.* (1995) reported using the non-chromogenic LAL test for testing pork carcasses, and found a high correlation between the coliform count and the LAL index (non \log_{10} transformed). Associations with two other indicators, total viable count and the *Staphylococcus aureus* populations per 100 cm², were low. They also indicated that a threshold of approximately 10^3 cfu of *E. coli* from a pure culture was the minimum for a positive LAL gelation reaction. However, as stated by the authors, the LAL test was used not for strict quantification, but as previously shown for the rapid microbial test (Siragusa *et al.* 1995), it was used for monitoring beef, pork and poultry carcasses (Bautista *et al.* 1995; Siragusa *et al.* 1995; Siragusa *et al.* 1996) as a rapid estimator of hygiene, a use which the data support.

Other workers have used the gelation-based LAL test for milk monitoring (Hartman *et al.* 1976; Svensson and Hahn-Hagerdal 1987; May *et al.* 1989). In the case of milk, the threshold of a positive reaction in the gelation test was 10^3 – 10^4 total viable bacteria per millilitre of milk sample. Hartman *et al.* (1976) utilized the gelation-based LAL test to discriminate mastitic milk due to Gram-negative mastitis from mastitic milk from predominantly Gram-positive mastitis by testing serially diluted samples of milk with the LAL gelation test and assigning a cut-off dilution as indicative of the condition. Similarly, the assay presented in the current work is based on dilutions; therefore, it is semi-quantitative for diagnosing process deviations. LAL-based microbial spoilage monitoring in marine fin fish was reported (Sullivan *et al.* 1983) and found to be highly correlated with the levels of total volatile bases and total viable bacterial counts. These same authors used 10-fold dilution series followed by doubling dilutions of the highest 10-fold diluted sample to increase accuracy of the LAL assay for determining fish quality.

Our approach of defining the LAL value based on a dilution titre precluded actually determining the quantity of endotoxin in a sample. Knowing the actual endotoxin level has little bearing on using the chromogenic LAL test as a rapid gauge of carcass microbial hygiene status. Future development or use of substrates for the LAL reaction that are fluorogenic, rather than chromogenic, might offer

greater sensitivity and concomitant linearity at lower contamination levels. However, at present, using commercially available chromogenic LAL assays offers a sound means for animal processors to test the efficacy of their process lines for producing hygienic carcasses and find gross process deviations in a time frame rapid enough to rectify the source of that contamination.

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