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Improved bioluminescent assay of somatic cell counts in raw milk

Valery G Frundzhyan[1], Inna M Parkhomenko[2], Lubov Y Brovko[3] and Natalia N Ugarova[1]*

[1] Chemistry Department and [2] Biology Department, Lomonosov Moscow State University, Moscow 119991, Russia
[3] Canadian Research Institute for Food Safety, University of Guelph, Ontario N1G 2W1, Canada

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Somatic cell count (SCC) in milk is considered to be a valuable indicator of cow mastitis. For assessment of SCC in milk, the bioluminescent assay based on determination of ATP from somatic cells ($[ATP_{som}]$) in milk was proposed earlier. However, this assay is still not widely used in practice owing to lower reliability compared with conventional methods such as direct microscopy and flow cytometry. We revised the bioluminescent SCC assay and developed a simple protocol based on determination of the total non-bacterial ATP concentration in milk. It was shown that the novel ATP-releasing agent Neonol-10 (oxy-ethylated iso-nonyl phenol) has superior performance providing 100% lysis of somatic cells while not disrupting bacterial cells of milk at a concentration of 1·5% w/w. There was high correlation (R^2=0·99) between measured bioluminescence and SCC as measured by direct microscopy. The observed detection limit of the bioluminescent milk SCC assay was as low as 900 cell/ml, time of analysis was 2–3 min per sample. The proposed method has high potential for on-site mastitis diagnostics.

Keywords: Bioluminescent assay, ATP, mastitis, milk, somatic cells, Neonol-10.

Somatic cell count (SCC), together with total bacterial counts (TBC), in bulk raw milk are important product-quality indexes to characterize the sanitary conditions of the dairy. In addition SCC in milk from individual cows is a tool for mastitis diagnostics. In healthy cows SCC in milk does not exceed 10^5 cell/ml (Hillerton, 1999). The increase of SCC in milk is a reliable indicator of mastitis. The following discrimination of cow's health status by the SCC in milk (cells/ml) has been suggested: $10^5 < SCC < 2\times10^5$, mastitis suspected cow (subclinical mastitis); $SCC > 2\times10^5$, sick cow (clinical manifestation of mastitis) (Ingalls, 1998). Increased SCC above $(3{\cdot}5–7{\cdot}5)\times10^5$ cell/ml in raw combined milk results in reduced compositional quality of milk thus leading to financial penalties and increased costs of milk production.

The methods used to assess SCC in milk include direct microscopy count (Pyörälä, 2003) or indirect methods based on determination of various indicator compounds in milk, particularly DNA or ATP (Pyörälä, 2003), that are originated from somatic cells. Somatic DNA based test (California Mastitis Test, CMT) relies on estimation of the amount of DNA gel formed after disruption of the somatic cell membrane (Ruegg et al. 2002). This simple semi-quantitative test is suitable for real-time monitoring of cows' health. However, its sensitivity is not enough to reliably diagnose subclinical cases of mastitis, and interpretation of results is highly subjective. CMT is not a test for large-scale milk monitoring (Pyörälä, 2003). Moreover, CMT is affected by different hardly predictable factors (lactation period, season, quality of fodder, etc.). Another method to monitor the amount of somatic cell DNA is based on the use of fluorescent DNA-specific dyes and flowcytometry (Miller et al. 1986). Available commercial instruments allow fast and fully automatic analysis of somatic cells in milk with high throughput capabilities. Bioluminescent ATP-assay was proposed for SCC assay in milk in the early 1980s (Emanuelson et al. 1988). This method is based on determination of the ATP originated from somatic cells in milk (Richardson et al. 1980) using firefly luciferin/luciferase system (Brovko et al. 1991). Owing to its sensitivity, specificity and simplicity it has potential for on-site use for mastitis diagnostics. Several commercial kits for bioluminescent milk SCC assay are available in the market (Sigma, Charm Sciences Inc., etc). However, despite high potential this assay is still not widely used for diagnostics of mastitis and milk quality. The goal of our research was to improve bioluminescent milk SCC assay to make it more user-friendly and reliable.

*For correspondence; e-mail: unn@enz.chem.msu.ru

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Materials and Methods

Milk sampling

Over 200 raw milk samples were obtained from individual udder quarters of cows from dairy farms in the Moscow region and were tested separately within 1–3 h of collection.

Instruments and supplies

The portable luminometer (photon-counter) LUM-1 was from Lumtek LLC (Russia). Polystyrene microcuvettes were from Costar (Spain). Filtravettes™ (polystyrene microcuvette with the bottom made of membrane filter, pore size 5·0 μm) were gifts of the New Horizons Diagnostic Corp. (USA). Microcentrifuge 3750 was from Eppendorf (Germany). Filtration cardboard (4 × 10 cm) was from Germes Agro (Russia).

Reagents

The ATP-reagent based on recombinant *Luciola mingrelica* luciferase and ATP-standard, both lyophilized, were from Lumtek LLC (Russia). Hanks solution and RPMI media were purchased from PanEco (Russia). KMAFAnM-agar was from Stavropol Experimental Biotechnology Plant (Russia). Trypan blue was from Sigma (USA). Reagents for somatic cell destruction (Somatic Releasing Agent, SRA) (New Horizons Diagnostics Corp., USA), Lubrol PX and Triton X-100 (Sigma, USA), Neonol-10 (oxy-ethylated isononyl phenol) (Nizhnekamskneftekhim, Russia), Synthanol DS-10 (Reakhim, Russia) were used. The ultrapure deionized water was prepared on Milli-Q (Millipore, France). Lyophilized ATP-reagent was reconstituted with deionized water according to the manufacturer's instructions. ATP-standard was dissolved in 1 ml of Hanks solution or reagent used for lysis of somatic cells, resulting in 10^{-11} mol/ml ATP solution.

Bioluminescent assay of ATP in milk samples

Microcuvette or Filtravette™ was placed into the measuring cell of the luminometer. Fifty μl of ATP standard solution and 50 μl of ATP-reagent were mixed rapidly in the microcuvette or Filtravette™ and the bioluminescent signal for ATP-standard (I_1) was recorded. Measurement of the bioluminescent signal for the milk sample was performed as above but instead of ATP-standard solution 50 μl of the pre-treated milk sample was used. The bioluminescent signal for the milk sample (I_2) was recorded. Concentration of ATP in the sample (mol/ml) was calculated according to Equation (1):

$$[ATP,\ mol/ml] = 10^{-11} \times (I_2)/(I_1) \quad (1)$$

where 10^{-11} is the concentration of the ATP-standard, mol/ml.

Table 1. Effect of different detergents on somatic and bacterial cells in milk samples ($n=10$)

Detergent	Final concentration of detergent in milk, %	Destruction of somatic cells in milk, %	Survival of milk bacteria, %
Hanks solution (control)	0	<0·1	100
Synthanol DS-10	1·0	43	100
Lubrol PX	1·0	90	100
Triton X-100	1·0	90	76
	0·5	82	93
Neonol-10	1·5	100	99
	0·5	83	100
SRA	90 (v/v)	100	90

Treatment of milk samples for determination of extracellular ATP concentration

One hundred μl of milk and 0·9 ml of Hanks solution were mixed. The microcuvette was placed into the measuring cell of the luminometer and 50 μl of tenfold-diluted milk was used for ATP assay using the method described above. Concentration of extracellular ATP ($[ATP_{extr}]$) in milk (mol/ml) was calculated according to Equation (1) and multiplied by 10 to take into account the dilution factor.

Treatment of milk samples for determination of total non-bacterial ATP concentration ($[ATP_{\Sigma}]$) *in milk*

One hundred μl of milk and 0·9 ml of the reagent for somatic cell destruction were mixed and the solution obtained was incubated at room temperature for 1 min. The $[ATP_{\Sigma}]$ value was measured as described in the previous paragraph. Besides $[ATP_{\Sigma}]$ determination, the live somatic and bacterial cells in the solution obtained were enumerated by microscopy and Standard Plate Count, respectively.

Determination of somatic ATP concentration ($[ATP_{som}]$) *in milk*

Concentrations of total non-bacterial ATP, $[ATP_{\Sigma}]$, and extracellular ATP, $[ATP_{extr}]$, in milk were determined as described above. The concentration of somatic ATP, $[ATP_{som}]$, in milk was calculated by subtraction using Equation (2):

$$[ATP_{som}] = [ATP_{\Sigma}] - [ATP_{extr}] \quad (2)$$

Somatic cell (SCC) count by direct microscopy

Raw milk samples were diluted (20–50)-fold with Hanks solution. Twenty μl of the diluted sample were mixed with 200 μl of 0·1% (w/v) trypan blue solution in saline and the number of cells was counted in a Goryaev chamber

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Table 2. The values of SCC and ATP concentration in milk samples ($n=5$); the total non-bacterial [ATP_{Σ}], extracellular [ATP_{extr}], and somatic [ATP_{som}] fractions

	$SCC\times10^{-5}$, cell/ml†	[ATP_{Σ}], nmol/ml	[ATP_{extr}], nmol/ml	[ATP_{som}], nmol/ml	$SCC\times10^{-5}$, cell/ml, calculated by the value:	
					[ATP_{som}]‡	[ATP_{Σ}]§
1	*2*	*3*	*4*	*5*	*6*	*7*
1.	0·8	0·19±0·01	0·11±0·01	0·08±0·04	1·2	0·8
2.	1·0	0·21±0·01	0·10±0·01	0·11±0·01	1·4	0·9
3.	1·1	0·25±0·02	0·22±0·02	0·03±0·01	0·7	1·0
4.	1·3	0·25±0·04	0·21±0·02	0·04±0·01	0·8	1·0
5.	1·4	0·28±0·05	0·14±0·05	0·14±0·06	1·6	1·1
6.	1·6	0·40±0·02	0·19±0·04	0·21±0·04	2·0	1·5
7.	1·6	0·62±0·04	0·40±0·04	0·24±0·05	2·2	2·2
8.	1·9	0·40±0·02	0·13±0·03	0·27±0·04	2·3	1·5
9.	2·3	0·63±0·08	0·30±0·05	0·33±0·05	2·6	2·2
10.	2·3	0·80±0·04	0·12±0·04	0·68±0·05	3·9	2·7
11.	2·8	0·83±0·11	0·09±0·03	0·74±0·11	4·1	2·8
12.	3·7	1·20±0·10	0·10±0·04	1·10±0·10	5·2	3·8
13.	4·7	1·41±0·10	0·11±0·03	1·30±0·10	5·7	4·3
14.	5·0	1·80±0·51	0·10±0·02	1·70±0·70	6·6	5·3
15.	5·7	1·80±0·11	0·10±0·03	1·70±0·10	6·6	5·3
16.	10·0	2·80±0·30	0·20±0·05	2·60±0·30	8·4	7·8
17.	10·0	3·60±0·20	0·10±0·03	3·50±0·20	10·0	9·6
18.	11·1	3·30±0·50	0·20±0·03	3·10±0·10	9·3	8·9
19.	16·0	5·70±0·40	1·10±0·04	4·60±0·60	11·7	14·2
20.	22·0	9·01±0·21	0·60±0·02	8·41±0·21	17·1	19·8

† Measured by direct microscopy cell count. SD did not exceed 15% of the mean in all samples (not shown)
‡ Calculated by Equation (5)
§ Calculated by Equation (6)

(2 grids, 5 lines on each). Live (untinted) and dead (tinted) cells were counted separately.

Bacterial counts in milk samples

These were assessed by the Standard Plate Count technique (37 °C, 72 h) using KMAFAnM-agar (composition, g/l: agar, 15; milk hydrolysate, 25; pH 6·8–7·0).

Statistical analysis

All measurements were performed in triplicate. Mean and standard deviation (SD), both for ATP concentrations and SCC, were calculated using EXCELL software. The results were logarithmically transformed and linear regressions (a, b and R^2 quotients for Equations 3 and 4) were used to assess the accuracy and reliability of the bioluminescent milk SCC assay.

$$lg(SCC)=a+b\times lg([ATP_{som}]) \quad (3)$$

$$lg(SCC)=a+b\times lg([ATP_{\Sigma}]) \quad (4)$$

Results and Discussion

ATP in milk exists as extracellular or free ATP, [ATP_{extr}], and intracellular ATP, originated from bacteria cells and somatic cells, [ATP_{bac}] and [ATP_{som}], respectively. The total non-bacterial ATP of milk, [ATP_{Σ}], comprises [ATP_{extr}], and [ATP_{som}]. To determine intracellular ATP by the bioluminescent method, the ATP has to be released from the cells, since the cell wall (both bacterial and somatic) is impenetrable to the firefly luciferase enzyme. Commercial bioluminescent milk SCC assay kits are based on [ATP_{som}] determination. In the first step of this assay, bacterial cells and extracellular ATP have to be removed from the milk analysed by centrifugation or filtration. After that the retained somatic cells are lysed by mild detergent and the ATP from somatic cells is released into the solution. Filtration and centrifugation of milk complicates the bioluminescent SCC assay in milk and can distort the results. We have observed that filtration of raw milk diluted tenfold with Hanks solution through a membrane filter that retains somatic cells resulted in partial damage of somatic cells and loss of [ATP_{som}] with the filtrate (results not shown). Centrifugation (5000 rpm, 5 min) of whole raw milk resulted in a (2–5)-fold increase of [ATP_{extr}] whereas somatic cells did not precipitate completely. We explained this phenomenon by the stress action of centrifugation on somatic cells that resulted in their partial destruction and release of ATP into solution. Thus, separation of somatic cells from the milk matrix by physical methods probably is unsuitable for bioluminescent milk SCC assay.

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We assessed the [ATP_{bac}] and [ATP_{som}] ratio in whole raw milk. Specific content of ATP in bacterial cells is equal to $\sim 10^{-18}$ mole/cell (Brovko et al. 1999). Thus, the [ATP_{bac}] in poor sanitary quality milk (TBC $\sim 10^{6}$ bacterial cell/ml) is $\sim 10^{-12}$ mol/ml. Specific content of ATP in somatic cell is $\sim 1{\cdot}5 \times 10^{-15}$ mol/cell (Bossuit, 1978). Somatic cell titre in healthy cows' milk is $<10^{5}$ cell/ml (Hillerton, 1999). Thus, even in the poor sanitary quality milk the [ATP_{som}] exceeds $\sim$150-times the [ATP_{bac}]. In mastitic cows' milk this ratio is considerably higher. Therefore the contribution of [ATP_{bac}] to the ATP pool of milk is negligibly low and removal of bacteria cells from milk is not necessary. Nevertheless, for accurate assessment of SCC in milk it is important to use detergents that effectively destroy all somatic cells and do not affect the bacterial cells.

Selection of detergent for ATP release from somatic cells

The structure of cell wall in bacterial and somatic cells is different. To destroy somatic cells, mild non-ionic detergents are used, for example, Triton X-100 or Triton X-100-based releasing cocktails (Brovko et al. 1991). Strong acids, ionic detergents or organic solvents are applied to destroy bacterial cells. However, it was shown before that Triton X-100 does not destroy lymphocytes completely (Lundin, 1984) but partially damages some bacterial species, for example, *Pseudomonas* (Theron et al. 1986). Therefore, to select the most appropriate ATP-releasing agent for somatic cells of milk, we compared the action of several neutral detergents, including Triton X-100, both on bacteria and somatic cells of milk (Table 1). All the detergents selected destroyed somatic cells and affected slightly milk bacteria, with Triton X-100 damaging up to 24% of bacteria in milk. In contrast to Triton X-100, treatment of milk with Neonol-10 (1·5% w/w in milk) destroyed somatic cells completely and did not damage the bacterial cells in milk. Thus, it was concluded that bacterial cells did not interfere with bioluminescent assay of SCC in milk when Neonol-10 (1·5% w/w) was used as an ATP-releasing agent. Another advantage of Neonol-10 as a releasing agent was that ATP solutions were relatively stable in the presence of the detergent at a concentration of 1·5% w/w. The decrease of the total non-bacterial ATP concentration in Neonol-treated milk, [ATP_{Σ}], after 5-min and 20-min storage at room temperature was 7% and 25%, respectively and no decrease in [ATP_{Σ}] was detected after 20-min storage on ice.

Effect of milk matrix on bioluminescent ATP assay in milk

It was observed that the high content of milk fat (3·5–10·0%) activated the ATP-reagent and increased I_2 value up to $\sim$50% (data not shown). This can be explained by the fact that lipids can activate firefly luciferase (Ugarova et al. 1987). To diminish the effect of the milk matrix on the assay results, milk samples were diluted tenfold with Hanks solution containing 1·65% Neonol-10 (final concentration was 1·5% w/w). This treatment completely eliminated the effect of the milk matrix on level of bioluminescence (I_2), while it did not damage either somatic or bacteria cells (see Table 1).

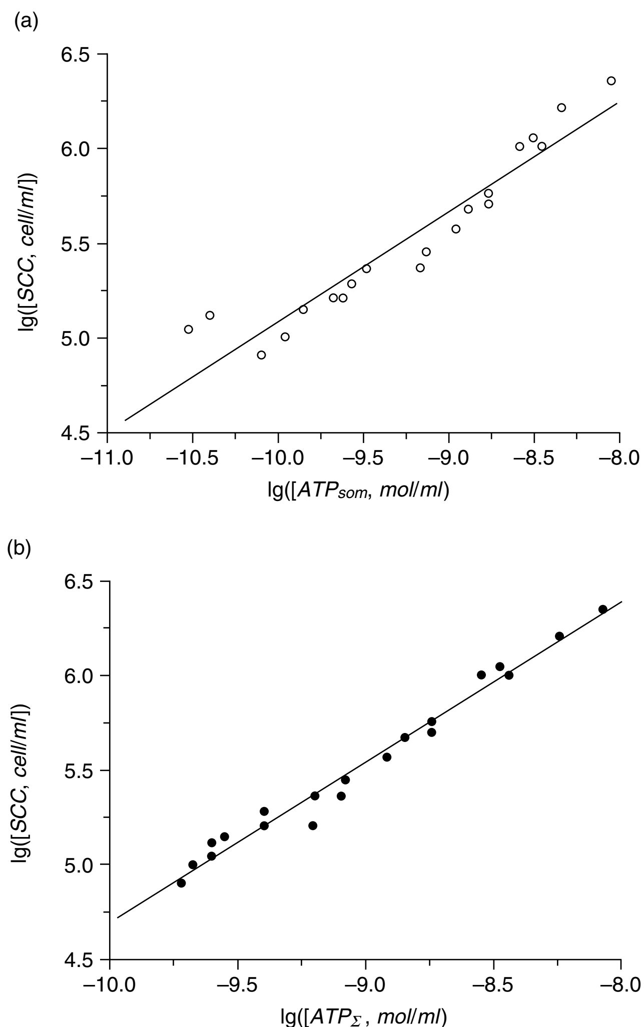

Fig. 1. Correlation between somatic cell count (SCC) and concentration of somatic ATP, [ATP_{som}] (a), and concentration of total non-bacterial ATP, [ATP_{Σ}] (b) in raw milk.

Correlation between SCC and [ATP_{som}] *or* [ATP_{Σ}] *in milk*

Twenty milk samples obtained from separate udder quarters of healthy cows (No. 1–3, Table 2), cows with subclinical mastitis (No. 4–10, Table 2) and sick cows (No. 11–20, Table 2) were analysed by measuring SCC using both direct microscopy count and bioluminescent assay of total non-bacterial ATP [ATP_{Σ}], free extracellular ATP [ATP_{extr}] and somatic ATP concentration [ATP_{som}] under optimized conditions (Table 2). The health status of cows was established prior to testing by the local veterinarian on the basis of clinical symptoms, CMT, visual and microscopic

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examination of milk. Our results showed that for all investigated samples concentration of free extracellular ATP varied in the range (0·09–1·10) nmol/ml and did not correlate with SCC as determined by direct microscopy ($R^2=0{\cdot}409$). On the contrary, concentration of total non-bacterial ATP [ATP_Σ] and somatic ATP [ATP_{som}] in milk increased simultaneously with the growth of SCC. Using the data presented in Table 2, two correlations were obtained: SCC *v.* [ATP_{som}] (Fig. 1a) and SCC *v.* [ATP_Σ] (Fig. 1b). Linear regressions are presented by Equations 5 and 6, respectively:

$$lg(SCC)=10.82+0.57\times lg([ATP_{som}]) \quad (5)$$

$$lg(SCC)=13.16+0.85\times lg([ATPS_\Sigma]) \quad (6)$$

The correlation coefficients (R^2)obtained were 0·95 and 0·99 for Equations (5) and (6), respectively. The better correlation between SCC as measured by the direct microscopy count and by bioluminescent assay ($R^2=0{\cdot}99$) was observed in the case of [ATP_Σ] assay (Table 2, columns 2 and 7). This could be explained by the fact that milk from cows with mastitis comprises both intact and damaged somatic cells that vary greatly in specific content of intracellular ATP. Therefore the somatic ATP concentration, [ATP_{som}], does not indicate the SCC of milk properly. The total non-bacterial ATP concentration, [ATP_Σ], as a sum of ATP both from damaged and intact somatic cells, as well as free ATP probably originating from the damaged cells, is proportional to SCC of milk to a greater extent. The proposed total non-bacterial ATP assay in milk is less laborious and more rapid than the analysis of somatic ATP; it does not include the step of free-ATP removal. The duration of the assay was ~2 min for each milk sample.

In conclusion, we have shown that measurement of total non-bacterial ATP concentration in milk provides a better indication of SCC than measurement of ATP originated directly from somatic cells. Bacterial cells and [ATP_{bac}] did not interfere with assessment of SSC of milk by bioluminescence. The mild detergent, Neonol-10 (final concentration in milk, 1·5% w/w), released ATP from somatic cells only and resulted in a relatively stable solution of ATP. Use of the standard ATP solution for internal calibration of bioluminescent signal resulted in greater accuracy of bioluminescent SCC assay as compared with earlier methods.

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