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Evaluation of Three Different Contact Plate Methods for Microbial Surface Sampling of Naturally Occurring Human Borne Microbial Contamination.

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Summary

The ability of irradiated 55 mm diameter tryptone soya agar contact plates to recover naturally occurring surface microbial contamination, using three different manual sampling procedures, was investigated. The investigation was completed by sampling hard surfaces contaminated with microbe-carrying particles (MCPs) dispersed from a person within heavily populated environments. This is more representative of the contamination that is found within cleanrooms and avoids issues resulting from the utilisation of standard commercial test organisms to distribute unicellular microbes onto surfaces, which are likely to be transferred to the plate with different efficiencies compared to the naturally occurring MCPs. It was determined that rolling the contact plate media over the surface, using firm pressure for either 1 or 5 seconds recorded little differences in the mean recovery efficiencies (53% and 48% respectively). However, both recorded significantly higher efficiencies than just a single
1 second press of the media onto the surface with firm pressure (16%).

Key words: Microbiological surface contamination, contact plates, RODAC plates, microbe-carrying particles (MCPs), environmental monitoring, recovery efficiency.

1. INTRODUCTION

For sterile products manufacturing, it is a requirement of Annex 1 of the European Union Guide to Good Manufacturing Practice (EU GGMP) [1] that microbiological monitoring of cleanrooms includes the use of 55 mm diameter contact plates for sampling defined surface locations. The Guide includes limits to be applied to this monitoring and the expectation is that supporting data for the recovery efficiency of the sampling method should be available.
Typically, circular RODAC (replicate organism detection and counting) plates (55 mm diameter, 24 cm^2 surface area) containing nutrient agar (between 15.5 and 16 ml) are used for sampling surfaces that are relatively flat. They are poured to give an agar meniscus that protrudes just above the rim, and are based upon plates that were originally reported on in 1964 [2]. They are easy to use and require minimal training and the full area of the nutrient agar is rolled over the surface to be sampled, facilitated by the convex media profile, or they can be applied to the surface for a few seconds ensuring uniform and steady pressure with no rolling action. Viable particles removed from the surface adhere to the agar and the lidded plates are then incubated and examined and the number of colony forming units (CFU) and types of micro-organisms recovered are reported and the results expressed as the number of CFU per plate.
The recovery efficiency will be affected by many factors, including the sort of plate media used, the type of micro-organisms present on the surface and the material and finish of the surfaces [3] to be sampled. It will also be influenced by the contact area of the plate media with the surface being sampled and may be influenced by the associated duration of this contact, both of which are associated with the plate sampling method. To reduce variations associated with these two parameters, there are commercially available plate holding devices which can be utilised for the sampling that apply the same pressure each time and also have an indicator of the specified contact time, that can be set for different durations. However, the surface sampling is typically a manual procedure that does not utilise such devices and the application method and contact time are reliant on the person undertaking the sampling and hence this is likely to be more variable.
In order to determine a convenient manual sampling procedure that can be routinely used to provide more consistency of practice, three different methods were tested. Surfaces associated with three different areas, each used on a daily basis by numerous people, were utilised. These surfaces are continually contaminated with naturally occurring microbe-carrying particles (MCPs), predominantly dispersed from personnel, in relatively large numbers, and are representative of the majority of the microbes recovered from cleanroom environments. Actual cleanroom surfaces were not utilised as the surface concentrations are too low and accurate results less likely be obtained.

The use of these naturally occurring MCPs also avoids issues resulting from the utilisation of standard commercial test organisms and a carrier medium to deposit suspensions of micro-organisms onto the test surfaces. Upon evaporation of the carrier medium, sub-micron unicellular microbes are distributed across the surfaces which are not representative of the larger MCPs present in cleanrooms which have an average aerodynamic diameter of about 8 µm to 12 µm [4,5,6]. On contact, these unicellular microbes may be transferred to the plate with different efficiencies compared to the larger size naturally occurring MCPs, for which the efficiency is likely to be greater. Bench and floor surfaces in laboratory and amenities areas, contaminated with MCPs, in sufficient numbers (typically greater than 20 MCPs) that could be used for the comparison of the three sampling methods, were identified from a preliminary study. From each of three adjacent but separate positions at 20 different locations, two sequential samples were taken from the exact same place using the three different sampling methods. Following incubation of the plates, the number of recovered CFUs were counted and the recovery efficiencies for each sampling method determined from the two samples, for each location, and an average efficiency then calculated. The resultant data was reviewed in order to determine if the recovery of surface microorganisms was influenced by the sampling method and the type of surfaces sampled and to also provide recommendations for the most appropriate sampling procedure for routine use to help to ensure consistency of practice.

2. METHOD TO DETERMINE SURFACE MICROBIAL COLLECTION EFFICIENCIES

Contact plates and incubation conditions

All plates utilised were Becton Dickenson, BD BBL™ IC-XT Trypticase™ Soy Agar medium with lecithin and polysorbate 80 surface neutralising agents, 55mm diameter RODAC™ LL. The plates have locking lid features and are gamma irradiated and sealed in triplicate polythene bags, sourced from an approved supplier and are routinely tested for their ability to recover microbial contamination. Although for the purpose of this investigation, Beckton Dickinson plates were used, the results are expected to be relevant to other similar contact plates.
Following sampling, all plates were immediately and simultaneously incubated, in the same validated incubator, at 30- 35^{0}C for 5 days and the number of microbial colonies counted and identification of the organisms from all plates completed.

Sampling methods

The following three different methods of contact plate sampling were used;

Method 1 - rolling the plate over the surface in a single motion, lasting 1 second, with firm force.

Method 2 - rolling the plate over the surface in a single motion, lasting 5 seconds, with firm force.

Method 3 - plate pressed onto the surface, with no rolling, with firm force

All of the sampling, for all three methods, was performed by the same person.

Sampling locations and surface materials

A total of 20 different surface locations, from tables, benches and floors associated with the following three separate areas, were identified;

1. Microbiological testing laboratory
2. Microbiological samples receipt area
3. Amenities area used for personnel breaks and food consumption

The floor surfaces were vinyl and the bench and table surfaces were ‘Trespa’ (synthetic resin). The sampling locations are described in table 1.

Table 1 Sampling locations

Sampling Location Reference	Location Description	
	Surface	Room
1	Middle of back bench	Microbiological testing laboratory
2	Floor by cupboard	
3	Floor between benches	
4	Floor in front of instrument	
5	Middle of right hand bench	
6	Middle of left hand bench	
7	Floor between benches	
8	Floor in front of fume cupboard	
9	Floor in front of biosafety cabinet	
10	Floor by entry door	
11	Floor by entry door	Microbiological samples receipt area
12	Floor by exit door	
13	Floor middle of room	
14	Floor in front of shelf	
15	Floor in front of table	
16	Table	
17	Floor by entry door	Amenities area
18	Floor in front of microwaves	
19	Floor in front of lockers	
20	Table	

Sampling procedure

From each of the 20 surfaces locations, three separate adjacent positions in close proximity to each other were used. At the first position, a sample was taken using Method 1 (sample A), and a second sample using the same method (sample B) was immediately taken at the exact same position. At the second adjacent (non-sampled) position, the sampling was repeated using Method 2. Finally in the third adjacent position, also not previously sampled, samples were taken in an identical manner using Method 3.
Plates were labelled with the sampling method, the location from which the sample had been taken and with the first (A) or second (B) sample reference. A total of 120 plates (40 for each sampling method) were utilised.

Determination of recovery efficiency

A mathematical model is described which may be used to assess the efficiency and consistency of a surface sampling method [7]. This is based upon multiple and two stage sequential sampling and the two stage sampling is a convenient method if the counts on the surface following the second sampling are relatively low. The recovery efficiency for the two stage sampling can be determined using equation 1 [7].

Recovery efficiency (%) = [1 – (B /A)] x 100 **Equation 1**

Where

B = total count from second sample
A = total count from first sample

3. TEST RESULTS

The results for the testing and the recovery efficiencies for each of the three different sampling methods are shown in table 2. All contaminated plates were subject to Matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectrometry identification and this confirmed the majority of microbes to be the expected Gram positive skin microbes and typical environmental microbes. Species of *Staphylococcus, Micrococcus* and *Bacillus* were most commonly identified, along with fewer *Microbacterium* and single isolates of other organisms such as *Dermacoccus* and *Okibacterium.* Shown in table 3 is a summary of the identification of the micro-organisms recovered from the surfaces, from both the first and second plate samples.

Table 2 Plate counts and recovery efficiencies for each sampling method

Sample Reference	Plate Count (CFU)								
	Sampling Method 1			Sampling Method 2			Sampling Method 3		
	Bacteria	Mould	Recovery Efficiency (%)	Bacteria	Mould	Recovery Efficiency (%)	Bacteria	Mould	Recovery Efficiency (%)
1A	23	0	87.0	41	0	73.2	57	0	71.9
1B	3	0		11	0		16	0	
2A	45	2	59.6	31	1	34.4	41	0	31.7
2B	19	0		21	0		27	1	
3A	41	1	47.6	29	1	26.7	42	0	50.0
3B	22	0		16	6		21	0	
4A	44	0	61.4	31	2	72.7	23	0	-30.4
4B	15	2		8	1		29	1	
5A	9	0	88.0	2	0	100.0[c]	1	0	-100.0
5B	1	0		0	0		2	0	
6A	5	1	83.3	7	0	57.1	9	0	-44.4
6B	1	0		3	0		13	0	
7A	49	0	46.9	41	0	68.3	37	0	24.3
7B	26	0		13	0		24	4	
8A	134	0	76.9	42	1	-48.8	50	0	48.0
8B	31	0		64	0		25	1	
9A	59	1	40.0	104	0	58.7	42	0	45.2
9B	33	3		40	3		23	0	
10A	44	0	34.1	41	3	81.8	32	0	34.4
10B	29	0		8	0		21	0	
11A	17	0	52.9	40	1	46.3	33	1	2.9
11B	8	0		18	4		33	0	
12A	29	0	13.8	46	8	64.8	20	3	56.5
12B	25	0		14	5		7	3	
13A	33	2	74.3	42	2	65.9	24	3	-3.7
13B	9	0		15	0		26	2	
14A	48	0	27.1	26	6	43.8	22	3	8.0
14B	30	5		16	2		23	0	
15A	30	0	56.7	34	0	76.5	15	0	40.0
15B	12	1		8	0		9	0	
16A	5	1	-16.7	4	1	80.0	5	0	60.0
16B	7	0		1	0		1	1	
17A	50	1	27.5	35	0	-25.7	21	0	-19.0
17B	34	3		44	0		25	0	
18A	33	0	66.7	30	0	40.0	13	1	-35.7
18B	11	0		18	0		19	0	
19A	13	3	62.5	13	0	-7.7	14	0	42.9
19B	6	0		14	0		7	1	
20A	17	0	64.7	12	1	46.2	6	1	42.9
20B	5	1		5	2		3	1	
Total A[a]	740			678			519		
Total B[a]	342			360			369		
Total A and B[a]	1082			1038			888		
Mean Recovery Efficiency[b]	53%			48%			16%		
Standard Deviation	26%			38%			44%		

Notes
a. Combined bacteria and mould counts
b. Mean of all of the individually calculated recoveries
c. The plate B count is 0 but there are counts with plate A and a 100% efficiency recorded

Table 3 Identification of recovered micro-organisms

Genus	Species
Micrococcus	*luteus, antarcticus*
Staphylococcus	*hominis, epidermidis, haemolyticus, ureilyticus, warneri, succinus, saprophyticus, equorum, saprophticus*
Microbacterium	*saccharophilum*
Corynebacterium	*tuberculostearicum, coyleae*
Bacillus	*myciodes, amyloliquefaciens, licheniformis, amyloliquefaciens, cereus, thuringiensis*
Mesobacillus	*thioparans, subterraneus*
Cytobacillus	*kochii*
Solibacillus	*silvestris*
Paenibacillus	*taiwanensis, glucanolyticus*
Brevundimonas	*vesicularis*
Okibacterium	*fritillariae*
Dermabacter	*hominis*
Curtobacterium	*flaccumfaciens*
Pantoea	*agglomerans*
Rathayibacter	*rathayi*
Arthrobacter	*(pseudarthrobacter)- sulforivorans, equi, chlorophenolicus, oryzae*
Gordonia	*hongkongensis*
Rothia kristinae	*kristinae*
Kocuria	*arsenatis, iridica, palustris, rhizophila*
Moraxella	*osloensis*

4. DISCUSSION OF RESULTS

Combined bacteria and mould data

Table 2 shows the bacteria and mould counts and it can be seen from this table that the total count for bacteria (2904) is much larger than for mould (104) and consequently, there are too few mould counts to estimate the sampling efficiencies of the three methods for moulds alone. Although there are likely to be differences in the recovery efficiency of moulds compared to bacteria, fundamentally due to differences in their sizes, it is reasonable to combine all of the counts, as the low mould counts are only a small proportion (3.5%) of the total counts. This small proportion will not have a significant influence to the mean recovery efficiency calculations. Additionally, mould may also be (rarely) recovered from the cleanroom environment, as well as a much higher relative number of bacteria, and so the inclusion of these low mould counts will be more representative of actual cleanroom environmental conditions. Table 3 shows the identification of the recovered micro-organisms and confirms them to be human borne and general environmental isolates, typical of what is recovered from pharmaceutical manufacturing cleanrooms.

Overall number of MCPs reported for each sampling method

Shown in figure 1 are the total counts (plates A and B) at each location for each of the three sampling methods. Method 1 showed the highest number of total counts (1082 and highest at 11 of the 20 locations). Method 2 had a similar total number of counts (1038) and Method 3 had the lowest number of counts (888 and lowest at 9 of the 20 locations). If the number of counts associated with just the first samples (sample A) are considered, counts of 740, 678 and 519 were recorded for Methods 1, 2 and 3 respectively. This is shown in figure 2 and shows a distribution similar to the total counts shown in figure 1. In terms of initial recovery, this suggests that Method 1 is superior to Method 2 and that Method 2 is superior to Method 3. Application of a Friedman test (adjusted for ties) gives a p-value of 0.009, which against the criterion of <0.05, is statistically significant. It has however been assumed that the three adjacent sampling positions at each location have similar microbial concentrations and that the higher counts associated with Methods 1 and 2, compared with Method 3, are due to the superior effectiveness of the sampling procedures. This is a reasonable assumption and the likelihood that all of the sampling locations associated with Method 3 were consistently lower than the adjacent locations is unlikely, as demonstrated by figures 1 and 2, where the distributions are generally consistently reduced at all locations, for both the first (sample A) and also the second (sample B) samples. Additionally, the two plate sampling procedure utilised, with sequential samples at the exact same position, will also be useful to address any local variations in surface concentrations associated with the different positions at the same sampling location. This provides further confidence that the results can be utilised to provide an accurate reflection of the actual recoveries associated with each sampling method.

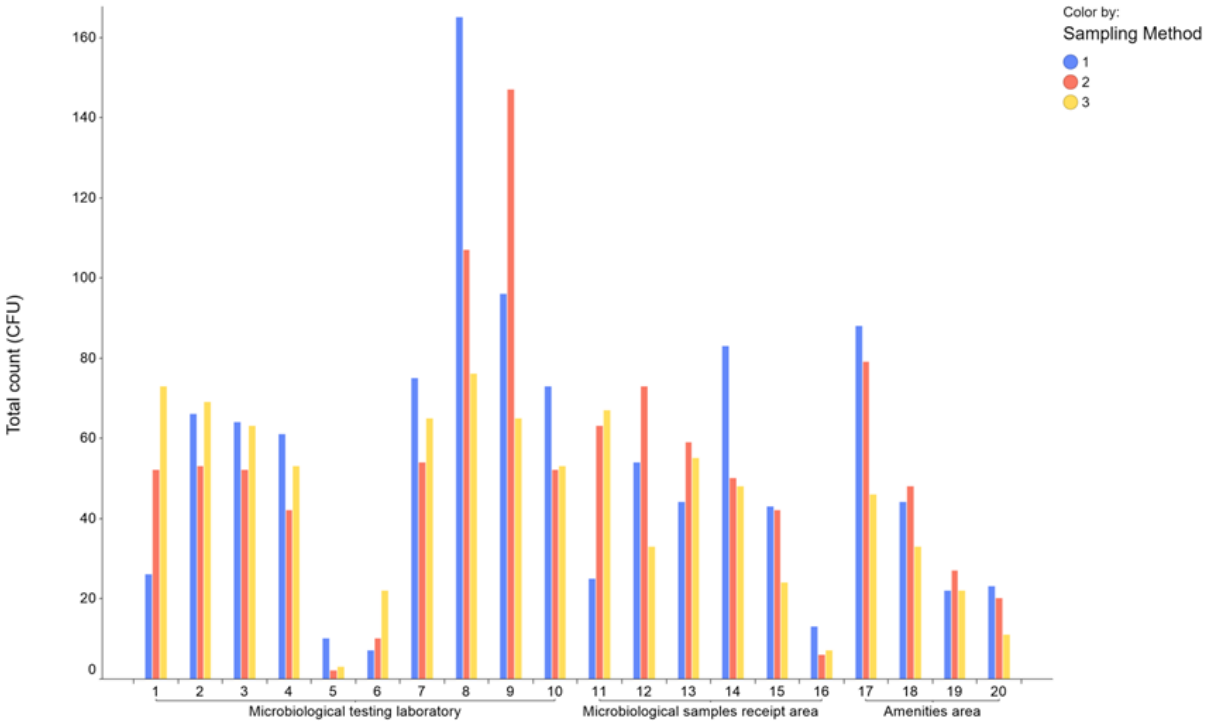

Figure 1 Total counts (samples A and B) and sampling locations

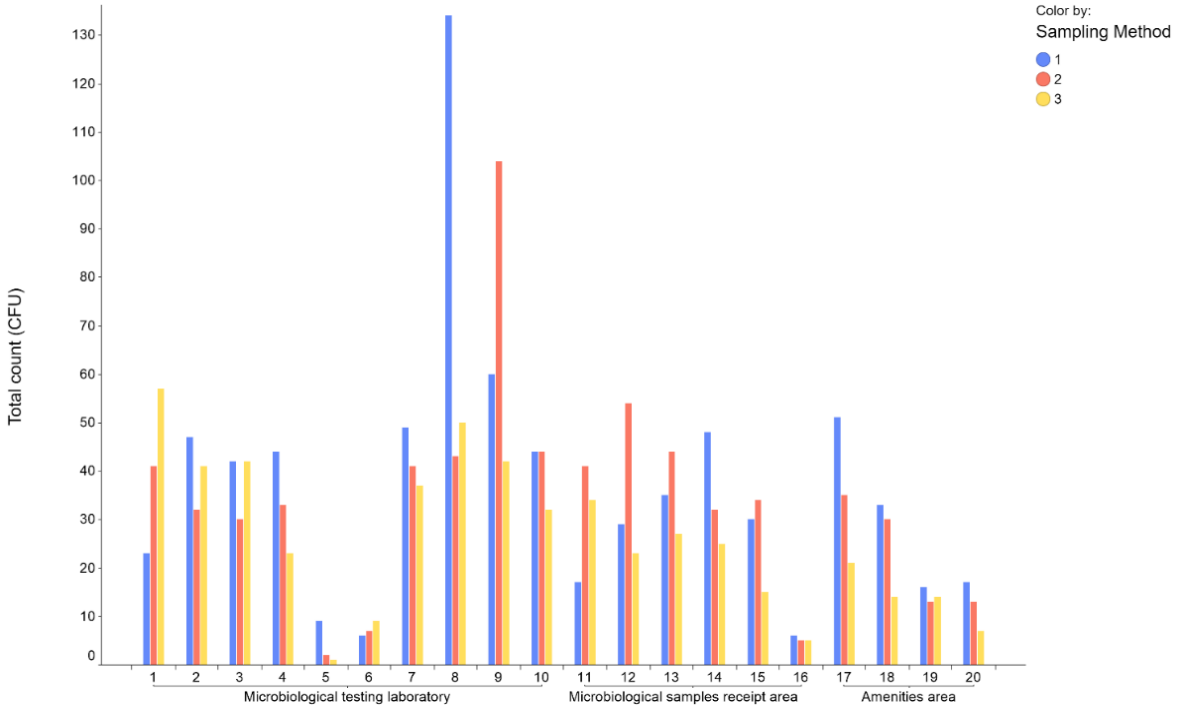

Figure 2 First sample counts (sample A) and associated sampling areas and their locations

Calculated recovery efficiencies

Shown in table 2 are the mean recovery efficiencies which are 53%, 48% and 16% for sampling Methods 1, 2 and 3 respectively. These were determined for combined bacteria and mould counts and to avoid bias from the samples that had the higher counts, the efficiencies were determined for each location and the mean efficiency calculated from all of the individual efficiencies. Shown in figure 3 are the individual recovery efficiencies, at each of the twenty surface locations, for each sampling method. This shows that for some of the sampling (17%), the count recorded for the second sample was higher than for the first sample and the calculated recovery efficiency is a value less than zero, which is not possible. These 'impossible efficiencies' are likely to be related to the acknowledged errors and inconsistencies that are associated with the microbiological testing methods and become more significant when dealing with very low counts. They will have an influence on the calculated recovery efficiencies but as all of the sampling is subject to the same microbiological testing method, these samples have been retained and all results considered as a collective set, and will provide a worst-case underestimate of the recovery efficiencies. It should also be noted that the majority of the impossible efficiencies occurred with Method 3 (60%) with lesser occurrences with Method 2 (30%) and Method 1 (10%).

One contributory reason for the reduced overall recovery efficiency of sampling Method 3 is likely to relate to the reduced area of contact between the surface being sampled and the plate media, as the procedure involves only pressing the media onto the surface with no rolling action that is utilised with Methods 1 and 2. Simple experiments completed at AZ Macclesfield involving replicating both sampling methods on coloured paper and determining the areas deposited by the wet media surface, indicated that sampling Method 3 has only approximately 89% of the contact area compared to Methods 1 and 2. However, even when this correction factor is applied to Method 3, it does not account for all of the reduced recovery efficiency compared with the other two sampling methods and other factors must therefore be applicable.

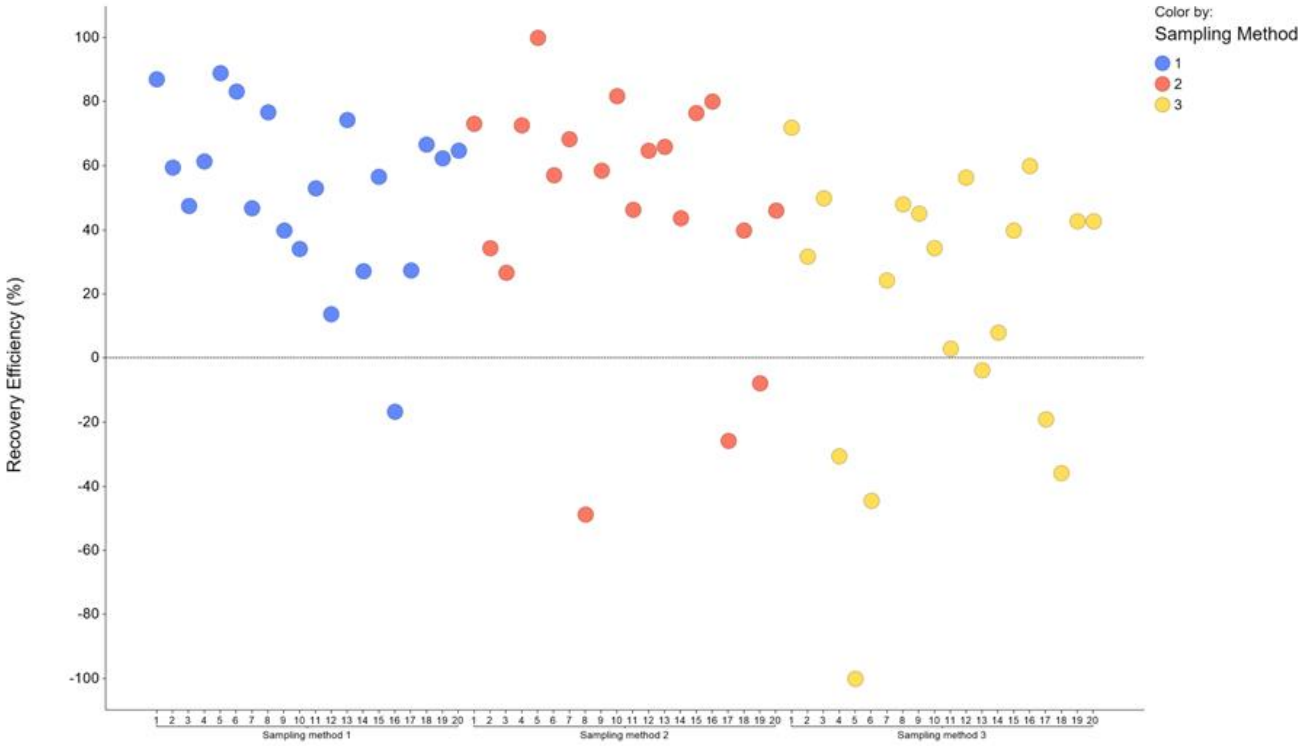

Figure 3 Recovery efficiencies for each surface location and each sampling method

Recovery from different types of surfaces

It is also useful to determine if there is any distinction between the recovery from the different surfaces. Table 2 confirms that the first plate counts are generally lower for the benches and tables compared to the floors where locations 5 and 6 (both microbiology testing laboratory), location 16 (sample receipt area) and location 20 (amenities area) are the four locations with the lowest counts. The bench at location 1 (microbiology testing laboratory) is an exception to this, with counts similar to the floor samples from the microbiology testing laboratory (locations 2 to 4 and 7 to10). Shown in figure 4 are the recovery efficiencies for each sampling method relevant to the surfaces that were sampled. Although there were only 5 bench and table samples compared to 15 floor samples, overall, these results suggest that the initial microbial concentrations are lower for the tables and benches than for the floor samples. However, the recovery efficiency of Methods 1 and Method 2 for bench and table samples are similar to the corresponding recovery efficiency for floor samples.

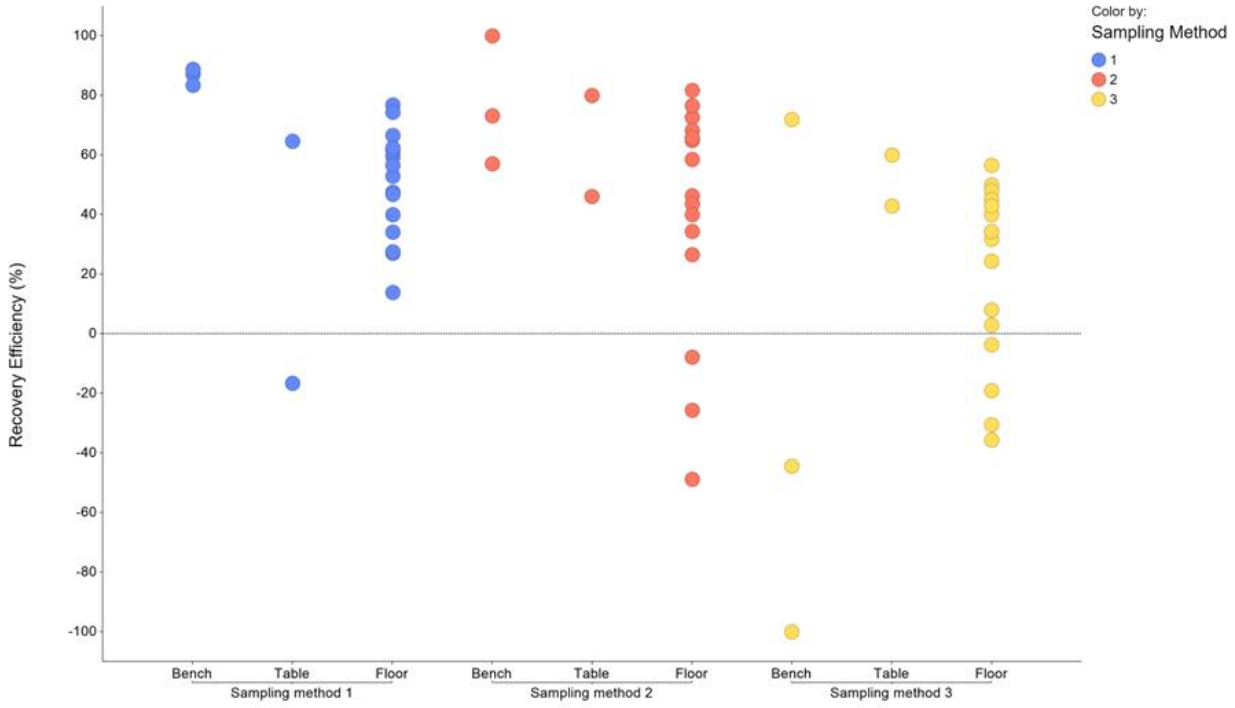

Figure 4 Recovery efficiency for bench, table and floor locations for each sampling methods

5. CONCLUSIONS

The recovery of naturally occurring surface microbial contamination with sterile tryptone soya agar 55 mm diameter RODAC plates, using three different manual sampling methods has been evaluated. It was shown that the two sampling methods (Methods 1 and 2) that rolled the plate media over the surface under consideration both had similar recovery efficiencies (53% and 48% respectively) and were better than the sampling method (Method 3) that just pressed the plate media onto the surface (16%). Method 3, as well as having a poorer recovery efficiency than the other methods, also showed a more variable range of efficiencies. Although sampling Methods 1 and 2 both rolled the plate media over the surface but with different contact times (1 second and 5 seconds, respectively) there was little difference in the recovery efficiency. It was determined that the reduced counts associated with sampling Method 3 were unlikely to be fully due to a reduced area of contact of the plate media with the surface sampled compared with Methods 1 and 2 (a reduction of 89%). Additionally, it was considered that there were no significant reductions in the surface microbial concentrations that were sampled using Method 3 that would account for the reduced efficiency. Issues associated with surface variations would, however, be offset by the two plate sampling procedure utilised that identically and sequentially sampled from the exact same position, at each sampling location. It was also noted that for the two different types of hard surfaces utilised (vinyl and 'Trespa') for the evaluation, there were no differences in the recovery efficiencies. Overall, it is concluded that there is little difference between Methods 1 and 2 but both are better sampling procedures than Method 3.

For such routine manual surface sampling of the cleanroom areas, the single use of sampling Method 1 would help to ensure consistency of practice. Due to known variations in the recovery efficiencies associated with different surfaces [3], further investigation of the recovery associated with different cleanroom surfaces, such as garment fabric, glove material and stainless steel should be

evaluated. This could be similarly done utilising naturally occurring surface microbial contamination and sampling Method 1 to provide comprehensive information regarding recovery efficiencies for different cleanroom surfaces.

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