Evaluation of Rapid Somatic Cell Counters Under Different Analytical Conditions in Ovine Milk

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ABSTRACT

A total of 31 individual ovine milk samples, ranging between 30 and 2600×10^3 cells/mL, were divided into 8 aliquots/milk with the objective of studying the overall accuracy of 2 rapid somatic cell count (SCC) counters, one based on cytometry on disk (Fossomatic 360) and the other on flow cytometry (Fossomatic 5000), under 4 types of preservation (without preservation, bronopol, sodium azide, and potassium dichromate) and 2 analytical temperatures (40 and 60°C). All analyses were carried out in duplicate. In addition, each sample was analyzed in quadruplicate by reference microscopic method using Pyronin Y-methyl green as a stain. A second experiment using 13 samples divided into 20 aliquots/sample, enabled repeatability to be studied depending on the values obtained in the SCC and on the SCC equipment used. Comparison of the methods was based on repeatability and accuracy studies (means comparison and regression studies vs. reference method). Both counters gave adequate repeatability and accuracy values in ovine milk, though the SCC obtained by Fossomatic 5000 was closer to the reference method and was somewhat more repeatable than Fossomatic 360. In the regression study, slope and intercept values were statistically different from their theoretical values (1.00 and 0.00, respectively) in the unpreserved samples but not in the preserved ones. In all cases, correlation coefficients very close to 1.00 were obtained. The preserved milk analyzed by flow cytometry gave optimal repeatability values ($s_r = 16.3$ to 19.7 and $s_r\% = 1.9$ to 2.4), and their logSCC means (5.62 to 5.64) were not different from the reference value (5.63). Bronopol was the optimal preservative for the Fossomatic method. Analytical temperature did not contribute significantly to SCC variation, although disk cytometry gave slightly more repeatable SCC at 40° than at 60°C.

(Key words: ovine milk, somatic cell count, Fossomatic method, direct microscopic method)

Abbreviation key: AZ = sodium azide, BR = bronopol, DMSCC = direct microscopic SCC, FSCC = Fossomatic SCC, F360 = Fossomatic 360, F5000 = Fossomatic 5000, PD = potassium dichromate, WP = without preservative.

INTRODUCTION

Ovine milk is very important in Mediterranean countries, some of which have well-established milk recording schemes for the SCC of milk (Gonzalo et al., 1994; El-Saied et al., 1998). Accuracy of milk SCC is very important to dairy farmers and to the dairy industry because it is a useful predictor of IMI in dairy ewes (González-Rodríguez et al., 1995; Gonzalo et al., 2002). The Fossomatic SCC method (FSCC) is the most widely used SCC method in milk-testing laboratories, and its performance has been standardized for bovine milk (Schmidt-Madsen, 1975; Heald et al., 1977; Schmidt-Madsen, 1979; IDF, 1995). Basically, the Fossomatic is a DNA-specific counter based on the principle of optical fluorescence (IDF, 1995). The ethidium bromide dye penetrates the cell and forms a fluorescent complex with the nuclear DNA. The sample is exposed to blue light, which excites the dyed cells, making these emit red light. These red light pulses are magnified, counted by a photo multiplier detector, and multiplied by the defined working factor to compute the number of somatic cells per milliliter. In cytometry on disk, the detector counts the number of cells passing by the microscope on a rotating disk. In flow cytometry, the sample is pumped through a flow cell of very small diameter that allows only one somatic cell to pass at a time. Automation of this process means that large numbers of samples can be analyzed per hour in milk-testing laboratories. However, in ovine milk, overall accuracy of automatic SCC methods has been uniquely tested using equipment with a lower speed of sampling processing (90 samples/h) and based on disk cytometry (Gonzalo et al., 1993, 2003). These studies allowed analytical conditions of ovine milk samples for the SCC to be defined in some cases. However, we are not aware of any studies of the performance of high-speed equipment

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for processing samples, based on disk and flow cytometry. These systems were designed for use with bovine milk and are periodically checked against bovine milk standards. Their performance using milk of other species, therefore, should be studied. In this sense, rapid incubation times and speed of sample processing (e.g., defatting, deproteinizing), characteristic of these devices, could hinder the correct reading of the SCC in ovine milk, which has more total solids than bovine milk. In addition, numbers of sheep per flock in the Mediterranean area are quite considerable (e.g., approximately 400 to 500 ewes per flock in the Castilla y León region), so automatic, high-speed SCC counters are necessary for periodic health checks of all milking ewes. Moreover, standardizing fast FSICC counters for ovine milk is essential in SCC laboratories to order to guarantee accuracy and reproducibility of results.

In addition, recent studies on SCC variation showed that preservation, analytical temperature, and storage type have significant influence on the SCC in ovine milk (Gonzalo et al., 2003). Some of these factors should therefore be considered, especially those used routinely in the laboratory, when studying overall accuracy of different automatic SCC systems in ovine milk.

At present, direct microscopic SCC (**DMSCC**) is the reference method recommended by the IDF (1995) for SCC as a quality control in automated SCC counters. This well-known reference method is based on staining the milk film with methylene blue stain. Its only negative aspect is its lack of specificity between cells and cytoplasmic particles. These particles are present in ovine milk in reduced concentrations (Schalm et al., 1971; Gonzalo et al., 2003), but they might interfere with milk SCC, so comparisons using DNA-specific dyes, such as pyronin Y-methyl green, improve the performance of DMSCC in ovine milk (Gonzalo et al., 2003).

The objective of our study was to assess performance of 2 rapid FSICC counters, one based on disk cytometry and the other on flow cytometry, under different analytical preservation and temperature conditions. Repeatability and accuracy were evaluated using the DMSCC method (pyronin Y-methyl green stain) as the gold standard.

MATERIALS AND METHODS

From Spanish Churra ewes in different stages of lactation, a total of 31 individual 350-mL milk samples, ranging between 30×10^3 and 2600×10^3 cells/mL, were divided into eight 40-mL aliquots that, in turn, were divided into 4 groups according to the preservative used: 2 aliquots with bronopol (**BR**) (0.2%), 2 aliquots with potassium dichromate (**PD**) (0.1%), 2 aliquots with sodium azide (**AZ**) (0.3%), and 2 aliquots without pre-

servative (**WP**). After heating in a water bath, one aliquot from each group was analyzed at 40°C and the other at 60°C, within 5 h after collection. After the aliquots had been prepared, they were refrigerated (5°C) until analysis. Analyses were carried out using 2 different counters of high-speed sample processing: Fossomatic 360 (**F360**; 360 samples/h), based on disk cytometry, and Fossomatic 5000 (**F5000**; 500 samples/h), based on flow cytometry. In both cases, the analyses were carried out in duplicate. The study was carried out at the milk-testing laboratory of the CENSRYA (National Center for Animal Breeding and Reproduction) in León (Spain). Before and during the experiment, the counters were subjected to quality control intercomparative tests led by CECALAIT (Poligny, France). These ring tests were based on bovine milk standards of known SCC because no ovine milk standards are available in the market. In the experiment, the 2 counters were adjusted to slope (b) = 1.00 and intercept (a) = 0.

In addition, the SCC of each original milk sample was determined in quadruplicate by the direct microscopic method, according to IDF regulation 148A (IDF, 1995). Within 7 h after collection, milk samples were heated to 40°C in a water bath maintained for 15 min at that temperature before being cooled to 20°C with careful stirring. Then, 0.01 mL of milk was spread on a 1 cm² (5 × 20 mm) area of a degreased microscopic slide and was dried in a horizontal position. The slides were previously treated with poly-L-lysine to increase the adherence of the film of milk. The method used to stain the film was Pyronin Y-methyl green, according to the methodology of Gonzalo et al. (2003). The working factor was 2200 in all cases.

This design enabled the study of repeatability and accuracy of the analytical conditions most frequently used in milk analysis laboratories. A total of 1116 analytical determinations were carried out, 992 by FSICC (360 and 5000) and 124 by DMSCC.

A second experiment was carried out to study repeatability of the FSICC counters only. Twenty subsamples per milk sample from 13 BR preserved ovine milk samples with varied numbers of cells (from 30×10^3 to 2100×10^3 cells/mL) were analyzed at 40°C by F360 and F5000, within 5 h after collection. The coefficients of variation for the results obtained from each sample were calculated for each SCC method.

Statistical Analyses

The DMSCC method and 16 FSICC analytical conditions were compared. These 16 conditions corresponded to 2 FSICC methods (F360 and F5000), 4 preservation types (WP, BR, PD, and AZ), and 2 analytical tempera-

tures (40 and 60°C). Representations of all 17 analytical conditions were: DMSCC for reference method; F360-WP-40, F360-BR-40, F360-PD-40, F360-AZ-40, F360-WP-60, F360-BR-60, F360-PD-60, and F360-AZ-60 for disk cytometry; and F5000-WP-40, F5000-BR-40, F5000-PD-40, F5000-AZ-40, F5000-WP-60, F5000-BR-60, F5000-PD-60, and F5000-AZ-60 for flow cytometry.

These analytical conditions were compared according to 3 types of statistical studies: means comparison, standard deviations of repeatability, and regression.

Means comparison was carried out by using the general linear model (GLM) procedure of SAS (1992). In the statistical model used, ewe effect was considered random and the analytical condition effect fixed:

$$Y_{ijk} = \mu + A_i + E_j + e_{ijk}$$

where Y_{ijk} = dependent variable logSCC, μ = mean, A_i = effect of 17 previously defined analytical conditions, E_j = effect of ewe ($n = 31$), and e_{ijk} = the residual effect in which 2 replicates were considered because analytical determinations were performed in duplicate.

Standard deviation of repeatability (s_r and its relative value: $s_r \times 100/\bar{x}$, where $\bar{x}$ is the arithmetic mean of the SCC) for each analytical condition was calculated according to international IDF standard 128A (IDF, 1999), using the formula:

$$s_r = \left(\frac{1}{2q} \sum_{i=1}^q w_i^2 \right)^{1/2}$$

where q = number of samples and w_i = absolute difference between duplicate results of FSCC methods. In the case of DMSCC, where the SCC was determined in quadruplicate, the standard deviation and coefficient of variation were calculated for each of the 31 individual milk samples. The mean values of the standard deviations of these samples were also calculated.

In the second experiment, repeatability was evaluated by standard deviation. Coefficients of variation for 20 aliquots/sample were calculated for 13 samples ranging between 30 and 2100×10^3 cells/mL.

Linear regression studies were performed to establish the relationship between DMSCC, used as the reference method, and each of the 16 analytical conditions considered for FSCC. As the SCC analytical conditions were carried out in duplicate for FSCC and in quadruplicate for DMSCC, the arithmetic mean of the 2 or 4 replicates was determined beforehand. Estimates were carried out from 31 observations (pairs of data) for each regression straight line. The corresponding intercept (a), coefficient of regression (b), and coefficient of determination (R^2) were estimated in all cases. Analyses of

differences between reference and instrumental results were performed by the t -test values observed for hypotheses $b = 1.00$ and $a = 0.00$ by using procedure REG in SAS (SAS, 1992).

Finally, a $2 \times 4 \times 2$ factorial experiment consisting of 16 treatment combinations was analyzed using data from 31 ewes. The analytical determinations were carried out in duplicate (replicates). The model (GLM procedure in SAS), where ewe was random and other factors were fixed, consisted of ewe (E_i), FSCC method (M_j), preservation (P_k), analytical temperature (T_l), and interactions: logSCC was $Y_{ijklm} = \mu + E_i + M_j + P_k + T_l + MP_{jk} + MT_{jl} + PT_{kl} + e_{ijklm}$.

RESULTS AND DISCUSSION

Table 1 shows the comparison of logSCC mean values obtained for DMSCC and 16 analytical conditions assayed by FSCC. LogSCC varied ($P < 0.05$) between 5.67 and 5.57, whereas the reference value was 5.63 (DMSCC). Flow cytometry (F5000) produced greater ($P < 0.05$) logSCC (5.62 to 5.67) than disk cytometry (F360: 5.57 to 5.61). The preserved milk analyzed by flow cytometry and the WP milk analyzed by disk cytometry produced SCC closest to reference values.

Regarding repeated SCC, Table 1 shows the standard deviation of repeatability for the SCC in the aliquots studied. The s_r (44.21) and $s_r\%$ (5.22) values for DMSCC aliquots were consistently greater than those assessed by FSCC, in agreement with the higher repeatability of the electronic methods vs. DSMCC demonstrated in bovine milk (Schmidt-Madsen, 1975). For FSCC, flow cytometry produced less variability among the replicates (s_r : 16.27 to 21.07 and $s_r\%$: 1.89 to 2.55) than disk cytometry (s_r : 17.81 to 35.28 and $s_r\%$: 2.31 to 4.56). Except in PD preserved milk, disk cytometry produced greater s_r values at 60°C ($s_r\%$: 3.25 to 4.56%) than at 40°C ($s_r\%$: 2.33 to 3.31%). These differences were less noticeable in the case of flow cytometry (Table 1). Milk preserved with BR and analyzed at 40°C by flow cytometry produced the lowest s_r values (16.27 and 1.89%), and its SCC was therefore the most repeatable. This analytical condition also was most accurate because its mean value (logSCC: 5.64) did not differ from the reference microscopic value (logSCC: 5.63).

A second experiment (Table 2) was carried out on BR preserved subsamples analyzed at 40°C, corresponding to 13 samples ranging between 30 and 2100×10^3 cells/mL. The study clearly demonstrated that coefficients of variation decreased as number of cells increased, in most cases to below 5%, according to the IDF standard (1995). Also, lower coefficients of variation (1.09 to 10.9%) were observed for F5000 than for F360 (1.64 to

Table 1. Least square means of logSCC obtained by direct microscopic (DMSCC) and Fossomatic (F) methods, geometric and arithmetic means of the SCC, and standard deviation of repeatability (s_r and $s_r\%$) for each of 16 analytical conditions.

Analytical condition ¹	LSM ² (logSCC)	SCC ($\times 10^3$ /mL) geometric mean	SCC ($\times 10^3$ /mL) arithmetic mean	s_r	$s_r\%$ ³
F5000-WP-60	5.667 ^a	465	826	21.07	2.55
F5000-WP-40	5.660 ^a	457	820	17.09	2.08
F5000-BR-40	5.638 ^b	435	859	16.27	1.89
F5000-BR-60	5.637 ^b	434	842	18.40	2.19
F5000-PD-60	5.631 ^{bc}	427	823	18.40	2.24
DMSCC	5.630 ^{bc}	426	846	44.21 ⁴	5.22
F5000-PD-40	5.629 ^{bd}	426	819	19.69	2.40
F5000-AZ-60	5.629 ^{bd}	425	821	16.91	2.06
F5000-AZ-40	5.624 ^{be}	420	821	16.59	2.02
F360-WP-40	5.615 ^{cde}	412	760	25.15	3.31
F360-WP-60	5.612 ^{def}	409	774	35.28	4.56
F360-BR-40	5.608 ^{efg}	406	797	19.46	2.44
F360-PD-40	5.595 ^{fgh}	394	779	19.33	2.48
F360-BR-60	5.592 ^{ghi}	391	785	25.54	3.25
F360-AZ-40	5.589 ^{hi}	388	764	17.81	2.33
F360-PD-60	5.587 ^{hi}	386	778	17.98	2.31
F360-AZ-60	5.573 ⁱ	374	768	27.27	3.55

^{a,b,c,d,e,f,g,h,i}Means within the same column bearing different superscript letters differ ($P < 0.05$).

¹F5000 = flow cytometry, F360 = cytometry on disk, WP = without preservation, BR = bronopol, PD = potassium dichromate, AZ = sodium azide, 60 = 60°C, 40 = 40°C.

²SEM of LSM = 0.007 for FSCC and 0.005 for DMSCC.

³ $s_r\% = s_r \times 100 / \text{SCC arithmetic mean}$.

⁴SD average of 31 ovine milk samples analyzed in quadruplicate.

30.85%), which confirmed that repeatability was greater for flow cytometry than for disk cytometry. On the whole, the coefficients of variation for the automatic counters were smaller than those for the DMSCC method, which was less repeatable.

Accuracy was estimated by means comparison and regression studies between instrumental and DMSCC results. Because of the repeatability produced by the DMSCC method, 4 analytical determinations were al-

ways carried out in each ovine milk sample. Table 3 lists the standard deviations of the 31 studied milk samples, showing coefficients of variation of $\geq 7\%$ in the samples with the $\text{SCC} \leq 700 \times 10^3$ cells/mL, which means that several analytical determinations must be carried out in the same sample to increase accuracy. This is important to the quality control of SCC instrumentation because a $\text{SCC} \leq 700 \times 10^3$ cells/mL includes the IMI discrimination threshold in ovine milk.

Table 2. Repeatability of the Fossomatic methods (20 subsamples/milk) in ovine milk samples with different SCC ($\times 10^3$ /mL).

Milk	F360-BR-40 ¹			F5000-BR-40 ¹		
	$\bar{x}$	SD	CV (%)	$\bar{x}$	SD	CV (%)
01	47	14.45	30.85	40	4.38	10.9
02	90	7.42	8.19	96	7.37	7.67
03	189	9.64	5.10	185	5.70	3.08
04	231	13.85	5.99	233	10.56	4.53
05	417	13.94	3.34	430	13.94	3.24
06	497	16.15	3.25	525	13.21	2.52
07	663	20.62	3.11	711	13.98	1.97
08	638	16.32	2.56	716	14.78	2.06
09	1218	33.67	2.76	1282	22.41	1.75
10	1249	23.51	1.88	1316	18.99	1.44
11	1619	26.54	1.64	1705	26.02	1.52
12	1784	29.59	1.66	1801	20.89	1.16
13	2175	37.82	1.74	2189	23.91	1.09

¹F360 = cytometry on disk, F5000 = flow cytometry, BR = bronopol, 40 = 40°C.

Table 3. SCC ($\times 10^3/\text{mL}$) arithmetic means, standard deviations, and coefficients of variation of the 31 ovine milk samples analyzed by direct microscopic method in quadruplicate.

Sample	$\bar{x}$	SD	CV (%)
01	33	6.27	19.00
02	36	4.57	12.70
03	42	6.40	15.23
04	45	11.52	25.60
05	75	7.77	10.36
06	111	22.96	20.69
07	111	12.01	10.82
08	106	20.55	19.39
09	130	23.56	18.12
10	170	10.78	6.34
11	202	16.91	8.37
12	208	28.88	13.88
13	312	40.02	12.83
14	430	29.92	6.95
15	653	46.77	7.16
16	724	50.46	6.97
17	963	14.80	1.54
18	970	31.48	3.24
19	997	30.87	3.10
20	1072	58.72	5.48
21	1328	79.35	5.97
22	1416	68.24	4.82
23	1472	70.98	4.82
24	1480	45.59	3.08
25	1508	109.4	7.25
26	1547	30.73	1.99
27	1642	69.31	4.22
28	1811	117.38	6.48
29	1860	119.54	6.43
30	2134	63.88	2.99
31	2651	120.94	4.56

Correlations between DMSCC and FSCC (Table 4) were very high in all cases, close to 1.00. These values coincided with values reported for bovine milk by other authors for different working factors (Grappin and Jeunet, 1974; Heeschen, 1975; Schmidt-Madsen, 1975; Heald et al., 1977; Schmidt-Madsen, 1979). They were also similar to those obtained by Gonzalo et al. (1993, 2003) in ovine milk. However, the slope and intercept of the WP samples were different ($P < 0.05$) from 1.00 and 0.00, respectively, indicating the need to preserve samples before the analytical determination of the SCC. In addition, the slope and intercept given by F360-AZ-60 differed ($P < 0.05$) from their theoretical values, which was compatible with the fact that this analytical condition was the least accurate when compared with DMSCC (Table 1). Both SCC devices achieved good exactness of calibration for the rest of the analytical conditions.

Ewe, FSCC method, preservation, and interaction method $\times$ analytical temperature contributed significantly to the SCC variation. Whereas this SCC variation was significant ($P < 0.001$) for the aforementioned method and preservation effects, it was of less importance ($P < 0.05$) in the case of method $\times$ temperature interaction. Flow cytometry gave greater ($P < 0.001$) logSCC values (5.64) than disk cytometry (5.60). In contrast, the WP samples produced greater ($P < 0.05$) values (5.64) than the BR (5.62), PD (5.61), and AZ (5.60) preserved samples. Higher SCC in the WP samples and lower SCC in the AZ preserved samples were demon-

Table 4. Coefficients of regression (b), intercept (a), and R^2 values based on regression analyses between direct microscopic and Fossomatic methods and analytical conditions.

Analytical condition ¹	b	SE	a	SE	R^2
F360-WP-40	1.079***	0.018	-0.424***	0.103	0.991
F360-BR-40	1.000 ^{NS}	0.014	0.022 ^{NS}	0.081	0.994
F360-PD-40	0.994 ^{NS}	0.018	0.070 ^{NS}	0.099	0.990
F360-AZ-40	0.995 ^{NS}	0.017	0.068 ^{NS}	0.094	0.991
F360-WP-60	1.054*	0.022	-0.284*	0.126	0.987
F360-BR-60	0.970 ^{NS}	0.020	0.204 ^{NS}	0.113	0.987
F360-PD-60	0.974 ^{NS}	0.016	0.189*	0.088	0.992
F360-AZ-60	0.951*	0.020	0.328**	0.112	0.987
F5000-WP-40	1.095**	0.031	-0.567**	0.178	0.977
F5000-BR-40	0.992 ^{NS}	0.013	0.034 ^{NS}	0.076	0.994
F5000-PD-40	1.018 ^{NS}	0.017	-0.099 ^{NS}	0.100	0.991
F5000-AZ-40	1.003 ^{NS}	0.023	-0.009 ^{NS}	0.133	0.984
F5000-WP-60	1.120***	0.027	-0.714***	0.152	0.983
F5000-BR-60	1.016 ^{NS}	0.017	-0.095 ^{NS}	0.094	0.992
F5000-PD-60	1.024 ^{NS}	0.016	-0.136 ^{NS}	0.088	0.993
F5000-AZ-60	1.015 ^{NS}	0.026	-0.081 ^{NS}	0.147	0.981

* $P < 0.05$.** $P < 0.01$.*** $P < 0.001$.^{NS}Nonsignificant.

¹F360 = cytometry on disk, F5000 = flow cytometry, WP = without preservation, BR = bronopol, PD = potassium dichromate, AZ = sodium azide, 40 = 40°C, 60 = 60°C.

strated in a previous study using FSCC in ovine milk (Gonzalo et al., 2003). The WP and BR preserved aliquots had the closest logSCC to that of the reference method (5.63) but were greater ($P < 0.05$) than the AZ preserved samples.

CONCLUSIONS

The 2 rapid SCC counters gave adequate overall accuracy in ovine milk; however, F5000, based on flow cytometry, was slightly more accurate and repeatable than F360, based on disk cytometry. Analytical temperature did not affect SCC accuracy, but it did affect repeatability, especially in F360. The regression study made evident the need to preserve samples before analytical determination of the SCC. Bronopol was the preservative of choice for FSCC analysis.

With regard to routine sampling from test day or bulk tank milk recordings, variations brought about by such methods may not be so important in determining the health status of sheep. However, uniformity of preservation and analytical temperature are essential for intra- and interlaboratory quality control tests, with the aim of guaranteeing reproducibility of SCC results.

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