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ICMSF Methods Studies. XV. Comparison of Four Media and Methods for Enumerating *Staphylococcus aureus* in Powdered Milk

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ABSTRACT

Four media were examined for their usefulness in enumerating *Staphylococcus aureus* inoculated (a) into milk that was then dried or (b) directly into dried milk powder. In all, seven strains of *S. aureus* were inoculated individually into each preparation and were enumerated after two periods of storage (18 to 19 d and 60 to 61 d). Fourteen laboratories from twelve countries participated in the comparison which found that direct plating on agar medium in 14-cm petri dishes may be as useful as enrichment followed by streaking. Plating on Baird-Parker medium or on Hauschild pork plasma fibrinogen medium and a MPN method using Giolitti and Cantoni's broth with Tween 80 were equally sensitive for enumerating *S. aureus* in dried milk powder. The use of Hauschild medium may eliminate the need for supplementary tests to confirm colonies as *S. aureus*, but in some cases was found to fail in some laboratories. Giolitti and Cantoni's broth without Tween 80 generally was less useful than the three other media for enumerating *S. aureus*. *S. aureus* inoculated into milk that was then dried survived longer than when inoculated into dried milk.

Milk powder is traded in large amounts in international markets and is frequently used for feeding high risk populations, e.g., infants, ill persons and aged. Therefore, reliable sensitive methods that can detect small numbers of potential pathogens are required for microbiological examination, especially because pathogens may increase in number when the milk is reconstituted.

The International Dairy Federation (IDF) had been concerned with microbiological methods for milk powder. IDF Standard 60A : 1978 (*13*) recommended that Giolitti and Cantoni (GC) enrichment broth be used to enrich *Staphylococcus aureus* in milk powder of any age, and phenol red lactose salt enrichment broth be used for powders less than 15 d after manufacture. The procedure with both broths was to inoculate 0.1 g (1 ml of 1:10 wt/vol) of powder in each of 3 tubes, incubate at 37°C for 24 to 48 h, and then streak on Baird-Parker (BP) agar. The powder was considered to contain *S. aureus* if colonies on BP from at least 2 of the 3 portions of 0.1 g were confirmed as *S. aureus*. The International Commission on Microbiological Specifications for Foods (ICMSF) criterion for milk powder considers as acceptable, 10 *S. aureus* per g (*10*) and several methods for enumerating *S. aureus* are described (*11*).

Before the studies reported here, members of IDF working group E24 participated in a series of collaborative assessments of media and methods for enumerating *S. aureus* in milk powder. As a result of these assessments, the decision was made to carry out a comparison of four media and methods for enumerating *S. aureus* in milk powder. These included: (a) direct plating on BP agar in 14-cm petri dishes (BP), (b) an MPN procedure using GC broth (GC), (c) an MPN procedure using GC broth with Tween 80 added (GCTw), and (d) direct plat-

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ing on Hauschild medium, a recently reported medium containing pork plasma and fibrinogen (PPF) (*9*).

MATERIALS AND METHODS

The statistical design and analysis were done in Ottawa, Canada, and the milk powders were prepared in Rennes, France. The participating laboratories were in 12 countries (see authorship list) and all analysts were accustomed to microbiological methods for analyzing dairy products.

Trials

The collaborative study was divided into three parts: Trial 1, Trial 2 and Trial 3. The objective in all three trials was to compare the efficiency of four culture media and methods for enumerating *S. aureus* in milk powder that had been inoculated in two ways, i.e., before drying and after drying. Additional objectives were: (a) to compare efficiency of the media for recovering debilitated cells of *S. aureus* from stored milk powder, (b) to assess the reproducibility of results by analysts who analyzed duplicate samples, and (c) to compare laboratories using the same media, methods and samples, for their ability to obtain the same results, i.e., testing reproducibility. This objective was tempered by uncontrollable conditions during transportation of samples. Nevertheless, the time lapse between the shipping of samples in a single group and the start of analysis was the same for all laboratories, i.e., exactly 2 wk.

In Trial 1, single samples from 14 batches of *S. aureus*-inoculated milk powder were analyzed 18 to 19 d after preparation. For practical reasons, the batches were prepared Thursday and Friday and samples prepared from those batches were shipped on the following Monday. The start of analysis was a fortnight later, also on a Monday (i.e., 18 to 19 d after preparation). In Trial 2, single samples from the same 14 batches were analyzed 6 wk later (60 to 61 d after preparation). In Trial 3, four samples from each of six batches of *S. aureus*-inoculated milk powder were analyzed (total of 24 samples per analyst). In Trial 3, the analysts were not aware that each batch consisted of duplicate samples at two levels of inoculation.

Fourteen laboratories participated in this study, i.e., 13 in Trials 1 and 2; 6 of 14 in Trial 3; and 7 of 13 in Trials that used PPF medium which will be discussed later.

Preparation of milk powders inoculated with S. aureus

The use of milk naturally contaminated with *S. aureus* was considered for this study, but rejected because of the problems of obtaining sufficient numbers and quantities of suitable samples. The procedure of Chopin et al. (*5*), with modifications described below, was used to artificially inoculate milk before and after drying.

Cultures. Seven strains of *S. aureus* isolated from milk and dairy products were used (Table 1). Each *S. aureus* strain was grown in peptone yeast glucose broth (*4*) at 37°C for 18 h.

Source of milk. A single batch of well-mixed spray dried skim milk powder (ca. 200 kg) was used. The milk powder contained fewer than 10 CFU of *S. aureus* per g. Portions were reconstituted with water, inoculated and dried, or the dried milk was inoculated directly.

Inoculation of batches 1 through 7 before drying. For each batch, 12 kg of milk powder was reconstituted with 18 kg of water to obtain concentrated milk (CM) with a solids content of 36 to 39%.

The CM was warmed to 37°C in the vat of an Anhydro spray drying pilot plant (Anhydro, Copenhagen, Denmark) (*4*) situated immediately before the spray drying tower. A suitable volume of *S. aureus* culture (from 13 to 2000 ml depending on the strain of *S. aureus* and the results of previous experiments) was added to each batch of CM and thoroughly mixed. The inoculated CM was then pumped from the vat into the spray drying tower, with the inlet temperature being 200°C and the outlet temperature being 90 ± 1°C. Viable *S. aureus* in each batch were enumerated 1 d after drying.

Inoculation of batches 8 through 14 after drying.

Inoculum. Suspensions were prepared from 18-h cultures of *S. aureus* that were centrifuged at $3000 \times g$ for 5 min, the pellets resuspended in reconstituted skim milk (10% wt/vol), recentrifuged and resuspended in skim milk.

Apparatus. The following apparatus, based on a principle described previously (*5*), was built for this experiment. A stainless steel cylinder contained an adjustable sieve at the bottom. The sieve could be moved to and fro by an electric motor. A nebulizer (a spare part of an atomic absorption spectrophotometer Varian Techtron model 1200) was attached below the sieve to produce an aerosol from the inoculum.

Basic inoculated powder (BIP). A suspension of *S. aureus* was added as an aerosol to a falling film of milk powder using the above apparatus. From 1.4 to 4.5 ml of suspension (depending upon the strain of *S. aureus* and the results of previous experiments) was used to inoculate 150 to 200 g of milk powder. As soon as the sieve and the nebulizer were operated, the milk powder started to fall and was thus mixed with the *S. aureus* aerosol. The inoculated milk powder was then collected through a funnel situated just below the sieve at a distance of 80 cm. The entire system was enclosed in a plastic isolator to avoid contamination of (and from) the outside. Powder thus inoculated is referred to as BIP. Viable *S. aureus* in BIP were enumerated 1 d after preparation.

Mixing of BIP with dried milk. Based on previous experiments, the BIP was prepared to contain ca. 10^6 CFU of *S. aureus* per g. BIP was then diluted with *S. aureus*-free milk powder to obtain about 3000 g of mixture containing ca. 5×10^4 CFU of *S. aureus* per g. This operation was performed in a cylinder-shaped mixer (20-L metal churn) revolving on a asymmetrical axis at 60 rpm for 5 min. The direction of rotation changed automatically at 12-s intervals. The initial counts in the diluted

TABLE 1. *Main characteristics of strains of* Staphylococcus aureus *used for inoculating milk powder samples (5).*

Characteristic	Strain						
	1036	1185	1186	1198	1237	1238	1247
Coagulase	+	+	+	+	+	+	+
Thermonuclease	+	+	+	+	+	+	+
Phosphatase	+	+	+	+	+	+	+
Fermentation of mannitol	+	−	−	+	+	+	+
Action on egg yolk	+	+	+	+	−	−	±[a]
Hydrolysis of Tween 40	+	+	+	+	−	−	+
Hydrolysis of Tween 60	+	+	+	+	−	−	+
Hydrolysis of Tween 80	+	−	+	+	−	−	−
Production of enterotoxin	ND[b]	−	−	ND	+C[b]	−	+A[b]

[a]On blood agar base containing 5% egg yolk, a stable mixture of egg yolk-clearing and egg yolk-negative colonies develops.
[b]ND, not determined; +C, enterotoxin C; +A, enterotoxin A.

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powder were calculated on the basis of the counts in BIP and the extent of dilution with the *S. aureus*-free powder.

The mixing was effective. *S. aureus* was enumerated in 10 subsamples, each of 2 g from a single lot, and the mean and standard deviation (log_{10} of CFU) were 3.45 ± 0.112. A similar standard deviation was noted when using broth cultures to assess the lot of Baird-Parker medium (Difco C481-01) used throughout this study.

For Trial 3, the same mixing device was used to prepare 1:3 dilutions of milk powder inoculated before and after drying as described previously.

Cleaning and disinfection

The inoculating chambers, mixer and drying tower were cleaned and disinfected after each strain of *S. aureus* had been used to inoculate a batch of CM or powder. The pipelines, the vat which contained the CM, and the atomizer were cleaned by circulating water at ≥90°C. The drying tower was disinfected by increasing the outlet air temperature to 110 to 115°C. This temperature ensured at least 99.9% kill of *S. aureus* surviving in the tower (5). Powder remaining in the tower contained fewer than 10 CFU of *S. aureus* per g. The nebulizer used for preparing BIP and its feeding circuit were cleaned with water and sanitized by circulating first ethanol and then sterile water. The plastic hood which covered the entire apparatus was removed and a new hood was installed. The funnel was washed and dried with a sterile cloth. The mixer (20-L churn) was autoclaved before use.

Diluent, culture media and confirmatory tests

With minor exceptions, all media and chemicals were from single lots. Prepared media, ingredients for media and tellurite were donated by Difco, Detroit, MI. Other chemicals and the filters were purchased. A shortage of bovine fibrinogen fraction I (BFI) necessitated using two lots from Calbiochem, La Jolla, CA, and one lot from Sigma, St. Louis, MO.

Diluents. Quarter strength Ringer's solution was used to dissolve samples of inoculated powder, and peptone (0.1%)-salt (0.85%) was used to prepare subsequent dilutions.

Baird-Parker medium (BP). The medium was prepared as described previously (2), with minor modifications. BP agar base in its dehydrated form without pyruvate (Difco C481-01) was used by all laboratories for all trials. Lot C481-01 was pretested in three laboratories by comparing it with BP medium and with nutrient agar, both laboratory-prepared. To make the comparison, each laboratory inoculated 20 petri dishes of each of the three media with suitable dilutions of 24-h cultures of two nutritionally-exacting strains of *S. aureus* and two strains with different egg yolk-clearing characteristics. Thus, there were 12 comparisons (3 laboratories × 4 strains of *S. aureus*). Analysis of variance indicated that the three media in general gave the same numbers of CFU. Pyruvate was added just before pouring plates because pyruvate may be unstable in dehydrated medium stored for long periods (6). The egg yolk emulsion was prepared from fresh eggs in each laboratory. The complete BP medium (28 ± 1 ml) was poured into a 14-cm petri dish. Plates were well-dried and inoculated within 30 min of drying.

Giolitti and Cantoni's enrichment broth (GC). The broth, tellurite and agar solutions were prepared and used as described previously (8), except that a 1% tellurite solution was used instead of a 10% solution as erroneously specified in the original publication.

Giolitti and Cantoni's enrichment broth with Tween 80 (GCTw). This broth was prepared and used as described above, but 1 g of Tween 80 was added to 1 L of the broth before autoclaving.

Hauschild's pork plasma fibrinogen (PPF) medium with Tween 80. The Hauschild medium combines the selectivity of BP medium and confirmation by the coagulase and thermonuclease (TNase) reactions into a single plating medium. The original medium (9) was modified slightly for our study to contain Tween 80 because some of the participants had found, in previous work, that addition of 0.1% Tween 80 increased the recovery of heat-injured *S. aureus* (3).

The Hauschild medium consists of three parts: (a) BP agar without egg yolk, (b) an overlay agar to detect coagulase, which contains pork plasma, bovine fibrinogen fraction I and soy anti-trypsin, and (c) a layer of toluidine blue O-DNA agar for detecting TNase. Because 14-cm petri dishes were used instead of 9-cm petri dishes, the volumes of medium and overlay agar used were increased.

BP agar without egg yolk (42 ± 2 ml) was poured per 14-cm petri dish. Plates were dried and used as described below.

Overlay agar consisted of three reagents, including:

1. Pork plasma. Lyophilized pork plasma was donated by NIZO, Ede, The Netherlands. It was prepared by aseptically collecting blood during slaughter and mixing it to obtain 5 volumes of 2% ammonium oxalate with 95 volumes of whole blood. The oxalated blood was centrifuged at $10{,}000 \times g$ for 30 min. The supernatant fluid was dispensed in 10-ml amounts and rapidly frozen during centrifugation in an Edwards EF6 freeze drier. Drying was continued for 48 h at 0.1 to 0.07 mm Hg. Each 10 ml of liquid plasma yielded about 1 g of dried plasma. For use, 1 g was reconstituted with 10 ml of distilled water.
2. Trypsin inhibitor solution. A 0.3% solution of soy trypsin inhibitor (Type S-1, Sigma Chemical Co.) was prepared in 0.05 M phosphate buffer, pH 7.0, and filter-sterilized.
3. Bovine fibrinogen fraction I (BFI) solution. A 0.8% solution of BFI (Bovine fibrinogen, fraction I, Calbiochem) was prepared in 0.05 M phosphate buffer, pH 7.0. The solution was filtered successively through Whatman No. 41 paper and a Millipore prefilter (Catalogue AP 25 04200) before being filter-sterilized.

The overlay agar was prepared by adding 2.5 ml of pork plasma and 0.5 ml of trypsin inhibitor solution to 47.5 ml of BFI solution and warming to 37°C. Immediately before use, the above mixture was added to 50 ml of sterile 1.4% agar solution warmed to 50 to 55°C.

Toluidine blue O–DNA agar was prepared by adding to 1 L of 0.05 M Tris (pH 9.0), 0.3 g of deoxyribonucleic acid (Difco 3231-10-3), 10.0 g of agar, 10.0 g of NaCl and 1.0 ml of 0.01 M solution of $CaCl_2$. The mixture was boiled until DNA was dissolved and the agar melted. Three ml of a 0.1 M solution of toluidine blue 0 was then added.

Confirmatory tests. Coagulase, catalase and TNase tests were performed as described previously (11). Brain heart infusion (Difco No. 0037-02-5), coagulase plasma EDTA (Difco No. 0803-66-0), deoxyribonucleic acid (Difco No. 3231-10-3) and other chemicals were each from a single lot and were distributed to each participating laboratory.

Reconstitution of milk powder sample and preparation of higher dilutions

The 10^{-1} dilution was prepared by adding 10 g of sample to a dilution bottle containing 90 ml of quarter strength Ringer's solution warmed in a water bath to 47 ± 2°C. The bottle was slowly swirled to wet the powder and then gently shaken 25 times in ca. 10 s through an excursion of about 30 cm. The bottle was replaced at 47 ± 2°C for 5 min and shaken occasionally (12,13). The 10^{-2} to 10^{-4} dilutions were prepared in peptone-salt dilution fluid at ambient temperature.

Inoculation and incubation of plates and tubes

Direct inoculation onto BP medium. Inoculum (1 ml) was spread on each previously dried 14-cm plate using a bent glass rod spreader. For each sample, duplicate plates were each inoculated with 1 ml of the 10^{-1} and 10^{-2} dilutions.

The inverted plates were incubated at 35 to 37°C for 45 to 48 h. At the end of the incubation period, all colonies that appeared to be *S. aureus* were counted.

Colonies of *S. aureus* were classified into three types according to their appearance and biochemical reactions (Table 2).

From plates inoculated with suitable dilutions, colonies representing the above types were picked (maximum of 10 per sample) according to their proportions.

Most Probable Number techniques using GC or GCTw

For each dilution (10^{-1}, 10^{-2}, 10^{-3} or 10^{-4}) of the milk powder sample, three tubes of GC or GCTw were inoculated (1 ml per tube). The inoculum was mixed with the medium, while avoiding the introduction of air. The agar solution was then poured over the liquid to form an agar overlay (or plug). The tubes were incubated at 37°C. All the tubes that were black after 24 h were streaked on BP agar. After 48 h, the remaining tubes (black or not black) were also streaked in a same manner.

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TABLE 2. *Classification of* S. aureus *colonies according to their appearance and biochemical reactions.*

Type	Colony description	Biochemical tests to confirm *S. aureus*		
		Coagulase	Catalase	TNase
I	Black, shiny, narrow white margin; clear zone extending into translucent medium; egg yolk positive.	3+ or 4+ 1+ or 2+		 +
II	Black, shiny, narrow white margin; clear zone absent or scarcely visible; egg yolk-negative or weakly positive.	3+ or 4+ 1+ or 2+	+ +	 +
III	Less black than I and II, rough, dry texture.	1+ to 4+	+	+

The colonies that developed on BP agar were classified into types I, II or III, and tested as described above. If the tested colonies were *S. aureus*, the corresponding tube was considered positive. If the colonies were not types I, II or III, or if no colonies were present, the tube was considered negative.

Direct inoculation onto PPF medium

The plates of PPF medium were dried and inoculated in the same way as the plates of BP medium, then covered with 20 ml of overlay agar and incubated. After 24 and 44 h, black colonies with halos (coagulase-positive) were recorded.

After 44 h the in situ TNase test was performed as described by Lachica (*14*), except that an increased volume of 22 ml of toluidine blue O-DNA-agar was poured on each plate.

Reasons for use of 14-cm plates

The maximum amount of reconstituted (10%) milk (RM) that can be spread on the surface of a 9-cm plate containing 10 ml of BP without seriously impairing selectivity and observation of the egg yolk reaction, is 0.3 ml (0.03 g of dried milk). A 14-cm plate containing 28 ± 1 ml of BP agar can, however, be spread with 1 ml of RM (0.1 g of dried milk) without loss of necessary qualities (R. Holbrook, personal communication). Thus, two 14-cm plates give a sensitivity for detecting low numbers of *S. aureus* equal to seven 9-cm plates.

Tasks of the analyst

A detailed instruction manual for preparing culture media, dilutions, and carrying out analyses was supplied to each analyst about 1 month before bench work started. All bacteriological examinations were done by one analyst in each laboratory, except in emergencies which were few. Preparatory tasks, however, could be done by a helper. Results were recorded in a uniform manner on a specially designed reporting form. Comments were made by the analyst in a section of the form, to describe any problems or departures from the protocol.

Statistical design and analysis

Trials 1 and 2 were statistically designed and randomized to comprise, for each batch, a split-plot experiment (*16*), with the main plots being laboratories and the methods being applied to sub-plots. This experiment was designed to (a) determine whether the relative performance of the methods is consistent from laboratory to laboratory, (b) examine difference in counts due to method, and (c) examine difference in counts due to laboratory.

Trial 3 was also designed and randomized, for each batch, as a split-plot experiment with a factorial arrangement of laboratories and level of contamination occupying the whole plots, and methods being applied to the sub-plots. Blind duplicates formed the basis for estimates of experimental error.

These two designs were complementary in the sense that the first method studied differences over a relatively large number of laboratories and strains, whereas the second was narrower in scope but studied one additional factor (level of contamination) and used a more precise source of error (based on results of blind duplicate samples) for statistical tests.

As a consequence of these designs, samples from each batch were randomly assigned to laboratories and, for each sample, the order of introduction of the inoculum into media was randomized. All samples were blind-coded so that their identities were unknown to the participating laboratories.

All statistical analyses were done separately for each batch, using analysis of variance techniques applied to the common logarithms of the observed counts per g. All tests of significance were performed at the 5% level.

RESULTS AND DISCUSSION

Comparison of the usefulness of methods BP, GC, GCTw and PPF

For most batches of inoculated powder, methods BP and GCTw yielded essentially the same counts. Method GC yielded counts that were usually significantly lower than the other methods (Table 3). Method PPF frequently failed to obtain useful results (see below); however, when it did not fail, the counts were comparable to those obtained with methods BP and GCTw (Table 4).

Additional observations were:

BP. Although some differences among laboratories analyzing the same material were apparent for all methods, this problem was less pronounced with method BP, which was reliable and easy to use in all three trials. The disadvantage of BP was the need to carry out coagulase and other tests. Of 4097 type I and type II colonies on BP suspected to be *S. aureus*, 3844 (94.8%) gave a 3+ or 4+ coagulase test and were not examined further. However, 213 colonies of types I and II required additional tests and only 6 (2.8%) were confirmed. Similarly, 209 of 324 (64.5%) of type III colonies were confirmed.

GC. Media in 2781 tubes became black within 48 h and 2570 of them (92.4%) yielded *S. aureus* when streaked on BP medium; 3315 did not become black, but 304 did yield *S. aureus*.

GCTw. Media in 3894 tubes became black within 48 h and 3508 of them (95%) yielded *S. aureus;* 2202 did not become black, but 206 did yield *S. aureus*.

Because the milk inoculum caused turbidity and precluded visual detection of bacterial growth in many tubes, it is probable that many of the non-black tubes did not contain bacterial growth. Hence, we did not calculate the percentage of non-black tubes that yielded *S. aureus*. These data support, but do not entirely agree with, the inference that growth of *S. aureus* in GC medium causes blackening (*8*).

There was variation among laboratories in detecting *S. aureus* from black tubes. The percentage of black tubes (combined data for GC and GCTw) that yielded *S. aureus* ranged from >99.8 to 83.7 (median 91.9%), depending on the laboratory.

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TABLE 3. *Trials 1 and 2. Comparisons of methods BP, GC and GCTw[a]. Results are from all laboratories except where indicated.*

Strain	Inoculated before drying				Inoculated after drying		
	Highest count		Lowest count		Highest count		Lowest count
1036	BP	GCTw	GC	Trial 1[b]	BP	GCTw	GC
				Trial 2	GCTw	GC	BP
1185	BP	GCTw	GC		GCTw	BP	GC
1186	BP	GCTw	GC		GCTw	BP	GC
1198	BP	GCTw	GC	Trial 1[b]	BP	GCTw	GC
				Trial 2	GC	BP	GCTw
1237	BP	GCTw	GC[c]	Trial 1[b]	GCTw	BP	GC
				Trial 2	GCTw	GC	BP
1238	BP	GCTw	GC[c]		GCTw	BP	GC
1247	GCTw	BP	GC	Trial 1[b]	GCTw	GC	BP
				Trial 2	GC	GCTw	BP

[a]Methods underscored by the same line did not differ at $\alpha = 0.05$ as determined by Scheffé's test (*16*).
[b]Results of method comparison differed with trial as indicated. In all other cases, results were the same for both trials.
[c]Results from one laboratory were omitted.

TABLE 4. *Comparison of methods BP, GC, GCTw and PPF[a]. Results are from four laboratories that used all four methods.*

Strain	Laboratory	Inoculated before drying				Laboratory	Inoculated after drying			
		Highest count			Lowest count		Highest count			Lowest count
1036	1[b]	BP	GCTw	PPF	GC	1[b]	GCTw	BP	PPF	GC
	2	PPF	BP	GCTw	GC	2	GCTw	BP	GC	PPF
	3	PPF	BP	GCTw	GC	3	BP	GC	GCTw	PPF
	4	BP	PPF	GCTw	GC	4	BP	PPF	GCTw	GC
1237	1[b]	GCTw	PPF	BP	GC	All[c]	PPF	BP	GCTw	GC
	2	PPF	BP	GCTw	GC					
	3	PPF	GCTw	BP	GC					
	4	PPF	BP	GCTw	GC					
1247	All[c]	PPF	BP	GCTw	GC	1[b]	BP	PPF	GCTw	GC
						2	PPF	BP	GCTw	GC
						3	PPF	BP	GCTw	GC
						4	GCTw	PPF	BP	GC

[a]Methods underscored by the same line did not differ at $\alpha = 0.05$ as determined by Scheffé's test (*16*).
[b]Results of method comparisons differed with laboratory as indicated.
[c]Results of method comparisons were consistent for all laboratories.

PPF. The formation of a coagulase reaction (halo) by a black colony in PPF medium is considered confirmation of *S. aureus*, and the production of TNase provides additional evidence. Of 51,593 suspect (black) colonies found by the seven laboratories using PPF agar, 97.6% had both halos and positive TNase reactions. Of 1259 anomalous colonies, 1070 (2%) were halo-positive but TNase-negative (category A), and 189 colonies (0.4%) were halo-negative and TNase-positive (category B). Most (838 of 1070) category A colonies were found in one laboratory and most (171 of 189) category B colonies were found in two other laboratories. These three laboratories had little previous experience with PPF medium, whereas the five laboratories with previous experience did not find such colonies, or found them only infrequently. Category A colonies had halos that appeared to be bacterial growth between the agar layers, but we have no explanation for category B colonies. It is suggested that in experienced hands, a halo surrounding a typical black colony on PPF medium used for *S. aureus* enumeration in dried milk obviates the need for a TNase test (A. Chopin, H. Asperger and A. Hauschild, personal communications).

Effect of die-off of S. aureus *in milk powder*

In order to obtain as uniform samples as possible at time of analysis, all samples were held at 25°C before shipping and at 23 to 26°C after arrival at 12 of the 14 participating laboratories, with the remaining two at ambient temperature (16 to 25°C in one and 22 to 28°C in the other). The greatest variation between preparation and analysis of samples was in time between shipping and receiving (median 3 d, average 4.1 d, range 2 to 12 d), temperature during shipping (unknown), and atmospheric

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temperature at the time of arrival (median 7.5°C, range -10°C to >30°C). These variations did not appear to influence the numbers of bacteria recovered. Immediately after preparation, the counts of *S. aureus* were about the same in samples inoculated before drying and samples inoculated after drying (Table 5). However, the rates of die-off during storage were very different for the two types of preparation. Milk inoculated before spray drying still contained 20 to 128% (median 69%) of the initial counts at Trial 1 (18 to 19 d after preparation) and 3 to 49% (median 28%) at Trial 2 (60 to 61 d after preparation). Conversely, milk inoculated after drying showed a rapid loss of *S. aureus*, with about 1% remaining at Trial 1 and usually less than 0.1% at Trial 2.

It is probable that *S. aureus* surviving in powder inoculated after drying were severely stressed. Despite stress, the plating media (BP and PPF) were as useful as the liquid enrichment medium (GCTw) for enumerating the small numbers of *S. aureus* that survived.

Detection of small numbers of S. aureus

Among the 14 laboratories, there were 132 analyses in which the number of *S. aureus* recovered was not over 150, and was usually less than 50 per g by both methods BP and GCTw. Although both methods are less precise when counts are this low (*7*), we feel that the following comparisons are still of interest. Of the 132 analyses, 53 gave higher recoveries by method BP, 51 gave higher recoveries by method GCTw, 3 gave equal recoveries by both methods, and 25 failed to detect *S. aureus*. At the lowest limits for detection (5 CFU/g for method BP and MPN of 4/g for method GCTw), method BP detected *S. aureus* in 5 analyses when method GCTw did not and, conversely, method GCTw detected *S. aureus* in 10 analyses when method BP did not. We can conclude that both methods were not different in their ability to detect small numbers of *S. aureus* in powdered milk.

Ability to analyze duplicate blind samples

The ability of analysts to obtain identical results with duplicate blind samples varied. Most laboratories would be considered to have obtained acceptable results for plate counts of duplicate samples (*1*), but two laboratories had such large differences in plate counts between a few duplicate samples to suggest laboratory error, and one of these two had results for two pairs of duplicate samples so inconsistent with those of the other five laboratories analyzing identically prepared samples, as to leave no doubt of laboratory error. Results for other samples by this laboratory were, however, consistent with those obtained by the five other analysts. An indication of the variation between counts obtained for duplicate blind samples is given in Table 6. Obviously, these variations were smaller when solid plating media were used (BP and PPF) than when MPN methods (GC and GCTw) were used.

Effect of the size of the inoculum

Samples with high inoculum were used to prepare samples with low inoculum by dilution of 700 g of "high inoculum" powder with 1400 g of *S. aureus*-free powder. Thus, one might expect that counts of the "high in-

TABLE 5. *Trials 1 and 2. Geometric means of CFU of* S. aureus *in dried milk after two periods of storage*[a,b].

	Inoculated before drying						Inoculated after drying					
			Method of analysis						Method of analysis			
Trial	Batch (strain)	Initial count[c]	BP	GC	GCTw	PPF	Batch (strain)	Calculated initial count[d]	BP	GC	GCTw	PPF
1	2(1036)	3,700	4,050	1,230	3,320	5,650	14(1036)	100,000	2,170	410	1,570	1,540
2			1,040	110	750	1,050			0[e]	10	10	0
1	5(1185)	8,500	9,870	2,490	5,630	14,920	12(1185)	38,000	1,930	1,180	2,080	1,070
2			4,140	460	4,340	4,510			30	20	30	10
1	7(1186)	30,000	19,110	2,680	8,880	25,190	11(1186)	100,000	760	470	1,130	670
2			5,320	400	3,200	3,720			50	30	70	30
1	4(1198)	24,000	30,940	1,790	11,520	33,200	8(1198)	13,000	180	50	150	150
2			6,750	350	4,060	4,100			0	0	0	0
1	6(1237)	15,000	10,420	1,340	4,060	27,340	10(1237)	50,000	150	50	170	180
2			6,190	270	2,990	4,920			0	0	0	0
1	1(1238)	39,000	10,920	1,420	7,990	32,020	9(1238)	41,000	130	70	190	150
2			6,570	410	2,430	6,670			0	0	0	0
1	3(1247)	31,000	6,320	2,200	9,420	13,450	13(1247)	16,000	90	110	260	120
2			880	350	1,320	1,410			0	10	0	0

[a]All 13 laboratories used methods BP, GC and GCTw; 7 of the 13 laboratories also used method PPF; all counts are geometric means except those under footnotes c and d, which are single counts on BP agar.

[b]Trial 1, stored for 18 to 19 d after inoculation; Trial 2, stored for 60 to 61 d after inoculation. Except during shipping, storage temperature was at or about 25°C.

[c]*S. aureus* added to liquid milk and CFU determined by A. Chopin 1 d after drying using BP agar. Note that counts under methods BP, GC, GCTw and PPF are geometric means from all participating laboratories, and were made 18 to 19 d after the initial counts for Trial 1 and 60 to 61 d for Trial 2.

[d]Calculated initial count based on numbers of *S. aureus* in BIP found by A. Chopin 1 d after inoculation.

[e]Below limits of detection, 5 CFU per g for methods BP and PPF and an MPN of 4 per g for methods GC and GCTw.

oculum'' powder would be 3-fold greater than counts of the ''low inoculum'' powder. In many instances ratios approximating 3:1 were obtained, but frequently they were not (Table 7). We have no explanation for this discrepancy. Others (*15*) have observed similar discrepancies in a collaborative analysis of *Salmonella* in chocolate.

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TABLE 6. *Trial 3. Comparisons between blind duplicate samples.*

Method	No. of comparisons	Distribution of % deviation from mean[a]				
		Median	25th percentile	75th percentile	Lowest	Highest
BP	72	11.7	4.5	34.9	0	96.7
GC	72	43.8	22.0	72.9	0	99.1
GCTw	72	37.9	12.9	62.3	0	97.2
PPF	48	13.2	6.2	31.8	0	90.8

[a]For each pair of blind duplicate samples, the deviation from the mean was expressed as a percentage of the mean. The percentiles were calculated for the resulting distribution of percent deviations.

TABLE 7. *Trial 3. Geometric means of CFU of* S. aureus *in dried milk containing high and low inocula*[a,b].

Inoculum	Strain	Inoculated before drying				Inoculated after drying			
		BP	GC	GCTw	PPF[c]	BP	GC	GCTw	PPF[c]
High	1036	2,113	161	1,131	2,048	40.8	22.6	27.6	21.8
Low	1036	298	27	151	189	27.5	15.7	19.3	8.1
High	1237	7,415	373	3,735	5,880	2,992	169	1,636	3,013
Low	1237	1,670	127	1,458	2,347	698	145	269	1,060
High	1247	1,634	320	1,143	2,180	2,819	463	1,340	2,986
Low	1247	625	121	531	895	891	156	695	1,239

[a]The samples were coded, and the analysts were unaware of strains of *S. aureus* and high and low inocula.
[b]The low inoculum dried milk was obtained by mixing 700 g of dried milk containing a high inoculum with 1,400 g of dried milk that was free of *S. aureus*.
[c]Four of six laboratories that participated in Trial 3 used method PPF.

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