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MEASURING EFFECTIVENESS OF TEAT PREPARATION

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Introduction

A proper udder health program is critical to achieving the goals of producing quality milk and a low incidence of clinical mastitis. A key factor in achieving these two goals is having clean teats prior to the attachment of the milking unit. Clean teats are a product of two variables: the degree of cleanliness of the teat prior to udder prep and the effectiveness of the prepping procedure to further clean and sanitize the teat skin at milking time. Successful udder health programs have consistent control over these two variables. Current methods to assess the effectiveness of udder cleanliness and teat preparation practices are subjective and prone to wide variations of interpretation between evaluators (Leslie et al., 2001). Dairy managers and consultants need rapid, cow-side tools to allow more objective evaluation of teat cleanliness prior to machine attachment.

The use of ATP bioluminescence to evaluate teat cleanliness has been investigated (Finger and Sischo, 2001; Leslie et al., 2002). ATP bioluminescence was originally developed by NASA to assist in determining the presence of extra-terrestrial life. Recently, ATP bioluminescence has been widely adopted by the food industry to monitor food processing plant hygiene. The method is based on the fact that all living cells contain adenosine triphosphate (ATP), the universal energy donor for metabolism (Griffiths, 1996). ATP is responsible for the production of light in lightning bugs. Within the abdomen of lightning bugs, an enzyme known as luciferase reacts with a substrate known as luciferin. The luciferase-luciferin complex converts the chemical energy associated with ATP into light (Griffiths, 1996). This simple biochemistry was harnessed into swab-based systems designed to detect light originating from ATP using a hand-held luminometer. Light is read in relative light units (RLU). In the food industry, obtaining a reading of 0 RLUs on a properly cleaned stainless steel surface is an achievable objective. A soiled surface, however, harbors ATP loads that may result in RLU levels in to the hundreds of thousands.

In their investigation, Finger and Sischo (2001) concluded that bioluminescence, compared to bacterial culture, was a conservative test in that it rarely classified cows that had not been cleaned as "clean". They also concluded that bioluminescence was able to demonstrate average changes in teat cleanliness as animals progressed through pre-milking udder preparation. It was the authors' conclusion that ATP bioluminescence could be a tool to demonstrate effectiveness of the pre-milking preparation routine. Their findings were supported by Leslie et al. (2002) who suggested that ATP bioluminescence has promise as an objective measure of udder preparation efficiency.

The objective of this research was to further investigate the use of ATP bioluminescence to assess teat cleanliness through a series of laboratory studies and field evaluations. Specifically, we sought to determine the influence of teat dip, milk, and manure on bioluminescence on porcine skin and *in vitro*; and to evaluate its application in assessing cleanliness of teat sides and ends prior to and following udder preparation.

Methods: Laboratory Evaluations

Evaluation of ATP bioluminescence to evaluate cleanliness of porcine skin: Sections of sterile porcine skin (Brennan Medical, St. Paul, Minn.) were aseptically placed on stainless steel mounting assemblies fitted with three apertures (2.3 cm, diameter) which served to represent teat ends. Fresh bovine feces was collected and diluted in sterile distilled water to ratios of 1:2 and 1:20 (feces:water). Porcine skin was inoculated with 200 µl of one of the fecal slurry dilutions. Slurries were uniformly spread over the skin within each aperture and incubated for 30 min at room temperature.

Porcine skin specimens were prepared to represent the typical microbiological and organic loads present at three stages of the udder preparatory process: pre-preparation, post-wash, and post-sanitization. Porcine skin representing the teat condition pre-preparation was prepared and inoculated as described with both fecal slurry dilutions without further treatment. Porcine skin representing teat washed with water was prepared and inoculated as described. Mounting assemblies were positioned vertically and sprayed with water (40 p.s.i.) for 20 sec to wash. To represent teat condition post-sanitization, porcine skin was prepared and inoculated as described. Two hundred microliters of a 1% iodine teat dip (Legend; Ecolab, St. Paul, Minn.) was dispensed on to the skin and exposed for 20 sec at room temperature after which the teat dip was removed using a disposable laboratory wipe. In order to determine the influence of milk on ATP bioluminescence measurement, controls in which milk combined with 5% fecal slurry and milk alone were prepared. Non-inoculated, untreated porcine skin served as the negative control.

Porcine skin was swabbed to recover ATP and microorganisms. Swabbing for ATP was performed using PocketSwab® Plus swabs (Charm Sciences). Swabbing for bacteriological analysis was performed using sterile cotton swabs wetted with sterile neutralizer buffer. Immediately after swabbing, bioluminescence was measured using a *FireFly* luminometer (Charm Sciences). Data was recorded as RLU/swab. Microbiological swabs were examined for mesophilic microorganisms appropriately. The experiment was performed in triplicate.

Comparison of RLU and CFU from porcine skin inoculated with serial dilutions of fecal slurry. Bovine feces was diluted in water to concentrations of 1:2, 1:20, 1:100, 1:200, 1:500, and 1:1000. Porcine skin sections were mounted as described above and two-hundred microliters of each slurry dilution were placed on each skin sampling area of three mounting assemblies. Samples were incubated at room temperature for 30 minutes to allow for attachment. Recovery of microorganisms and ATP was performed as described.

Influence of iodine teat dip on bioluminescence from pure ATP. A solution of ATP (Sigma) was prepared. Aliquots of 0.1 ml were added to 9 ml of sterile water or 1% iodine teat dip (see figure

1). After a 15-sec exposure time, 1 ml was transferred to water or 0.1% sodium thiosulfate to neutralize iodine. PocketSwab Plus swabs were dipped into the solutions and analyzed immediately for bioluminescence measurement.

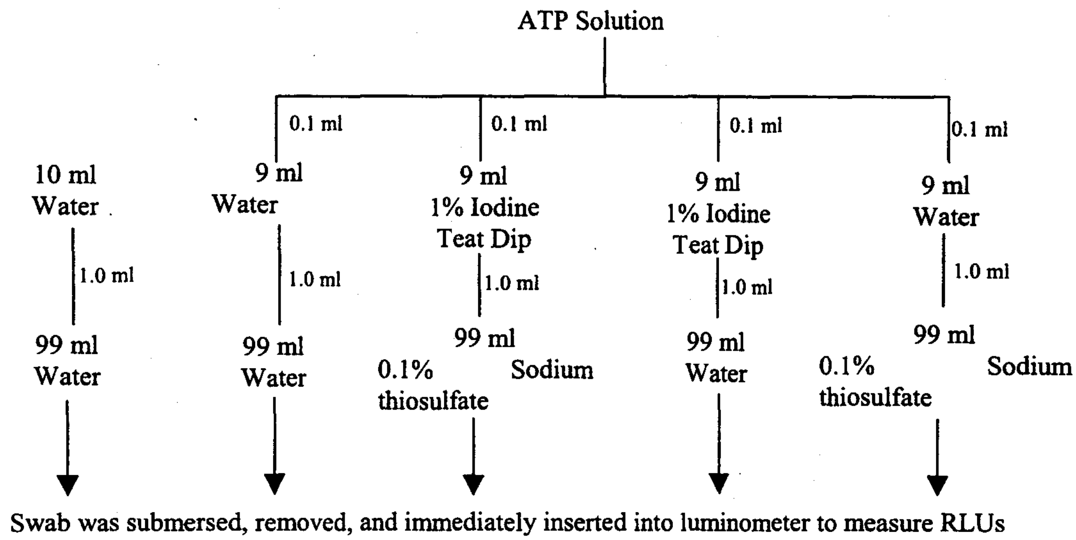

Figure 1. Schematic of procedure to determine influence of teat dip on ATP bioluminescence

Influence of iodine teat dip on bioluminescence from bacterial suspensions. Suspensions of *Escherichia coli* ATCC 11229 and *Staphylococcus aureus* ATCC 6538 were prepared. One milliliter of bacterial suspension was added to 9 ml of 1% iodine teat dip or sterile water in a sterile 50-mL centrifuge tube and the contents were mixed. Treatment solutions (Table 1) were held at room temperature. After 15 seconds of exposure, 1 ml was removed and added to 9 ml of 0.1% sodium thiosulfate or sterile water. Immediately, a PocketSwab Plus swab was dipped into the secondary treatment suspension and RLU was measured. Colony forming units were enumerated as described. The experiment was performed in duplicate.

Table 1. Treatment combinations

Treatment	Primary Treatment	Secondary Treatment
1	1% Iodine Teat Dip	0.1% Sodium thiosulfate
2	1% Iodine Teat Dip	Water
3	Water	Water
4	Water	0.1% Sodium thiosulfate

Methods: Field Evaluations

Test populations. The experiments were conducted at a commercial dairy. Teat cleanliness was evaluated on animals from three groups: pre-fresh heifers, pre-fresh cows, and post-fresh milking animals. The pre-fresh heifers were dipped three to five times weekly with a sanitizing teat dip (1% iodine). The pre-fresh cows were a test population of a separate study and had not undergone udder sanitization since dryoff (>2 weeks prior to testing). Post-fresh milking animals were prepped three times daily at each milking with a 1% iodine teat dip as both a predip and a post-milking teat dip.

Sample Collection. The right hind teat was selected for swabbing unless visible milk was present on the end of the teat, in which case the left hind teat was selected. Finger and Sischo (2001) reported no significant difference in bacterial counts from swabs of all four teats out of over 100 cows. Swab samples were taken from teat sides by passing the swab over the skin from the base of the teat to its end. Careful attention was made to consistently apply moderate pressure to the swab. Preliminary observations have shown that the higher the pressure applied to the swab, the greater the amount of ATP collected. Areas swabbed by ATP swabs did not overlap with areas swabbed with culture swabs. Teat ends were swabbed by passing the swab across the teat end over the orifice to the canal. Culture swabs were sampled prior to ATP swabs, and their paths were perpendicular to each other. Control studies conducted in our laboratory have indicated that no ATP is present in the neutralizing broth contained in the culture swabs. Figure 2 depicts the sampling plan performed in the initial experiment. A second field evaluation was conducted using only post-fresh milking animals. In this experiment, teat ends and sides of post-fresh milking animals (n=26) were swabbed prior to and following preparation using PocketSwab Plus swabs only.

Data Analysis. Bioluminescence and microbiological swabs were processed as described. Data was subjected to analysis of variance (ANOVA) and Tukey's test for comparisons of multiple means, where appropriate, using MiniTab.

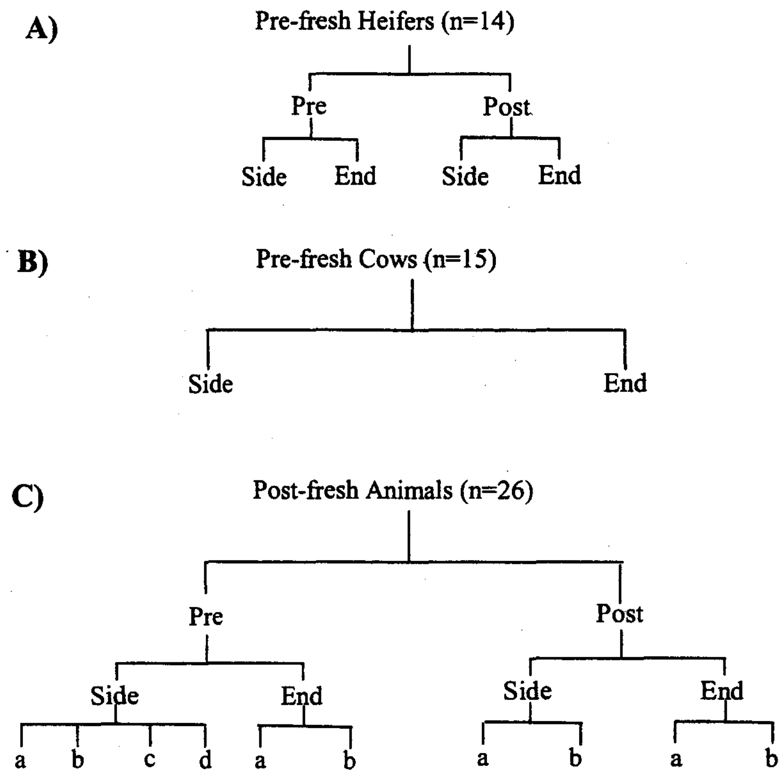

Figure 2. Sampling plan for teat swabbing. A), B): ATP and culture swabs were performed for each side and end; C) ATP swabs were performed for side a, b, c and end a, while culture swabs were performed for side d and end b.

Results and Discussion

Evaluation of ATP bioluminescence to assess cleanliness of porcine skin. A similar trend was observed in changes of RLU and CFU values on porcine skin at the three stages of preparation (Fig. 3). Discrepancies between RLU and CFU values were greatest from skin which had been treated with teat dip. This observation suggests that high levels of ATP remained on skin surfaces, while populations of viable bacteria decreased substantially. The negative control (skin only) demonstrated the presence of ATP in the absence of microorganisms on skin surfaces. Since the porcine skin used was sterilized by ionizing radiation by the manufacturer, and no viable microorganisms were recovered from its surface, it is likely that the origin of this ATP was somatic cells. Milk was demonstrated to harbor a high level of ATP in the absence of detectable microorganisms.

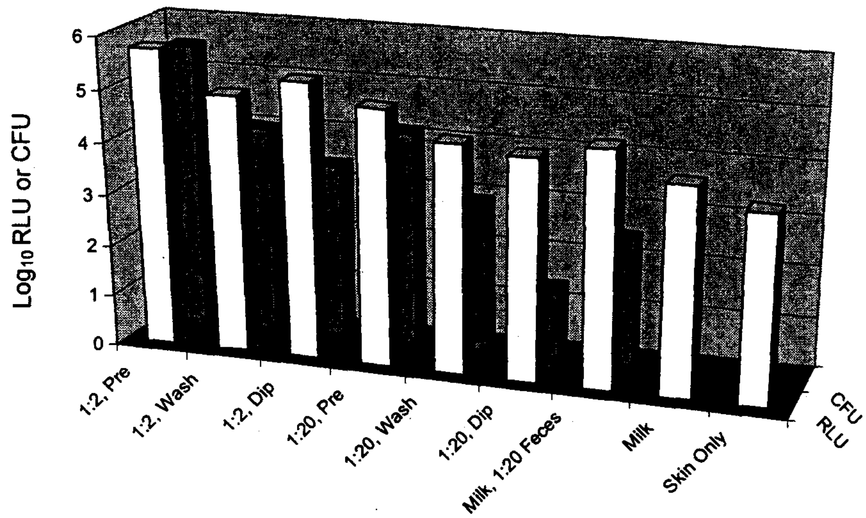

Figure 3. Relative light units ($\log_{10}$ RLU) and colony forming units ($\log_{10}$ CFU) obtained from porcine skin following inoculation with 1:2 or 1:20 bovine fecal slurry with a) no further treatment (No Treat); b) water wash only (Wash); and c) application of teat dip only (Dip).

Comparison of RLU and Colony Forming Units from porcine skin inoculated with serial dilutions of fecal slurry. Relative light units and colony forming units (CFU) obtained from swabs of skin inoculated with serial dilutions of bovine feces are represented in figure 4. Good correlation (R^2=0.8244) was detected between the two measurements although correlation broke down at CFU levels below 2 $\log_{10}$, a finding supported by Finger and Sischo (2001).

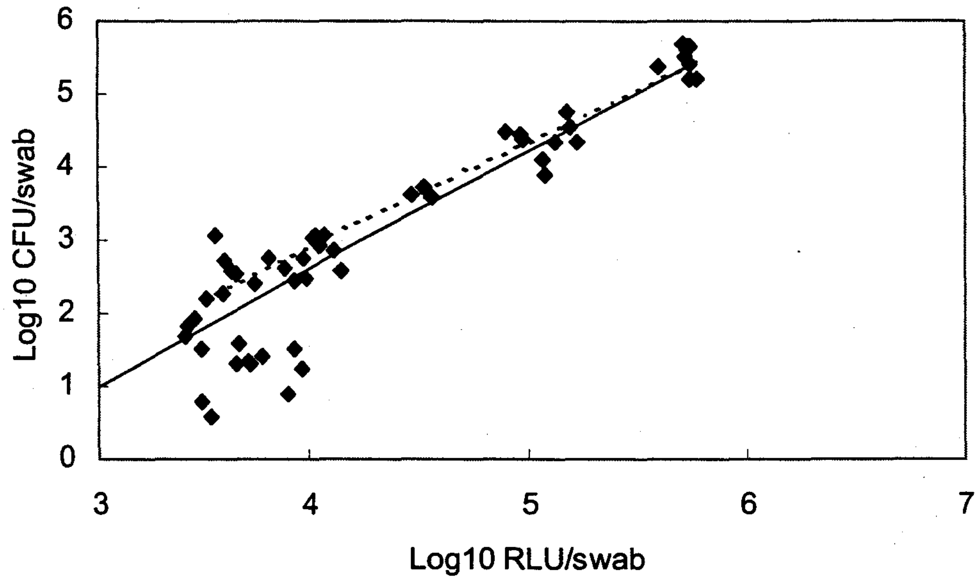

Figure 4. Scatterplot of $\log_{10}$ CFU/swab versus $\log_{10}$ RLU/swab; regression line of all points (solid line, R^2=0.8244); regression line of points at >2 $\log_{10}$ CFU/swab (dashed line, R^2=0.9417).

Influence of iodine teat dip on bioluminescence from pure ATP. Exposure of ATP to 1% iodine had no significant influence on bioluminescence (Figure 5). RLU levels were similar between the positive control and the solution of ATP exposed to iodine followed by water, suggesting that the iodine did not interfere with the enzymatic reaction responsible for the conversion of ATP to light. Other researchers have noted a quenching effect of sanitizers other than iodine on ATP bioluminescence (Velazquez and Feirtag, 1997; Lappalainen, 2000). Quenching was not observed by the 1% iodine teat dip used in these experiments.

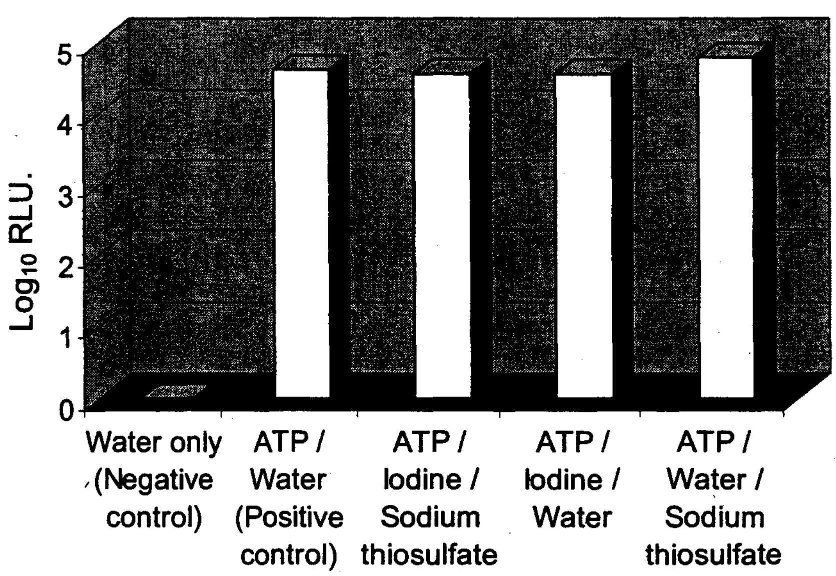

Figure 6. Influence of iodine on bioluminescence.

Influence of iodine teat dip on bioluminescence from bacterial suspension. No survivors were detected by culturing methods following treatment with 1% iodine (Figure 6). RLU levels remained constant through all treatments and did not correlate to CFU counts. Injury or lysis of bacterial cells by treatment with iodine may result in leakage of ATP to the outlying suspension. Thus, the ATP swab would collect an equivalent amount of ATP, independent of the number of viable and nonviable cells in the suspension. These data suggest a potential for false positive results. In the field, however, thorough drying of teats with a clean towel following dipping, will remove ATP sources. Drying is regarded as the most important step in both the cleaning and stimulation of teats (Johnson, 2000). Improperly cleaned and dried teat ends result in a high prevalence of environmental mastitis. Thus, failure by milkers to clean and dry teat ends may be detected by ATP bioluminescence.

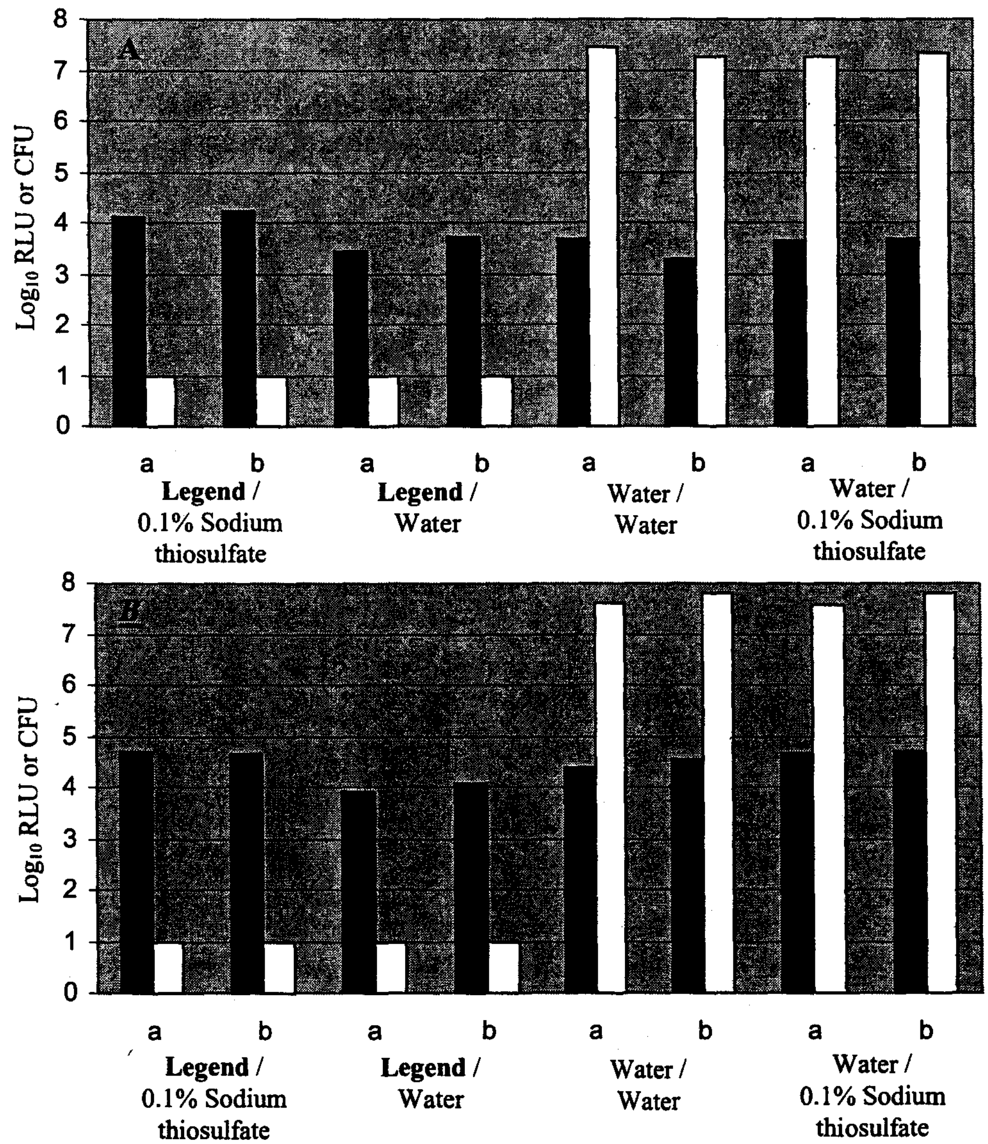

Figure 6. Comparison of RLU (solid columns) and CFU (open columns) obtained from *S. aureus* (A) and *E. coli* (B) in pure suspension.

Laboratory evaluation of the use of ATP bioluminescence demonstrated the potential opportunities and limitations in its use as an assessor of teat cleanliness. Trends in levels of RLU were similar to levels of CFU detected on porcine skin following three stages of preparation. Good correlation between CFU and RLU was demonstrated on skin contaminated with fecal dilutions, however, this correlation diminished at low levels of microorganisms and following the application of 1% iodine. Therefore, field evaluations were conducted under the following hypotheses: 1) there is little correlation between RLU and CFU on teat surfaces; and 2) significant decreases in RLU values can be obtained on teat surfaces following thorough udder preparation.

Field Evaluations. Results from the initial field evaluation are presented in figure 7 and table 2. Little correlation was detected between bioluminescence and microbial numbers obtained from teat swabs ($R^2 = 0.259$). Non-microbial sources of ATP contribute to the lack of correlation. On teat surfaces, these sources include teat skin, milk, and organic matter. ATP bioluminescence in the food industry is used to measure and monitor food processing plant hygiene prior to production start up after surfaces have been cleaned. In this application, ATP is used to measure cleanliness. It is accepted that RLU values do not reflect the microbial bioburden on swabbed surfaces. However, properly cleaned surfaces harbor less and, often times, no ATP compared with soiled surfaces. Therefore, in the food processing industry, a thorough cleaning and sanitizing program is expected to result in low ATP levels.

Changes in RLU and CFU levels on teat sides and ends as influenced by udder preparation are presented in table 2. Statistically significant reductions in RLU levels were matched with significant reductions in CFU levels, with the exception of results from teat ends on post-fresh animals. A reduction in microbiological counts was observed, while ATP bioluminescence levels were not reduced. Because non-microbial ATP and ATP from dead microorganisms on teat skin

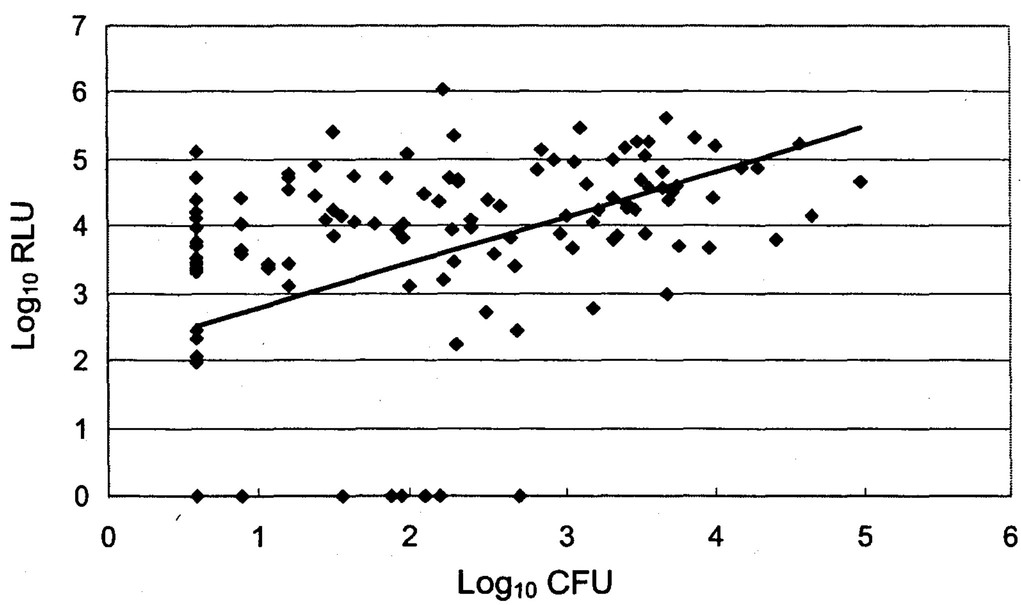

Figure 7. Scatterplot of log10 RLU vs. log10 CFU obtained from swabbing pre-fresh heifers, pre-fresh cows, and post-fresh cows. $R^2 = 0.259$.

contribute to the overall RLU reading, ATP bioluminescence is a more conservative assay of cleanliness than is conventional microbiological swabbing. That is, ATP bioluminescence will not classify a teat that has not been physically cleaned as clean (Finger and Sischo, 2001), even though traditional microbiological enumeration may result in only small populations of viable microorganisms. The failure of milkers to aggressively wipe teat ends can result in a heavy organic burden on the teat ends. However, another source of ATP is milk, which may be present on teat ends due to excellent stimulation. Since all these materials harbor high levels of ATP, one must be careful to not assume that high RLU values from teat ends are always diagnostic of poor teat preparation. On the return visit the dairy, decreases in RLU on both teat sides and ends following udder preparation were detected (Figure 8).

Table 2. ATP bioluminescence and populations of microorganisms recovered from teats of heifers and cows before and after udder preparation[1].

Cow Group	*Teat Area*	Udder Prep Stage	Log_{10} RLU	Log_{10} CFU
Pre-fresh heifers	Side	Pre	2.95 A	2.44 A
		Post	0.94 B	0.53 B
	End	Pre	2.45 A	1.85 A
		Post	2.75 A	1.74 A
Pre-fresh cows	Side	Pre	4.63	3.25
	End	*Pre*	4.48	3.38
Post-fresh animals	Side	*Pre*	3.73 A	1.91 A
		Post	2.63 B	1.23 B
	End	Pre	4.20 A	2.32 A
		Post	3.95 A	1.50 B

[1]Letters following values indicate statistical significance. Values in columns within the same cow group and teat area followed by the same letter are not significantly different ($\alpha = 0.05$).

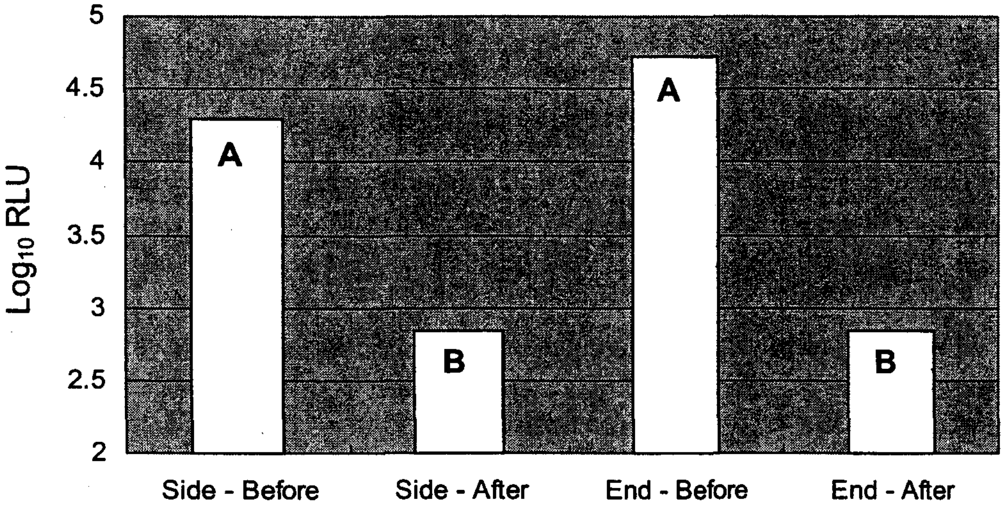

Figure 8. ATP bioluminescence on teat sides and ends before and after udder preparation.

During the initial evaluation, medial, lateral, and posterior sides of teats were swabbed to compare bioluminescence readings. No significant differences were determined ($\alpha = 0.05$; data not shown). Pre-preparation cleanliness of teat sides and ends of the three animal groups investigated ranked, from dirtiest to cleanest: pre-fresh cows, post-fresh animals, and pre-fresh heifers. The pre-fresh cows were a test population of a separate study and had not undergone udder sanitization since dryoff (>2 weeks prior to testing). Teats of pre-fresh heifers were sanitized three to five times weekly. Observations of bioluminescence were in agreement with and were able to quantify what would be considered a logical ranking of cleanliness of these three groups.

Conclusion

A commonly used method for assessing teat cleanliness following udder preparation is the cotton ball test. In this practice, a cotton ball is passed over the teat side or end before it is visually inspected. A score is subjectively assigned to the condition of the cotton ball, dependent on the amount and type of materials (e.g. manure, teat dip, etc.) that were collected. ATP bioluminescence may offer a more quantitative method of evaluating teat cleanliness than the cotton ball test. Additionally, results of bioluminescence may be used by dairy management and evaluators to reinforce proper pre-milking routine by milkers.

Results of our preliminary field evaluations indicate that ATP bioluminescence, although not accurately representing bacterial loads on teat surfaces, is able to show changes in teat side and end cleanliness following udder preparation. These changes do not necessarily mimic changes in microbiological culture swabs. Further investigations are currently being conducted to develop a cow-side protocol to consistently and objectively evaluate teat cleanliness and effectiveness of udder preparation routines using ATP bioluminescence.

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