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MICROBIAL LOAD MONITORING OF MEAT ANIMAL AND POULTRY CARCASSES USING A RAPID FILTRATION MICROBIAL ATP BIOLUMINESCENCE ASSAY

GR Siragusa, CN Cutter, WJ Dorsa, M Koohmaraie
United States Department of Agriculture, Agricultural Research Service, US Meat Animal Research Center, Clay Center, NE 68933 USA

Introduction

Microbial ATP testing of meat animal carcasses provides a means of rapidly assessing the microbial load of the carcass in order for proactive measures to be taken for improving the animal conversion process. The benefits of such monitoring are obvious; the presence of high microbial loads on carcasses is indicative of potential fecal contamination, and any deviations in the animal conversion process resulting in an increase in the microbial ATP levels is a signal that a process breakdown has occurred. It was previously demonstrated that microbial ATP bioluminescence could accurately gauge the levels of microbial contamination on beef carcass surface tissue and that the general method would indicate efficacy of antimicrobial interventions such as spray washing (1). This research used a method that required approximately one hour to complete.

The next challenge was to apply the same general assay principles of firefly luciferin/luciferase based ATP determination to a format that allowed much greater speed. The assay presented in this paper was developed and field tested for use with beef, swine and poultry carcasses (2). It has also been employed for monitoring in a lamb processing plant. Other workers have presented similar versions of microbial ATP (mATP) assays for carcass monitoring (3, 4, 5). The major challenge in using mATP as a means of determining total microbial populations in food samples is the separation of non-microbial ATP from microbial ATP. The basis of the described rapid microbial ATP (R-mATP) assay is the use of a filtration device in which somatic ATP is extracted and then removed within the same device; extraction of bacterial ATP is followed by its quantification.

Materials and Methods

Rapid Microbial ATP Assay (R-mATP): The steps of the R-mATP assay are as follows. 50 µL of the carcass sponge sample was added to a Filtravette™ (New Horizons Diagnostics, Columbia, MD, USA) followed by 100 µL of a somatic ATP extraction reagent, NRS™ (Nucleotide Releasing agent for Somatic cells; Lumac BV, Perstorp Analytical, Netherlands). The fluid was then aspirated through the Filtravette™ using a vacuum manifold (New Horizons Diagnostics, Columbia, MD, USA) and trap. Another 150 µL of NRS™ were added and pulled through the device. At this stage, the Filtravette™ retained bacteria and other cellular debris on the filter's surface, somatic cell ATP and free ATP having been removed by the action of the somatic cell extractor, NRS™. The Filtravette™ ,with its extracted contents, was placed in the holding drawer of the Profile™-1 Model 3550 Microluminometer (New Horizons Diagnostics, Columbia, MD, USA). Thirty µL of the microbial ATP extractant, NRB™ (Nucleotide Releasing agent for Bacteria; Lumac BV, Perstorp Analytical, Netherlands) were added, followed immediately by 30 µL of luciferin/luciferase reagent (New Horizons Diagnostics, Columbia, MD, USA) reconstituted to the manufacturer's recommended volume with Lumit buffer (Lumac BV, Perstorp Analytical, Netherlands). The fluid was mixed by rapidly aspirating three times with the micropipettor; the drawer to the luminometer was closed and light emission was integrated over 10 s. The microbial ATP level was recorded as relative light units (RLU) taken directly from the luminometer's digital readout. Microbial ATP content is reported as the $\log_{10}$ RLU normalized to 1 mL sample volumes (log RLU mL^{-1}). Each assay was performed in duplicate and the average RLU value

calculated. All reagents were used at room temperature and were checked for contaminating ATP before use. The time required to perform the steps of the R-mATP after sampling is approximately 90 s.

Beef and Pork In-Plant Carcass Samples: Beef carcass samples were obtained from three different commercial processing plants including graded beef (from heifers and steers) and ground beef (from cows and bulls). Samples were taken both immediately before and after the final spray wash, but before chilling. Randomly chosen carcass sites were sampled mainly from the brisket area. Sampling was done by one of two means. First, a 500 cm^2 sampling area was delineated on the brisket area with a rectangular stainless steel template. Alternatively, a sampling area was drawn with edible ink and the dimensions measured with a ruler. Samples from the marked areas were taken using an ATP-free, sterile, microbiological sampling sponge (NASCO, Fort Atkinson, WI, USA) packaged in a Whirlpak™ bag. The sponge was moistened with 25 mL of a sterile sponge solution composed of 0.085% (w/v) NaCl + 0.05% (v/v) Tween 20 adjusted to pH 7.8. The solution was expressed from the sponge as it was removed from the Whirlpak™ bag using a sterile glove. The sponge was wiped firmly over the sample area 10 times in both the vertical and horizontal direction. The sponge was then placed into the bag containing the residual sponge solution, held in an ice chest at approximately 8 - 10°C. Analyses were performed within about 2-3 h of taking the sample.

Pork carcass samples were obtained from three large swine processors. The method of sampling for pork carcasses was the same as for beef except that a 50 cm^2 template was used. Pork carcass samples were from skinned or scalded carcasses.

To determine the levels of microbial contamination on beef carcasses immediately after hide removal, a set of in-plant samples were taken from the two different plants within 45 s of the hide being removed and assayed for aerobic plate counts. Microbial contamination levels were determined from samples from two different plants taken randomly from carcasses with (n=237) and without (n=163) signs of visible faecal contamination, both before and after the final carcass wash. Upon returning to the laboratory (usually within 3 h), the sponge samples and their contents were aseptically transferred to a filtered Stomacher bag (Spiral Biotech, Bethesda, MD, USA) and stomached for 2 min in a Stomacher Model 2000 (Tekmar, Inc., Cincinnati, OH, USA). The filtered stomachate was transferred to a sterile tube and held at room temperature until analyzed. Repeatability of the in-plant sample tests was determined as the correlation coefficient of the regression line between the duplicate determinations.

Poultry Sampling: A total of 320 poultry broiler carcasses were obtained from three different plants. Eighty poultry broiler carcasses were selected from each of the following same four sites or critical control points within each plant; post defeathering, post evisceration, post wash, and post chill. Carcasses were chosen from the processing line to represent a variety of levels of contamination ranging from those that were visibly contaminated with feces to no visible faecal contamination. In the plant, the entire exterior surface of each bird was sponged using a Speci-Sponge (NASCO, Fort Atkinson, WI, USA) moistened in 25 mL of buffered peptone water (Difco, Detroit, MI, USA) containing 0.5% (v/v) Tween 20 and 0.5% (w/v) glucose. The solution was expressed from the sponge as it was removed from the bag using a sterile glove. The sponge was wiped firmly over the entire outside surface of the bird carcass, turning the sponge at least two times. The sponge was then placed into the bag containing the residual sponge moistening solution, held at between 8° to 10°C and analyzed within 2 h of sampling. Sponges and all fluid contents were transferred to a filtered stomacher bag (Spiral Biotech, Bethesda, MD, USA) and stomached in a LabBlender 400 stomacher (Tekmar, Cincinnati, OH, USA) for 2 min. The sample was withdrawn from the filtered side, transferred to a conical centrifuge tube and analyzed.

Bacterial Plate Counts: Samples were serially diluted in buffered peptone water (BBL, Cockeysville, MD, USA). For aerobic colony counts, samples were either spiral-plated (Model D Spiral Plater; Spiral Systems Instruments, Bethesda, MD, USA) or pour-plated with tryptic soy agar (BBL, Becton and Dickinson, Cockeysville, MD, USA) and incubated for 36 h at 35°C. Bacterial counts were converted from colony forming units (CFU) per mL to $\log_{10}$ CFU mL^{-1}.

Statistical Analysis: Linear regression analysis was performed on scatterplots of the mATP values (RLU) versus the viable microbial counts using SAS (Cary, NC, USA) and InStat2 version 2.0 statistical analysis package (GraphPad Software, San Diego, CA, USA). Likelihood ratios were calculated according to Sackett *et al.* (6). Cut-off values and accuracy and repeatability determinations were calculated as previously described (1).

Results and Discussion

Laboratory Studies: Initial laboratory studies on diluted bovine and porcine faecal samples indicated that the use of microbial ATP analysis for gauging levels of carcass contamination was as repeatable and nearly as accurate as standard 36 h plate count methods (Table 1).

Table 1. Repeatability and accuracy estimates from laboratory studies evaluating the R-mATP assay on in-vitro faecal dilutions.

Method	Species	Accuracy	Repeatability
R-mATP[1]	Bovine	-0.88	0.99
Aerobic Plate Count[2]	Bovine	-1.01	0.96
Total Plate Count[3]	Bovine	-1.17	0.96
R-mATP[1]	Porcine	-0.84	0.93
Aerobic Plate Count[2]	Porcine	-1.22	0.96
Total Plate Count[3]	Porcine	-1.10	0.98

[1] $\log_{10}$ RLU mL^{-1}; [2] $\log_{10}$ CFU mL^{-1}; [3]aerobic plus anaerobic

Using the newly developed R-mATP assay, the correlation between cell numbers and the ATP content of various pure cultures (Table 2) was shown to be very satisfactory.

Table 2. Correlation between the R-mATP assay and plate counts of selected bacterial species.

Species	Correlation (r)
Listeria innocua	0.97
Pseudomonas fluorescens	0.98
Lactobacillus plantarum	0.91
Enterococcus faecalis	0.90
Escherichia coli	0.99

Field Trials: Scatterplots of data collected from beef and pork plants indicate a high level of agreement between the R-mATP method and the standard 36 h aerobic plate count (Figures 1 and 2). No significant differences were detected between regression lines obtained from different plants (data not shown).

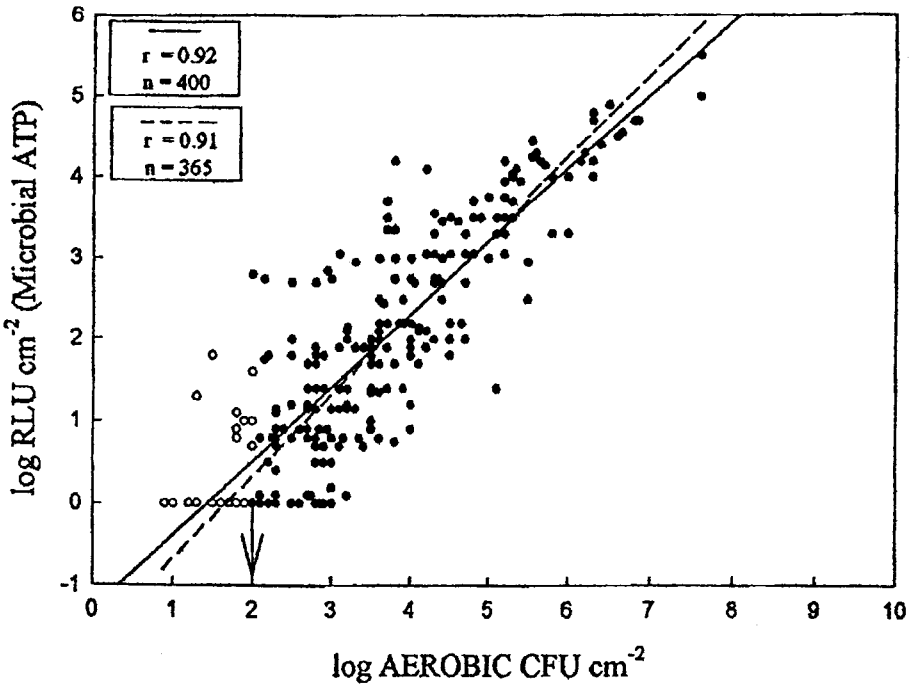

Figure 1. Scatterplot of R-mATP values and aerobic plate counts from beef carcass in-plant sponge samples. The solid line is the regression including all data points. The dashed regression line is calculated from data points above the lower limit of assay sensitivity ($\log_{10}$ 2.0 aerobic cfu cm^{-2}, indicated by the arrow). Data points below the threshold are open symbols and those above the threshold are solid symbols.

Analysis of poultry carcass sponge samples indicated a reasonable agreement between the standard 36 h plate count method and the 5 min R-mATP test (Figure 3). As in the case of beef and pork samples the between plant regression lines were remarkably similar (data not shown).

Likelihood ratio based odds of a sample having a plate count of a certain minimum level as indicated by the R-mATP test result are given in Table 3. These odds are calculated from the same dataset used to plot the scattergraph (Figure 3).

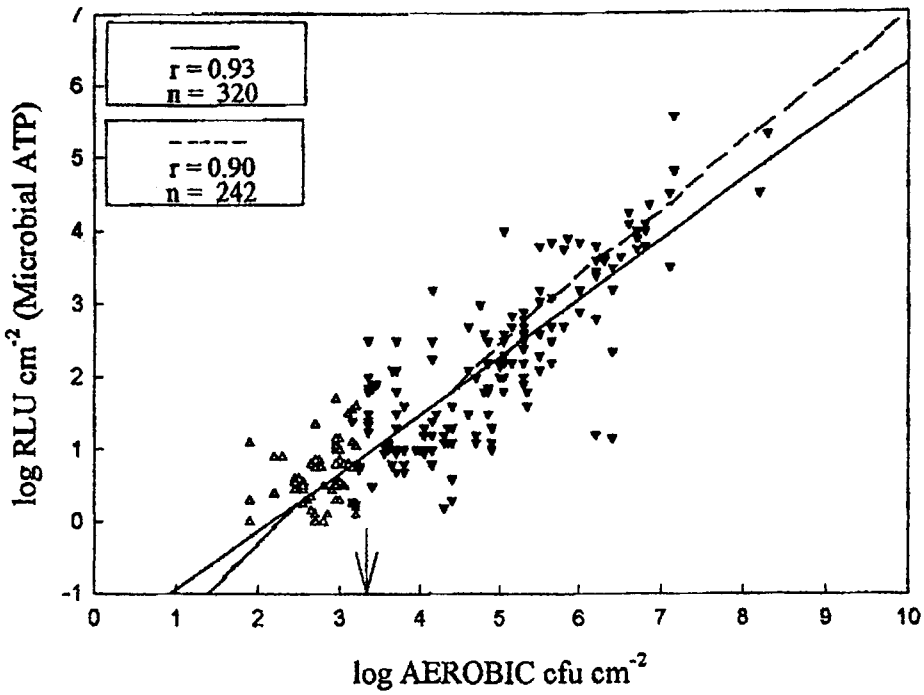

Figure 2. Scatterplot of R-mATP values and aerobic plate counts from pork carcass in-plant sponge samples. The solid line is the regression including all data points. The dashed regression line is calculated from data points above the lower limit of assay sensitivity ($\log_{10}$ 3.2 aerobic cfu cm^{-2}, indicated by the arrow). Data points below the threshold are open symbols and those above the threshold are solid symbols.

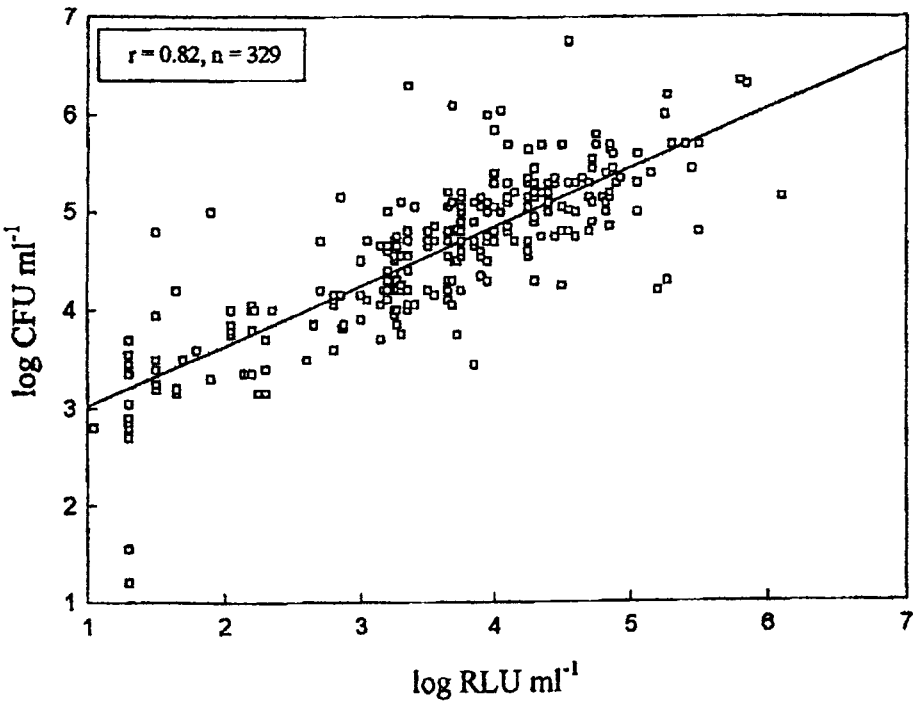

Figure 3. Scatterplot of R-mATP assay values (log RLU/mL) and 35°C aerobic colony counts (log CFU mL^{-1}) from poultry carcass in-plant sponge samples.

Table 3: Post-test probabilities (calculated from likelihood ratios) of the R-mATP test result predicting aerobic microbial population of poultry sponge samples.

R-mATP result[1]	Aerobic count[2]	Post-test probability
≥ 2.5	≥ 3.5	98%
≥ 3.5	≥ 4.0	97%
≥ 4.0	≥ 4.5	92%
≥ 5.0	≥ 5.0	75%

[1] log RLU mL^{-1}; [2] log CFU mL^{-1}

The R-mATP test is a filtration based method which utilizes differential extraction and physical removal of non-microbial ATP in a sample to segregate the bacterial or microbial ATP content. This method requires about 5 min to complete, including sampling. The actual analytical time requires less than 90 s. Many factors are known to affect the results of microbial ATP tests that are based on firefly luciferin/luciferase systems, including cell physiology status, environment, analytical considerations, extraction efficiency, presence of inhibitors and others (7). Despite these adverse factors, the R-mATP demonstrated a high degree of reliance in estimating microbial load and in being highly repeatable. The effects of environmental stress on cell ATP content and variability was likely a significant factor in analyzing poultry broiler samples. Poultry carcasses are scalded, flamed, chlorinated and chilled at various points in the process. This is not the case for beef and pork carcasses. The general agreement between the standard plate count and the R-mATP test result was more favorable for beef and pork samples than poultry (3, 4, 8). The utility of this method lies not necessarily in determining the actual aerobic plate count, since other non-aerobic microorganisms could contribute ATP to the sample, but in quickly assessing the relative microbial load of a carcass; hence the likelihood ratio is a useful tool in using the R-mATP test data as the basis for decision making.

Using a simple filtration device offers the added advantage of removing soluble inhibitors that might be present in the sample. It has been proposed that this test format be adapted for hygiene monitoring, water testing and for other foods. Certainly its versatility is only limited by sample filterability. The sample preparation described above utilizes a filtered stomacher bag to accomplish a coarse glass pre-filtration of the material prior to being put in the Filtravette™. It has been documented that pre-filtration of the material is an important step for ATP bioluminescence assays that incorporate filtration steps (3).

Finally, this method offers the rapidity needed to assess critical control points in regards to microbial levels on carcasses. Most previously described methods for microbial ATP testing rely on steps to differentially extract non-microbial ATP and its subsequent enzymatic hydrolysis. While this method is appropriate from some sample types such as those with greatly more somatic ATP than microbial ATP, it still requires considerably longer than 5 min to complete. Sponge samples decrease the loads of somatic ATP versus the somatic ATP content of excised or ground meat samples. Sponge sampling has been shown to be an adequate means of sampling carcass surfaces (9). HACCP monitoring dictates that feedback and assessment be timely and allow proactive measures to be taken when process deviations are found (10). The R-mATP test requires about 5 min to complete, including time to sample the carcass. This time decreases when multiple samples are analyzed in batch. Therefore this test, while not real-time, provides information quick enough for food processors to detect deviations and take corrective action.

Future research will focus on low level or low microbial density detection and the modification of the R-mATP assay for refrigerated samples.

† Mention of a trade name, proprietary product or specific equipment is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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