FOOD BIOLOGICAL CONTAMINANTS

Validation of the Hygicult® E Dipslides Method in Surface Hygiene Control: A Nordic Collaborative Study

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A collaborative study with *Enterobacteriaceae* was conducted to validate Hygicult® E dipslides by comparison with violet red bile glucose agar (VRBGA) contact plates and swabbing, using stainless steel surfaces artificially contaminated with microbes at various levels. Twelve laboratories participated in the validation procedure. The total number of collaborative samples was 108. The microbial level in each sample was assessed in triplicate by using the 3 above-mentioned methods. No *Enterobacteriaceae* were used at the low inoculation level. At the middle inoculation level, the percentages detached from the test surfaces were 16.6 with the Hygicult E method, 15.3 with the contact plate method, and 14.6 with swabbing; at the high innoculation level, the percentages were 14.5, 15.8, and 9.8, respectively. The percentage of acceptable results after the removal of outliers was 97.2. Repeatability relative standard deviations ranged from 33.4 to 44.9%; reproducibility relative standard deviations ranged from 45.2 to 77.1%. The Hygicult E dipslide, VRBGA contact plate, and swabbing methods gave similar results at all 3 microbial levels tested: <1.0 colony-forming units (CFU)/cm^2 at the low level, 1.2–1.3 CFU/cm^2 at the middle level (theoretical yield 8.0 CFU/cm^2), and 1.2–2.0 CFU/cm^2 at the high level (theoretical yield 12.5 CFU/cm^2).

Methods for controlling hygiene indicators are needed to obtain information on hygiene levels in the food industry. Data values are needed when hygiene-level results for different periods or sites are compared. Control of bacteria in the processes may be verified by aerobic bacterial counts as a direct measure of cleanliness or *Eschericia coli* counts as an indirect measure of fecal contamination (1). *E. coli* is a coliform, and its presence in food products indicates poor hygiene as a result of direct or indirect fecal contamination (2). Coliforms are not a taxonomic group, and their detection is dependent on the method used. Coliforms are members of the family *Enterobacteriaceae*, which is a taxonomic group (3). Therefore, the use of *Enterobacteriaceae* as hygiene indicators instead of coliforms yields much more precise results. The risk for the presence of pathogens in cases with elevated counts of *Enterobacteriaceae* is obvious (2).

The bacteria in the *Enterobacteriaceae* family are Gram-negative, aerobic or facultatively anaerobic, nonspore-forming, and rod-shaped, and they produce acid and/or gas from glucose fermentation and other carbon hydrates, that is, coliforms ferment lactose. The family *Enterobacteriaceae* is subdivided into a number of genera, e.g., *Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella,* and *Enterobacter*. Factors selective for *Enterobacteriaceae* are most often obtained by mimicking the environment of the intestine by using bile salts or more defined components with similar properties (4). For example, violet red bile glucose agar (VRBGA), violet red bile agar (VRBA), and violet red bile MUG (4-methylumbelliferyl β-D-glucuronide) agar contain bile salts No. 3 and crystal violet as selective agents. Gassner's agar contains metachrome yellow, eosin–methylene blue agar contains eosin and methylene blue, and deoxycholate agar contains sodium deoxycholate (4).

Hygiene monitoring on industrial premises is currently based on conventional cultivation using swabbing or contact plates. Validation of practical methods is needed to study sur-

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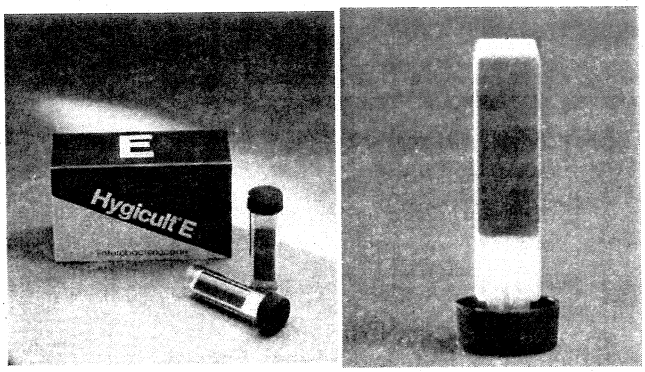

Figure 1. (Left) Hygicult E kit and (right) growth on Hygicult E dipslide.

face hygiene on industrial premises (5). Techniques 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 (6–10).

The aim of the present Nordic collaborative study was to evaluate the reliability of the Hygicult® E dipslide (Figures 1, left and right, and 2, left), which was developed for the rapid monitoring of microbiological hygiene of surfaces and solid and liquid materials (11). The Hygicult E dipslides contain VRBGA on both sides of a plastic frame that is connected to the cap by a hinge joint. This method was compared with the contact plate method (Figure 2, right), which was specially developed for taking microbiological surface samples, and the swabbing method, which is the standard method used for monitoring surface hygiene on microbially soiled surfaces (12–16) in terms of repeatability (RSD_r) and reproducibility (RSD_R) standard deviations. AOAC requirements were considered during planning and arranging the present study and reporting the results (17–20); statistical data handling was performed according to AOAC guidelines (21).

Collaborative Study

Twelve laboratories participated in this collaborative study, which was planned at the research institute VTT Biotechnology. In the validation process, 3 methods (Hygicult E dipslides, VRBGA contact plates, and swabbing) were compared by testing their suitability for detection of *Enterobacteriaceae* on artificially contaminated stainless steel surfaces. Sampling with the various methods was performed out in triplicate. Incubation was performed at 37 ± 1°C for 24 h. The test series performed at each participating laboratory is shown in Figure 3. This validation was performed jointly with the validation of Hygicult TPC (14). Each collaborator repeated this procedure for each of the 9 randomly coded samples.

Trial 1: Preliminary Samples

The purpose of the preliminary study was to acquaint the collaborators with the procedure used in the actual collaborative study. Comments on the description of the procedure were collected to obtain a manuscript containing the procedure for the sound performance of the collaborative experiments. The precollaborative study was performed with one bacterial sample. Ten primary data points were obtained by each laboratory in the preliminary test. These results were obtained from triplicate determinations for each of the 3 methods as well as for the control cultivation of the bacteria in suspension. The precollaborative test was performed out in December 1998 by the 12 laboratories participating in the collaborative study.

Trial 2: Collaborative Samples

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

Principle of Methods

The purpose of using the Hygicult E dipslide, VRBGA contact plate, and swabbing methods in this validation test was to assess the yields of various cultivation-based methods used in surface hygiene. All of these methods are based on the cultivation of *Enterobacteriaceae* detached from the surface. In the sampling techniques under study, the transfer of microbes from the surfaces differs. In the Hygicult E dipslide and VRBGA contact plate methods, microbes are transferred by pressing the surfaces with the agar that enables the growth of *Enterobacteriaceae* only. In the swabbing method, the surface is swabbed and microbes are transferred with the swab to the diluent by mixing on a vortex mixer. This diluent solution is cultivated on *Enterobacteriaceae*-specific VRBGA plates.

Preparation, Packaging, and Delivery of Test Materials

The inocula for the validation tests consisted of commercial reference cultures of *Bacillus cereus* (22), *E. coli* (23), *Enterobacter cloacae* (24), and *Staphylococcus warneri* (25) obtained from the Foundation for the Advancement of Public Health and Environmental Protection in The Netherlands. The sample in the preliminary test was a bacterial mixture of *B. cereus* 5000, American Type Culture Collection (ATCC) 9139,

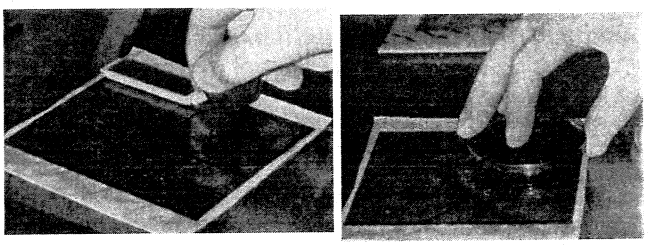

Figure 2. The sampling in the collaborative study was performed by pressing (left) the dipslide Hygicult E and (right) the VRBGA contact plates against the artificially contaminated stainless steel surface.

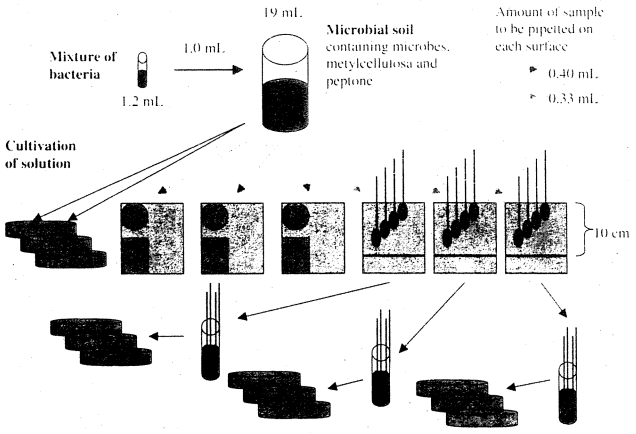

Figure 3. Test series performed in the validation of the Hygicult E dipslide, VRBGA contact plate, and swabbing methods.

and *E. coli* 500, WR 1 containing approximately 3000 colony-forming units (CFU)/mL, of which 2560 CFU/mL was *E. coli*. The various samples for the final tests had bacterial contents of approximately 325 CFU/mL, containing no *Enterobactericeae* (Table 1); 3750 CFU/mL, of which 2790 CFU/mL was *E. coli*; and 16 250 CFU/mL, of which 4660 CFU/mL was *E. cloacae*. Each inoculum was deep-frozen (–70°C) in skim milk in cryovials, and the bacterial soils were provided in triplicate to each collaborator. These samples were blind-coded, which meant that the collaborators did not know which of the 3 samples were replicates. It had not been revealed to the collaborators that the samples had 3 different bacterial compositions. The final soils were prepared in 19 mL methylcellulose (MC) as described by Salo et al. (14). The deep-frozen microbial suspensions in cryovials (1 vial for the preliminary test and 9 vials for the final test) were packed in dry ice and delivered frozen by courier to the collaborators (14). The materials used in evaluating the methods were delivered to all participating laboratories.

Experimental

Apparatus and Glassware

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

(**b**) *Empty sterile Petri dishes.*—90 mm diameter (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 (37 ± 1°C), vortex mixer, colony counter, pipets (0.1 and 1 mL), pH meter, dispenser, and scissors or scalpels.

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

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 ± 1°C for 15 min. Peptone saline was used for the MC diluent and for the dilution series. (*2*) *Sterile MC-based diluent.*—Containing 1000 mL peptone saline diluent and 6.0 g MC (Tylose® MH 300, Fluka, Buchs, Switzerland), autoclaved separately at 121 ± 1°C for 15 min. MC was added aseptically after autoclaving, and the diluted solution was dispensed in a disposable 50 mL tube (19 mL/tube). The MC diluent was used for preparing the microbial soil.

(**b**) *Agars.*—VRBGA, containing 7.0 g peptone, 3.0 g yeast extract, 1.8 g bile salts mixture, 5.0 g lactose, 5.0 g dextrose, 5.0 g NaCl, 15.0 g agar, crystal violet, and neutral red. The pH of the agar was 7.0–7.5. VRBGA is available in bottles and culture medium plates (Orion Corp., Orion Diagnostica, Espoo, Finland).

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

(**d**) *Contact plates.*—Contact agar dishes containing VRBGA (Orion Corp., Orion Diagnostica).

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

Table 1. Theoretical bacterial contents of the various microbiological soils used in the validation procedure

	Microbial soil 1, low, *S. warneri*		Microbial soil 2, medium, *B. cereus* and *E. coli*		Microbial soil 3, high, *E. cloacae* and *S. warneri*	
Parameters	CFU/mL	CFU/cm^2	CFU/mL	CFU/cm^2	CFU/mL	CFU/cm^2
Total count, mean	325	1.41	3750	10.7	16250	43.6
No. of *Enterobacteriaceae* (mean percentage)	0	<0.04 (0)	2790	7.96 (74.4)	4660	12.5 (28.7)

Table 2. Statistical evaluation of the number of *Enterobacteriaceae*, based on VRBGA contact agar plate, Hygicult E dipslide, and swabbing methods[a]

Parameters	Microbial soil 1, low			Microbial soil 2, medium			Microbial soil 3, high		
	VRBGA contact agar plate	Hygicult E dipslide	Swabbing	VRBGA contact agar plate	Hygicult E dipslide	Swabbing	VRBGA contact agar plate	Hygicult E dipslide	Swabbing
No. of labs	12 (12)	12 (12)	12 (12)	12 (10)	12 (12)	12 (12)	12 (12)	12 (11)	12 (12)
No. of determinations	36 (36)	36 (36)	36 (36)	36 (30)	36 (36)	36 (36)	36 (36)	36 (33)	36 (36)
Mean theoretical yield, (CFU/cm^2)	<0.04 (<0.04)	<0.04 (<0.04)	<0.04 (<0.04)	7.96 (7.96)	7.96 (7.96)	7.96 (7.96)	12.5 (12.5)	12.5 (12.5)	12.5 (12.5)
Mean surface yield (CFU/cm^2)	0.04 (0.04)	0.06 (0.06)	0.133 (0.133)	1.68 (1.22)	1.32 (1.33)	1.17 (1.17)	1.97 (1.97)	2.16 (1.81)	1.22 (1.22)
Recovery, %	100 (100)	150 (150)	333 (333)	21.2 (15.3)	16.6 (16.6)	14.6 (14.6)	15.8 (15.8)	17.3 (14.5)	9.75 (9.75)
Precision parameters[b]									
s_r	0.0 (0)	0.0 (0)	0.0 (0)	0.797 (0.662)	0.595 (0.595)	0.426 (0.426)	0.864 (0.864)	1.02 (0.722)	0.527 (0.527)
RSD_r, %	0.0 (0)	0.0 (0)	0.0 (0)	47.3 (39.3)	44.9 (44.9)	36.5 (36.5)	43.8 (43.8)	47.4 (33.4)	43.2 (43.2)
r	0.0 (0)	0.0 (0)	0.0 (0)	2.26 (1.87)	1.68 (1.68)	1.2 (1.2)	2.44 (2.44)	2.9 (2.04)	1.49 (1.49)
s_R	0.0 (0)	0.0 (0)	0.121 (0.121)	1.39 (0.761)	1.02 (1.02)	0.669 (0.669)	1.4 (1.4)	1.89 (1.34)	0.878 (0.878)
RSD_R, %	0 (0)	0 (0)	90.5 (90.5)	82.4 (45.2)	77.1 (77.1)	57.4 (57.4)	70.9 (70.9)	87.3 (62.1)	72.1 (72.1)
R	0 (0)	0 (0)	0.341 (0.341)	3.93 (2.15)	2.89 (2.89)	1.89 (1.89)	3.96 (3.96)	5.34 (3.79)	2.49 (2.49)

[a] Values in parentheses were calculated after outliers were removed.

[b] s_r = Repeatability standard deviation; RSD_r = repeatability relative standard deviation; r = repeatability; s_R = reproducibility standard deviation; RSD_R = reproducibility relative standard deviation; R = reproducibility.

Application of Soil to Surface

Each cryovial containing the frozen sample was thawed in a water bath at 37 ± 1°C for 120 ± 5 s immediately before each trial (14). The prepared microbial soil mixtures were pipetted onto the stainless steel surfaces (AISI 304, 2B) as described by Salo et al. (14; Figure 3) and dried on the surfaces for 5 min; after drying, sampling was performed by using swabs, contact agar plates, and dipslides.

Hygicult E Dipslide Method

Hygicult E ready-made dipslides (17 cm^2 area) with suitable agar for enterobacterial counts were firmly pressed for 3–5 s against the surface tested. The dipslides were incubated at 37 ± 1°C for 24 ± 1 h.

Contact Agar Plate Method

Contact agar plates (55 mm diameter, 23.8 cm^2 area) filled with VRBGA were firmly pressed for 3–5 s against the surface tested. The plates were incubated as described for the Hygicult E dipslide method (37 ± 1°C for 24 ± 1 h).

Swabbing Method

Each test surface with an artifically soiled area of 10 × 10 cm^2 was firmly and thoroughly sampled with 4 swabs as described by Salo et al. (14). The test tubes containing the swabs and 10 mL peptone saline were mixed well, and a logarithmic dilution series was prepared for each sample. The undiluted and diluted solutions were sealed, by using the pour and spread plate techniques with VRBGA (14). The agar plates were incubated at 37 ± 1°C for 24 ± 1 h.

Theoretical Value of Microbial Soil

Conventional cultivation of the suspensions was performed to obtain the theoretical amount of *Enterobacteriaceae* pipetted onto the test surfaces. A dilution series was composed of microbial soil, cultivated onto VRBGA plates, and incubated at 37 ± 1°C for 24 ± 1 h.

Counting Colonies, Reporting, and Collecting Results

After the test process and incubation, the number of colonies was counted from agar plates and from Hygicult E dipslides as CFU/cm^2. In the case of confluent growth agars, the results were reported according to the key provided with the Hygicult E dipslides. The results of the swabbing method were reported as numbers in the dilutions. These scores, as well as the colonies developed on contact plates (area of the contact plate, 23.8 cm^2) or dipslides (area of the dipslide, 17.0 cm^2), were calculated as numbers of bacteria per cm^2 area (14). The collaborators were asked to transfer the results obtained to the forms provided by VTT and to return the forms to VTT for statistical analysis and reporting.

Statistical Approach

The 3 methods (Hygicult E dipslide, contact plate, and swabbing), each with 3 levels of microbial soil, produced

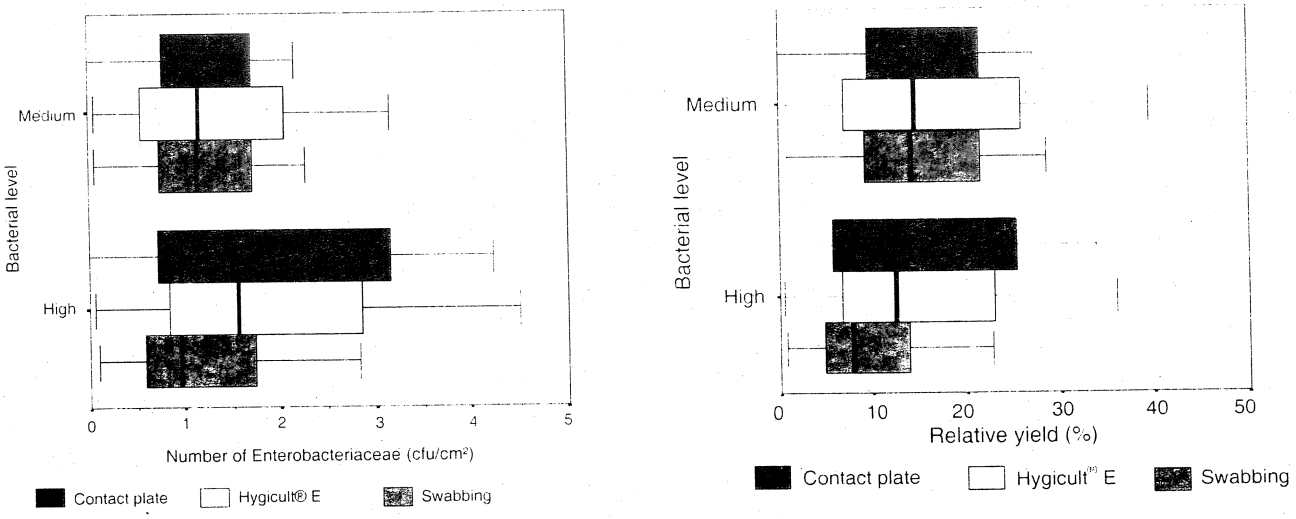

Figure 4. Comparison of VRBGA contact plate, Hygicult E dipslide, and swabbing methods according to the number of *Enterobacteriaceae* on artificially contaminated stainless steel surfaces. Incubation was performed at 37°C for 24 h: (left) median results and standard deviations of the methods and (right) yield percentages counted by using the theoretical yield obtained from the cultivation of unspread microbial soil.

9 collaborative series in each of the 12 laboratories. The arithmetical average of the 3 (nonblind) parallel determinations mentioned above was counted as the basic result in the present study. The following procedure was adopted:

(*1*) Results for all 3 methods for each of the 3 levels were treated separately and examined for the presence of outliers. The Cochran C, Grubbs test cycle (21) for outliers was followed, in which a total of 3/108 (2.8%) of the laboratory-level results were rejected as outliers.

(*2*) Standard precision parameters, yield, and recovery were calculated for each of the 9 series both before and after outlier removal. Recovery was defined as the percentage yield of the theoretical amount of bacteria obtained by conventional cultivation of the suspension. At the low bacterial level, the theoretical yield was zero, by definition, and the results for yield reflected the detection levels of the methods and the number of false-positive results.

Results

The yields, recovery percentages, and precision parameters for the different methods are given in Table 2 and are based on raw data and on data after outlier removal (the results obtained after outlier removal are used in the following discussion). At the low bacterial level, the theoretical yield was zero, by definition. The wide range of recoveries, which varied from 100 to 333% for the low-level soil samples is due to false-positive results (Table 2). Both yields and precision parameters reflect the detection levels of the methods and are not comparable to the higher levels. In Figure 4, left, the effect of the method on yield and, in Figure 4, right, the effect on recovery are illustrated for the 2 higher microbial soil levels. Systematic differences between the methods with respect to precision or to yield could not be detected.

Detection of the *Enterobacteriaceae* (*E. cloacae* and *E. coli*) occurred at a slightly lower level than did the detection of the total bacterial count (bacteria including *B. cereus* and *S. warneri*) in these trials (Table 3). Bacterial properties of *Enterobacteriaceae* such as peritrichous flagellae may affect the yield in sampling, especially in swabbing (3). Recoveries from the surfaces soiled with the medium contamination level (total count 10.7 CFU/cm^2 and *Enterobacteriaceae* count 8.0 CFU/cm^2) with the Hygicult E dipslide method were 19.3% for the total bacterial count and 16.6% for the *Enterobacteriaceae* (Table 3). Recoveries with the contact agar plate method were 17.8% (total count) and 15.3% (*Enterobacteriaceae*) and with the swabbing method, 20.2% (total count) and 14.6% (*Enterobacteriaceae*). The recoveries from heavily contaminated surfaces were, with the Hygicult E dipslide method, 18.4% (total bacteria) and 14.5% (*Enterobacteriaceae*), with the contact agar plate method, 16.3 and 15.8%, respectively, and with the swabbing method, 20.9 and 9.8%, respectively.

Discussion

Monitoring of hygiene indicator microbes is an important part of maintaining the hazard analysis and critical control point (HACCP) system (26). Common contaminants on food contact surfaces are enterobacteria, lactic acid bacteria, micrococci, streptococci, and pseudomonas; these bacteria are also common biofilm producers (5). The biofilms cause problems such as contamination, energy losses, and blockages in both industrial water systems and the food processing industry (5). Early detection of contamination and tracking of the contamination source save money and reduce health risks (26).

Table 3. Recovery (%) of total bacteria and *Enterobacteriaceae* from stainless steel surfaces contaminated with medium- and high-level microbial soils

	Microbial soil 2, medium		Microbial soil 3, high	
Method	Total bacteria	*Enterobacteriaceae*	Total bacteria	*Enterobacteriaceae*
Hygicult dipslides	19.3	16.6	18.4	14.5
Contact agar plates	17.8	15.3	16.3	15.8
Swabbing	20.2	14.6	20.9	9.8

Foodborne pathogens and foodborne disease-causing agents are subdivided into 3 main groups according to the hazards they present and the bacteria present that occur in the family *Enterobacteriaceae*: (*1*) severe hazards caused by *Salmonella choleraesuis, Salmonella typhi, Salmonella paratyphi A, Shigella* spp., (*2*) moderate hazards with potentially extensive spread caused by pathogenic *E. coli* and *Salmonella* spp., and (*3*) moderate hazards with limited spread (5). It is somewhat alarming that *Salmonella typhimurium* readily produces biofilms, causing severe disinfection and cleaning problems on surfaces in the food industry (27, 28). The bacteria in the *Enterobacteriaceae* family are therefore suitable hygiene indicators; they can be cultivated quickly and easily, and they directly or indirectly indicate the presence of fecal contaminations (1, 26). Ready-made contact agars for detection of *Enterobacteriaceae* enable hygiene control in places without standard laboratory facilities, such as autoclaves and laminar flow hoods (15).

This collaborative study focused on comparing the yield of *Enterobacteriaceae* detached from the test surface by the swabbing, VRBGA contact agar plate, and Hygicult E dipslide methods with the known number of *Enterobacteriaceae* and other microbes spread onto the test surface. The study was performed by using 3 microbial soils containing 1 or 2 of 4 different microbes; 2 of the strains used belonged to the *Enterobacteriaceae* family. The experimental arrangement was limited to simulate sampling from stainless steel surfaces after cleaning when the pH of the environment is neutral. With these experimental arrangements, no significant effect on yield was observed between the 3 test methods. The results for these methods showed that detection of contamination on artificially soiled stainless steel surfaces was approximately 14% of the theoretical yield. Similar results were obtained in studies performed by Niskanen and Pohja (12) and Eginton et al. (29). Swabbing of highly contaminated surfaces in this study gave the lowest recovery (9.8%), and the recovery for surfaces with the medium contamination level was 14.6%. The recoveries for Hygicult E dipslides and contact plates varied from 14.5 to 16.6%. The sample at the low microbial level contained no *Enterobacteriaceae*. According to these results, we conclude that the methods investigated do not differ in practical terms either in yield or in precision.

Recommendation

The authors recommend the use of the Hygicult E dipslide method for detecting *Enterobacteriaceae* from process surfaces instead of the contact agar plate or swabbing method.

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