

Validation of the Microbiological Methods Hygicult Dipslide, Contact Plate, and Swabbing in Surface Hygiene Control: A Nordic Collaborative Study

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A collaborative study on total aerobic bacterial count was conducted to validate the Hygicult® TPC dipslide against contact plates and swabbing, using stainless-steel surfaces artificially contaminated with different microbes at various levels. Twelve laboratories took part in the validation procedure. The total number of collaborative samples was 108. The microbial level in each sample was assessed in triplicate using the 3 above-mentioned methods under 3 different incubation conditions (at $25 \pm 1^\circ\text{C}$ for 48 and 72 h and at $30 \pm 1^\circ\text{C}$ for 48 h). Surface sampling methods detached 25–30% at the lowest (theoretical yield, 1.4 cfu/cm^2), 18–20% at the middle (theoretical yield, 10.7 cfu/cm^2), and 16–21% at the highest (theoretical yield, 43.6 cfu/cm^2) levels of microbes from the test surfaces. The percentage of acceptable results after removing outliers was 89%. Repeatability standard deviations ranged from 27.2 to 74.6% and reproducibility standard deviations ranged from 42.1 to 97.5%. There were no significant differences between results obtained at different incubation temperatures (25 and 30°C) or incubation times (48 and 72 h) for all 3 methods. The Hygicult TPC dipslide, contact plate, and swabbing methods gave similar results at all 3 microbial levels tested: $0.35\text{--}0.43 \text{ cfu/cm}^2$ at the lowest level, $1.9\text{--}2.2 \text{ cfu/cm}^2$ at the middle level, and $7.1\text{--}9.1 \text{ cfu/cm}^2$ at the highest level.

niques and practices in the food industry have evolved to aid formal quality control systems in meeting international or company standards for total quality management systems as well as customer demands (1, 4–7). HACCP (hazard analysis critical control point) systems and good hygiene practices are very important in meeting these obligations at an affordable cost, without compromising safety, quality, or service to the customer (8–10). The common purpose is to identify microbial risks by controlling process surfaces and surfaces in food production areas (11). In hygiene control, the total number of bacteria and enterobacteria provides an estimate of the level of contamination risk during production (12–14). Hygiene monitoring in industrial premises is currently based on conventional cultivation using swabbing or contact plates. There is a need for validation of practical methods to study surface hygiene in industrial premises (15).

Sampling from the surface is very difficult. Microbes are attached to the surface and if they are removed violently, for example, using strong agitation with beads, they may not be detectable in methods based on multiplication in agar (16). It is not possible to perform research methods such as microscopy because a small piece of surface is needed in the evaluation (15). In practice, the process surfaces are made of various materials whose condition and roughness vary. The amount and type of soil and microbial contamination also affect the results obtained.

The swab method was standardized in 1997 as a quantitative and semiquantitative surface sampling method (17, 18). Numerous methods for identification of certain bacteria and enumeration of total bacteria have been validated by AOAC INTERNATIONAL (19, 20). The contact plate, dipslide, and swab methods have not been studied previously through a collaborative validation process. Other methods used in surface hygiene monitoring are ATP-bioluminescence, protein detection methods, staining combined with microscopy, and image analysis (15).

Microbiological hygiene in food production and processing aims to protect the consumer from pathogenic agents and assure food quality (1–3). Tech-

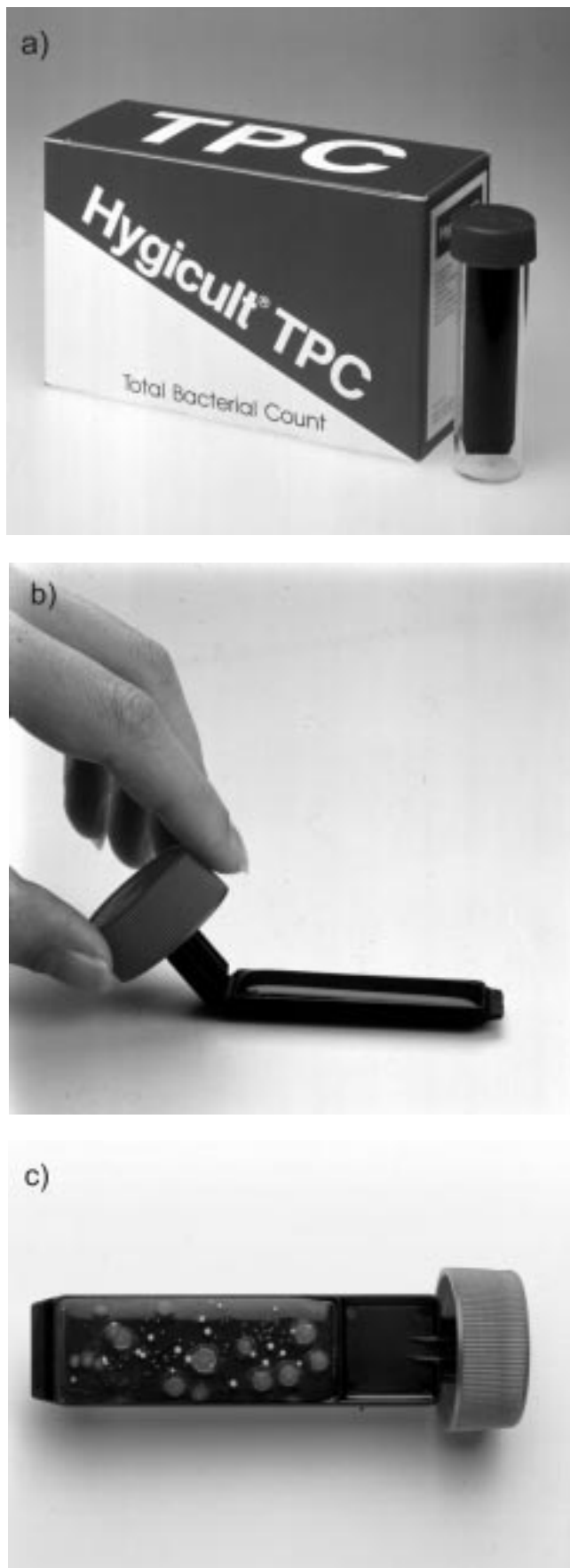


Figure 1. a) Hygicult TPC kit, b) model contact sampling, and c) growth on Hygicult TPC dipslide.

The aim of the present Nordic collaborative study was to evaluate the reliability of the Hygicult TPC dipslide (Figures 1 and 2a), which was developed for the rapid monitoring of microbiological hygiene of surfaces and solid and liquid materials (21). This method was compared with the contact plate method (Figure 2b), which was specially developed for taking microbiological surface samples (12), and the swabbing method (Figure 2c), the standard method (17) used for monitoring surface hygiene (12, 17, 22) on microbially soiled surfaces in terms of repeatability and reproducibility (23). The AOAC requirements have been considered during planning, arranging, and reporting the present study (19, 20, 24, 25). Statistical data handling was performed according to the guidelines given in the AOAC standards (26).

Collaborative study

Twelve laboratories participated in this collaborative study, which was planned at VTT Biotechnology (VTT). In the validation process, 3 methods (Hygicult TPC dipslides, contact plates, and swabbing) were compared by testing their suitability for hygiene control of artificially contaminated stainless-steel surfaces. Sampling with various methods was performed in triplicate. Samples were incubated at 2 temperatures and for 2 incubation periods. The test series performed at each participating laboratory is shown in Figure 3. Collaborators repeated this procedure for each of the 9 randomly coded samples.

Trial 1: Preliminary Samples

The purpose of the preliminary study was to acquaint collaborators with the procedures used in the actual collaborative study. Comments on the procedure description were collected to obtain a procedure manuscript for sound performance of the collaborative experiments. The precollaborative study was performed using one bacterial sample. The number of primary data obtained in each laboratory in the preliminary test was 28. These results were obtained from triplicate determinations for each of the 3 methods using 3 different incubation times and temperature combinations as well as control cultivation of the bacteria in the suspension. The precollaborative test was performed in December 1998 by 12 laboratories.

Trial 2: Collaborative Samples

The second trial was the final test for all the collaborative samples in the validation procedure, in which all 9 samples were randomly coded. Three replicates of each of 3 different bacterial solutions dispensed on stainless-steel surfaces were used as microbial soil for comparing the Hygicult TPC dipslide, contact agar, and swabbing methods. The collaborative test was performed in February 1999 by 12 laboratories. All participating laboratories returned their results to VTT on time.

Principle of Methods

The purpose for using the Hygicult TPC dipslide, contact agar, and swabbing methods in this validation test was to assess the yields of various cultivation-based methods used in

surface hygiene. All these methods are based on cultivation of microbes detached from the surface. In the sampling techniques under study, the transferral of microbes from surfaces differs. In the Hygicult TPC dipslide and contact agar methods, the microbes from the surfaces studied are transferred by pressing the agar onto the surface. In the swabbing method, the surface is swabbed and microbes are transferred with the swab into the diluent by agitation on a Vortex mixer. This diluent solution is cultivated on agar plates.

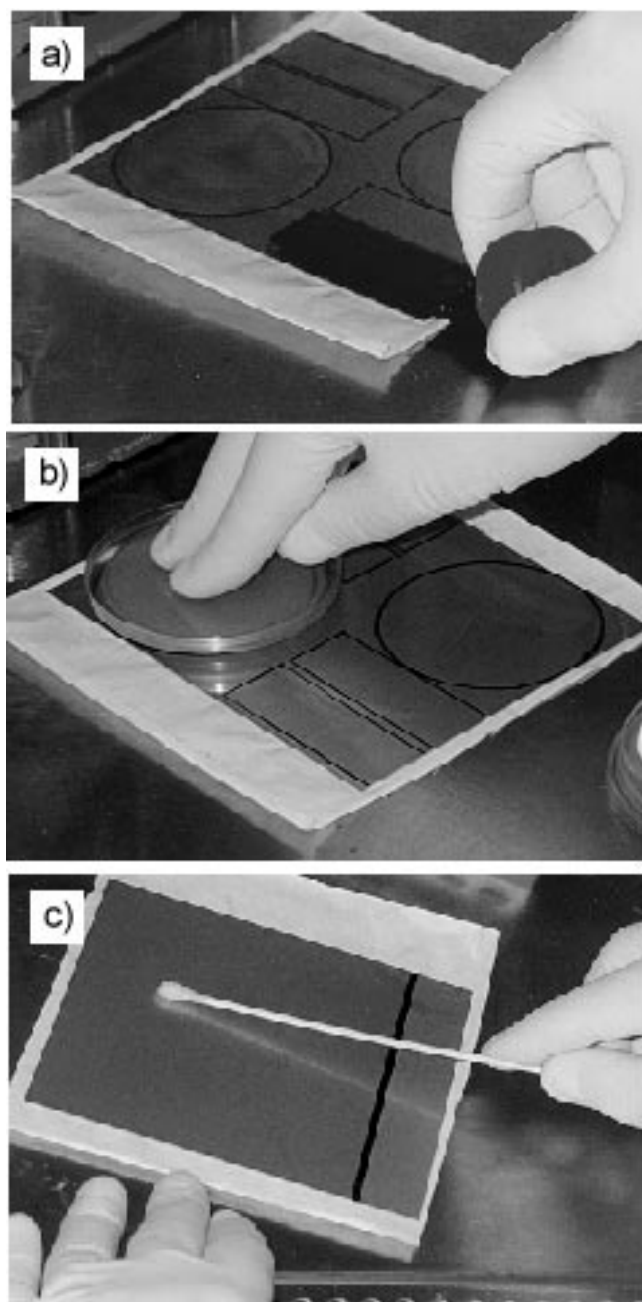


Figure 2. The sampling in the collaborative study was performed by pressing a) the dipslide Hygicult TPC, and b) the TSA contact agar plates against the artificially contaminated stainless-steel surface as well as c) swabbing the surface.

Preparation and Packaging of Test Materials

The inocula for the validation tests consisted of commercial reference cultures of *Bacillus cereus* (27), *Escherichia coli* (28), *Enterobacter cloacae* (29), and *Staphylococcus warneri* (30) obtained from the Foundation for the Advancement of Public Health and Environmental Protection, The Netherlands. The sample in the preliminary test was a bacterial mixture of *B. cereus* 5000, ATCC 9139 and *E. coli* 500, WR 1 containing ca 3000 colony-forming units (cfu)/mL, while the various samples for the final tests had bacterial contents of ca 325 cfu/mL (*S. warneri* 500, WR 51), 3750 cfu/mL (*B. cereus* and *E. coli*), and 16 250 cfu/mL (*E. cloacae* and *S. warneri*). Each inoculum was deep-frozen (-70°C) in skim milk in cryovials. Each of the 3 inocula was provided in triplicate to each collaborator as blind-coded samples.

The base solution for microbial soils was also prepared at VTT. Methylcellulose diluent (MC) was added to peptone-saline and the solution was autoclaved and dispensed into plastic tubes in 19 mL portions. The stainless-steel surfaces were wiped with alcohol to remove grease, packed individually, and autoclaved. All culture media were supplied from manufacturers in ready-to-use form. The materials used in evaluating the methods were delivered to all participating laboratories. Other equipment needed in this study, e.g. pipets, dispensers, incubators, water baths, and colony-counting equipment, are normally available in microbiological laboratories.

Distribution of Samples

The deep-frozen microbial suspensions in cryovials (one vial for the preliminary test and 9 for the final test) were packed into dry ice and sent frozen by courier to the collaborators. Each collaborator was asked to send to VTT, responsible for performance of the validation, a message describing the state in which the samples had arrived at the participating laboratory. Only one delivery melted during transport, and new samples were provided to that laboratory. The other material needed in the validation test was sent to collaborators before the microbial suspensions were sent.

Experimental

Apparatus and Glassware

(a) *Sterile stainless-steel plates.*—AISI 304, 2 B; 10×12 cm (Stala, Lahti, Finland).

(b) *Empty sterile Petri dishes.*— \varnothing 90 mm (Oriola, Espoo, Finland).

(c) *Sterile cottonwool swab sticks.*—LP112298 Italiana (Milan, Italy).

(d) *Z-shaped rod.*—Radiation-sterilized (Servant, Tampere, Finland).

(e) *Laboratory equipment.*—Autoclave, water bath for melting and tempering the agars, incubators ($30 \pm 1^{\circ}\text{C}$ and $25 \pm 1^{\circ}\text{C}$), Vortex mixer, colony counter, pipets (0.1 and 1 mL), pH meter, dispenser, scissors, or scalpels.

(f) *Laboratory glassware.*—Test tubes for dilution series.

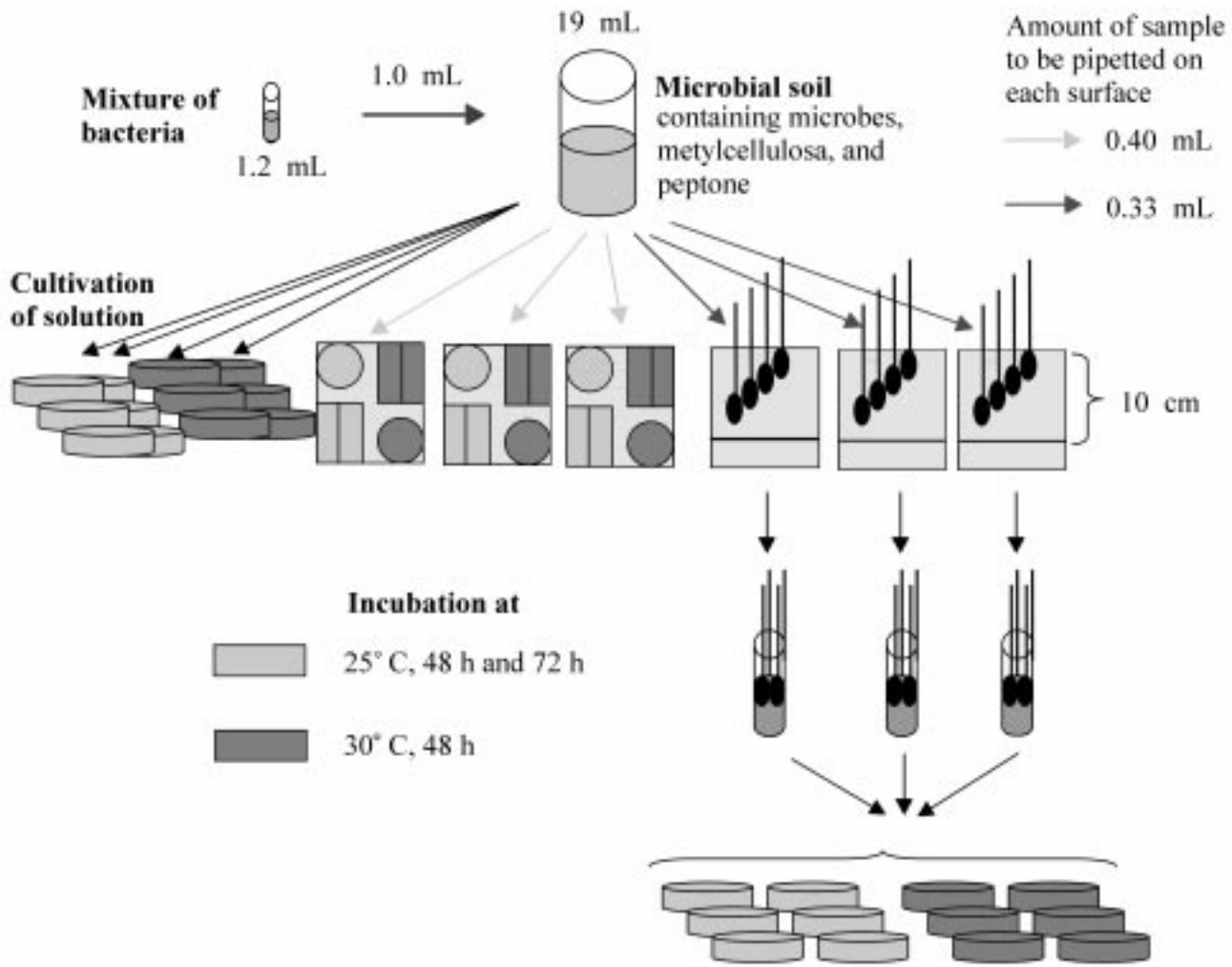


Figure 3. Test series performed in validation process of Hygicult dipslides, contact plate, and swabbing methods.

Reagents and Diluents

(a) *Diluents.*—(1) *Peptone saline diluent.*—(LabM, Bury, UK) containing 8.5 g NaCl and 1.0 g peptone in 1000 mL distilled water autoclaved at $121 \pm 1^\circ\text{C}$ for 15 min. Peptone saline was used for MC diluent and for dilution series. (2) *Sterile MC-based diluent.*—Made of 1000 mL peptone saline diluent and 6.0 g MC (‘Tylose’[®] MH 300, Fluka, Buchs, Switzerland), autoclaved at $121 \pm 1^\circ\text{C}$ for 15 min separately. MC was added aseptically after autoclaving and the diluted solution was dispensed in a disposable 50 mL tube (19 mL per tube). MC diluent was used for preparing microbial soil.

(b) *Agars.*—Soybean-casein digest agar (TSA), containing 15.0 g pancreatic digest of casein, 5.0 g papaic digest of soybean meal, 5.0 g NaCl, and 15.0 g agar in 1000 mL distilled water. TSA was available in bottles (Orion Diagnostica, Espoo, Finland) and culture medium plates (bioMerieux, Marcy l’Etoile, France).

(c) *Dipslides.*—Hygicult TPC dipslides, Orion Diagnostica.

(d) *Contact plates.*—Contact agar dishes containing TSA (BioMerieux).

(e) *Bacterial mixture tube.*—Bacterial strains were reference materials from the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

Reactivation of Microbe Samples

Each cryovial containing a frozen sample was thawed in a water bath at $37 \pm 1^\circ\text{C}$ for 120 ± 5 s immediately prior to starting a trial. A 1.0 mL volume from the vials was pipetted into a tube containing 19.0 mL MC diluent to obtain the microbial soil for the tests.

Soiling of Surface

The microbial soil mixture was pipetted onto the stainless-steel surfaces (AISI 304, 2B), in amounts of 400 μL soil on an area of 10×12 cm^2 and 330 μL soil on 10×10 cm^2 , and

evenly spread on the surfaces with a z-shaped rod. The soil was then dried on the surface for 5 min, after which sampling was performed using swabs, contact agar plates, and dipslides. The soil did not dry completely in 5 min. The participants were asked to perform soiling in a laminar-flow hood to obtain more even results in drying.

Hygicult TPC Dipslide Method

Hygicult TPC ready-made dipslides (area 17 cm²) with suitable agar for total counts of aerobic bacteria were firmly pressed for 3–5 s against the surface tested. The dipslides were incubated at 25°C for 48 and 72 h and at 30°C for 48 h.

Contact Agar Plate Method

Contact agar plates (Ø 55 mm, area 23.8 cm²) filled with TSA were firmly pressed for 3–5 s against the surface tested. The plates were incubated as described for the Hygicult dipslide method.

Swabbing Method

The swabs, 4 for each surface, were removed from their sterile wrapping and inserted into 10 mL of the peptone-saline diluent to moisten. Each swab was pressed against the tube wall to remove excess liquid, leaving the swabs moist but not saturated. Each test surface with an artificially soiled area of 10 × 10 cm² was firmly and thoroughly sampled with the swab placed at an angle of ca 30° to the surface and rubbed rigorously 4 times using a new swab each time. The direction of rubbing was changed from one time to the next. Each of the swabs was inserted into the test tube and the sticks were cut off under sterile conditions. The test tubes were mixed well, then a logarithmic dilution series was prepared for each sample. The undiluted solution was sealed using the pour plate technique with TSA. In the spread plate technique, 0.1 mL of the 10⁻¹ and 10⁻² dilutions of the sample were pipetted in duplicate onto the ready-made agar dishes of TSA. The agar plates were incubated at 25 ± 1°C for 48 and 72 h and at 30 ± 1°C for 48 h.

Theoretical Value of Microbial Soil

Conventional cultivation of the suspensions was performed to obtain the theoretical amount of bacteria pipetted onto the test surfaces. A dilution series was composed of microbial soil, cultivated onto TSA plates, and incubated at 25 ± 1°C for 48 and 72 h and at 30 ± 1°C for 48 h.

Plate Counting

The number of colonies was counted from agar plates and from Hygicult dipslides as cfu/cm². In the case of confluent growth agars, the results were reported according to the key provided with the Hygicult TPC dipslides (in counting of contact plate and dipslide results containing high numbers of colonies, a key chart with models of cell densities is needed).

Reporting and Collecting of Results

The results of the swabbing method were reported as numbers in the dilutions (Equation 1). These scores were then cal-

culated as numbers of bacteria per cm² area according to Equation 2:

$$N_v = \frac{\Sigma C}{(n_1 + 0.1n_2 + 0.01n_3)d} \quad (1)$$

where N_v = number of colonies per mL sample; ΣC = sum of colonies counted on all the dishes retained; n_1 = number of dishes retained from the first solution used (which in practice can be the neat solution followed by successive dilutions); n_2 = number of dishes retained from the second solution used; n_3 = number of dishes retained from the third solution used; and d = dilution factor corresponding to the first solution used: d is 1 if the neat solution is the first solution used and 0.1 if the first dilution is used, etc.

$$N_A = \frac{N_v V}{A} \quad (2)$$

where N_A = number of colonies per cm²; V = sample volume (= 10 mL); and A = area swabbed (= 100 cm²).

The number of colonies counted on contact plates or dipslides was reported as number of bacteria per cm² area (Equation 3):

$$N_A = \frac{\Sigma C}{nA} \quad (3)$$

where N_A = average number of colonies per cm² of sample; ΣC = sum of colonies counted on all the replicate plates retained; n = number of plates retained; and A = area of the contact plate (= 23.8 cm²) or the dipslide (= 17.0 cm²).

Collaborators were asked to transfer the results obtained to the forms provided by VTT and to send the forms back to VTT for statistical analysis and reporting.

Statistical Analysis

A large number of results from different combinations of incubation time (48 and 72 h), incubation temperatures (25 and 30°C), and the 3 methods (Hygicult TPC dipslide, contact plate, and swabbing), each with 3 levels of microbial soil, was used in the analysis of the results. The arithmetical average of the 3 (nonblind) parallel determinations mentioned above is counted as the basic result in the present study. The following procedure was adopted:

(a) All 9 combinations of the above factors for each of the 3 levels were first treated separately and examined for outliers. The Cochran C, Grubbs test cycle (26) for outliers was followed. The number of outlying laboratories in the 27 collaborative series are presented in Table 1. Out of 321 laboratory-level results, 32 (10%) were rejected as outliers.

(b) Standard precision parameters, yield, and relative yield were calculated for each of the 27 series both before and after outlier removal. Relative yield is defined as the percentage of yield out of the theoretical amount of bacteria obtained by conventional cultivation of suspension.

Table 1. Statistical evaluation of total aerobic bacterial counts based on contact agar plate, Hygicult TPC, and swabbing methods

Parameters	Microbial soil 1, low			Microbial soil 2, medium			Microbial soil 3, high		
	Contact plate	Hygicult TPC	Swabbing	Contact plate	Hygicult TPC	Swabbing	Contact plate	Hygicult TPC	Swabbing
Pooled raw data									
No. of labs	12	12	12	12	12	12	12	12	12
No. of determinations	108	108	108	105	105	105	108	108	105
Mean of theoretical yield (cfu/cm ²)	1.41	1.41	1.41	10.7	10.7	10.7	43.6	43.6	43.6
Mean of surface yield (cfu/cm ²)	0.783	0.562	0.637	2.98	2.29	2.17	9.25	8.03	9.09
Recovery, %	55.5	39.8	45.2	27.8	21.3	20.2	21.2	18.4	20.9
Precision parameters ^a :									
s _r	0.937	0.699	0.793	0.836	1.41	0.807	2.22	2.51	3.04
RSD _r , %	120	124	124	28.0	61.9	37.2	24.0	31.2	33.4
r	2.62	1.96	2.22	2.34	3.96	2.26	6.20	7.02	8.50
s _R	1.24	0.847	0.943	2.88	2.18	1.45	5.99	4.29	5.07
RSD _R , %	159	151	148	96.5	95.5	66.9	64.8	53.4	55.8
R	3.48	2.37	2.64	8.06	6.11	4.06	16.8	12.0	14.2
Pooled data without outliers									
No. of labs	10	10	10	10	12	12	10	12	12
No. of determinations	90	90	90	87	99	105	90	108	105
Mean of theoretical yield (cfu/cm ²)	1.41	1.41	1.41	10.7	10.7	10.7	43.6	43.6	43.6
Mean of surface yield (cfu/cm ²)	0.426	0.349	0.429	1.91	2.07	2.17	7.12	8.03	9.09
Recovery, %	30.2	24.7	30.4	17.8	19.3	20.2	16.3	18.4	20.9
Precision parameters:									
s _r	0.174	0.148	0.320	0.701	1.45	0.807	1.94	2.51	3.04
RSD _r , %	40.9	42.6	74.6	36.8	70.2	37.2	27.2	31.2	33.4
r	0.488	0.415	0.895	1.96	4.07	2.26	5.43	7.02	8.50
s _R	0.210	0.199	0.381	1.29	2.02	1.45	2.99	4.29	5.07
RSD _R , %	49.3	57.1	88.9	67.5	97.5	66.9	42.1	53.4	55.8
R	0.588	0.558	1.07	3.60	5.64	4.06	8.38	12.0	14.2

^a s_r = repeatability standard deviation, RSD_r = repeatability relative standard deviation, r = repeatability value, s_R = reproducibility standard deviation, RSD_R = reproducibility relative standard deviation, R = reproducibility value.

(c) The effect of incubation temperature and incubation time on the yield was examined by a mixed analysis of variance (ANOVA) model, with laboratories as a random effect, and analysis method, temperature, and incubation time as fixed effects. Systematic differences in precision parameters due to incubation time or temperature were also examined, although formal statistical tests were not performed, due to the relatively low number of cases.

(d) Once it was established that incubation time and temperature did not significantly affect the results, the series were pooled within each method, and precision parameters and yields were recalculated for each method and bacterial soil level.

Results

Effect of Incubation Temperature and Incubation Time

The results from the ANOVA (before and after outlier removal) showed that incubation temperature and incubation time, accounting for method and laboratory effects, did not have statistically significant effects on yield. To be more precise, no significant effect was found for either factor prior to outlier removal, but a singular effect of temperature at the lowest bacterial soil level was found once each bacterial level was analyzed separately. We concluded that incubation time

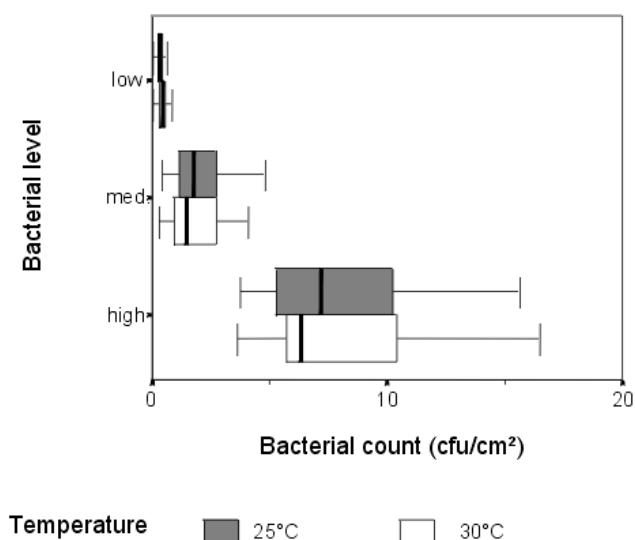


Figure 4. Comparison of incubation temperatures of 25 and 30°C after 48 h incubation based on total aerobic count of artificially contaminated stainless-steel surfaces sampled with 3 methods.

had no effect on yield and that temperature had an overall effect on yield, except possibly, at very low bacterial soil levels.

The results are illustrated in Figures 4 and 5, which show the spread of yields in box plot format. Figure 4 shows the effect of temperature for all methods, for 48 h incubation time. Figure 5 shows the effect of incubation time for all methods, at an incubation temperature of 25°C. In both cases, the results show almost completely overlapping distribution.

The effect of incubation temperature and time on precision parameters was based on examination of estimated precision parameters for similar combinations as above. No systematic effects could be detected.

Comparison of Methods

Once it was found that incubation time and temperature did not influence the results, the collaborative series were pooled and the method comparison below was based on pooled data. The effect of method on yield is illustrated in Figure 6a and the effect on recovery in Figure 6b. The ANOVA described above showed a small number of significant differences in yield between methods at low and high bacterial levels, but these are not systematic across bacterial soil levels and not significant from a practical point of view.

The yields, recovery percentages, and precision parameters for the different methods are given in Table 1 as raw data and outliers. Removing the outliers had a clear effect on both yield and precision, especially at the lowest bacterial soil level. It is obvious that both repeatability (repeatability relative standard deviation, RSD_r) and reproducibility (reproducibility relative standard deviation, RSD_R) variances of the methods increase with higher bacterial soil levels. Striking systematic differences between the methods could not be detected, although it

could be stated that in the present data, the contact plate method has the lowest repeatability and reproducibility values.

Discussion

The hygiene of surfaces, instruments, and equipment in the food industry essentially affect the quality of the final product. To ensure high quality in the final product, reliable detection of microorganisms on industrial contact surfaces is needed.

Contact methods are easy to use and labor-saving, because it is not necessary to transfer the microbes from the swab to the cultivation medium (22). Commercially available contact agars enable hygiene control in places without standard laboratory facilities such as autoclaves and laminar-flow hoods. These methods are also based on the detachment of surface-bound microorganisms, which is also the limiting factor in the swab method (16).

This collaborative study was performed using 3 microbial soils containing 1 or 2 of the 4 different microbes. The samples were incubated at 2 different temperatures using 2 different incubation periods. With these experimental arrangements, no significant effect on yield was observed at different tested incubation times and temperatures. During actual sampling, the variety of microbes and, thus, the optimum incubation time and temperature varies (22). Incubation of microbes at temperatures similar to that of the sampling site allows potentially risky microbes to grow on the sampler (22). Incubation at ambient temperature saves investment costs for incubators in small- and medium-sized food companies that do not have such equipment.

According to the results of the contact agar plate, Hygicult TPC dipslide, and swabbing methods, detection of contamination on artificially soiled stainless-steel surfaces was approxi-

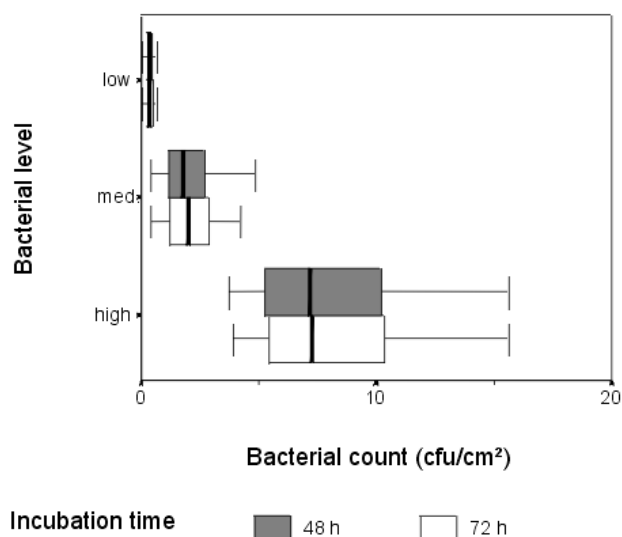


Figure 5. Comparison of incubation at 25°C for 48 and 72 h based on total aerobic count of artificially contaminated stainless-steel surfaces sampled with 3 methods.

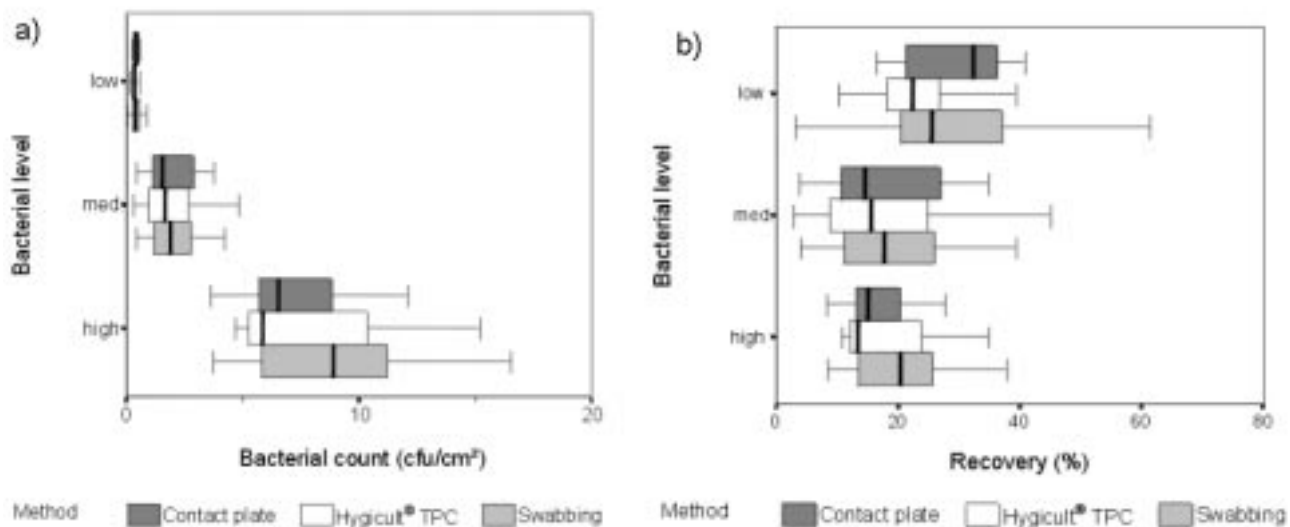


Figure 6. Comparison of contact agar plate, Hygicult TPC dipslide, and swabbing methods based on total aerobic count of artificially contaminated stainless-steel surfaces. Incubation was performed at 25°C for 48 and 72 h and at 30°C for 48 h: a) Median results and standard deviations of the methods and b) yield percentage counted using theoretical yield obtained from cultivation of unspread microbial soil.

mately 20% of the theoretical yield. Recovery from slightly contaminated surfaces (1.4 cfu/cm²) was an additional 10% higher than from heavily contaminated surfaces (10.7 cfu/cm² and 43.6 cfu/cm²). It has been reported that cells counted by direct microscopy consistently give results one log unit higher than with the cultivation method (5). Techniques based on swabbing and contact agars provide only limited information on actual surface hygiene (16). It has been clearly shown that even vigorous swabbing only detaches a small part of the actual biofilm and the cells within it (23, 31). In addition to the sampling method, microbial yield at sampling is also dependent on various factors such as surface material and topography, consistency of microbial soil, number and age of microbes, etc. (4, 14, 15). This collaborative study focused on comparing the yield of microbes detached from the test surface by swabbing, contact agar plate, and Hygicult TPC dipslide, with a known microbial load spread onto the test surface. According to these results, it can be concluded that the methods presented do not differ in practical terms either in yield or in precision.

Recommendations

We recommend the Hygicult TPC dipslide method to be used for detecting microbes from process surfaces instead of contact agar plates or swabbing.

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- E. Vatonen, Food and Environmental Laboratory in Pori, Pori, Finland
- A. Woivalin, Food and Environmental Control Laboratory in Tampere, Tampere, Finland

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