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Study Title: Evaluation of the ProtoCOL 3 instrument for enumeration of bacterial and fungal colonies on agar plates

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SUMMARY

The ProtoCOL 3 instrument (Synoptics) was evaluated for enumeration of bacterial and fungal colonies on agar plates. These plates comprised surface spread plates and spiral plates produced from the following organisms:

Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Kocuria rhizophila, Enterococcus faecalis, Mannheimia haemolytica, Bacillus subtilis, Streptococcus pneumoniae (all bacteria) *Candida albicans* (a yeast) and *Aspergillus brasiliensis* (a mould). These species were selected because they produce colonies of differing shapes and sizes and also require different agar formulations for growth. Thus, both translucent and opaque agars were included in the evaluation.

Spiral plates and conventional surface spread plates were prepared for each of these organisms. For each plate that yielded discrete, countable colonies, the accuracy of colony count results obtained using ProtoCOL 3 was assessed by comparison with manual reading of the same plates. Manual plate reading was the reference method. For each plate type (spiral and spread) the comparison between ProtoCOL 3 and manual counts was analysed using a two-tailed t-test for paired samples. In the case of spiral plate data, the log cfu/ml figures were used for analysis; for conventional spread plate data, the count per frame was used. Results obtained using the t-test were $p = 0.105$ for spiral plate data and $p = 0.143$ for spread plate data. Thus, the t-test did not identify significant differences between methods, for either plate type, at the 95% confidence level.

Therefore, based on spiral plate data and spread plate data obtained from the ten microbial species used in the present study, no statistically significant differences could be identified between the ProtoCOL 3 instrument and conventional manual colony counting, for the enumeration of colonies on agar plates.

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1. INTRODUCTION

1.1 Study Objectives

To evaluate the ProtoCOL 3 instrument (Synoptics) for enumeration of bacterial and fungal colonies on agar plates.

1.2 Description of Test Methods

Agar plates used in the evaluation comprised surface spread plates and spiral plates produced from the following organisms:

Bacteria: *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Kocuria rhizophila, Enterococcus faecalis, Mannheimia haemolytica, Bacillus subtilis, Streptococcus pneumoniae*

Yeast: *Candida albicans*

Mould: *Aspergillus brasiliensis* (formerly *Aspergillus niger*).

These species were selected because they produce colonies of differing shapes and sizes and also require different agar formulations for growth. Thus, both translucent and opaque agars were included in the evaluation. Spiral plates and conventional surface spread plates were prepared for each of these organisms. For each plate that yielded discrete, countable colonies, the accuracy of colony count results obtained using ProtoCOL 3 was assessed by comparison with manual reading of the same plates. Manual plate reading was the reference method.

2. RESPONSIBILITIES

2.1 Name and Address of Test Laboratory

Don Whitley Scientific Limited (DWS)
14 Otley Road
Shipley
West Yorkshire
BD17 7SE

2.2 Name and Address of Sponsor

Synoptics Ltd
Beacon House
Nuffield Road
Cambridge
CB4 1TF

3. MATERIALS AND METHODS

3.1 Test apparatus

The ProtoCOL 3 instrument was supplied by Synoptics Limited, Cambridge, UK. The serial number of the instrument provided for this evaluation was P3PC/2017.

3.2 Bacterial strains

Colony count performance of the ProtoCOL 3 was assessed using the following bacterial and fungal strains, which were cultured on the agar media shown below:

Key: PCA – Plate Count Agar CBA – Columbia Blood Agar
SDA – Sabouraud Dextrose Agar

DWC code	Identification	Description	Agar
DWC 9874	*Pseudomonas aeruginosa*	Type strain - ATCC 9027	PCA
DWC 9313	*Escherichia coli*	Type strain - NCTC 12241	PCA
DWC 15848	*Staphylococcus aureus*	Type strain - ATCC 29213	PCA
DWC 25216	*Kocuria rhizophila*	Type strain - DSM 348	PCA
DWC 9314	*Enterococcus faecalis*	Type strain - NCTC 12697	PCA
DWC 21825	*Mannheimia haemolytica*	Type strain - ATCC 33396	CBA
DWC 10090	*Bacillus subtilis*	Type strain - NCIMB 8054	PCA
DWC 25963	*Streptococcus pneumoniae*	Type strain - ATCC 9619	CBA
DWC 0081	*Aspergillus brasiliensis*	Type strain - ATCC 16404	SDA
DWC 9875	*Candida albicans*	Type strain - NCPF 3179	SDA

Each strain is held within the DWS culture collection and identified by its unique “DWC” code, as shown above. While not in use for experimental work, strains were routinely stored in cryoprotective suspensions at a nominal temperature of -80°C.

3.3 Culture media, media supplements and other reagents)

(Abbreviations for subsequent use are shown in the right-hand column)

Plate Count Agar (Oxoid; CM0325) - PCA
Sabouraud Dextrose Agar (Oxoid; CM0041) - NA
Columbia Agar Base (Oxoid; CM0331)
Maximum Recovery Diluent (Lab M; LAB103) - MRD
Horse blood, defibrinated (Southern Group Laboratory; 9011B)

Each culture medium was prepared in accordance with the manufacturer's instructions and the DWS Media Preparation Manual. After autoclaving and cooling to 47±2°C, Columbia Agar was supplemented with 5% v/v defibrinated horse blood before pouring into sterile petri dishes.

3.4 Procedure: preparation of agar plates for colony counting

3.4.1 Each bacterial culture was subcultured from frozen stocks onto the appropriate agar medium, as indicated in 3.2 above.

3.4.2 Agar plate cultures were incubated at 37°C for approximately 24 h (bacterial strains), at 30°C for approximately 48 h (*C. albicans*) or at 30°C for 7 days (*A. brasiliensis*).

3.4.3 For each strain, with the exception of *A. brasiliensis*, cells were collected from 3 to 5 colonies on the incubated plate and the collected material was suspended in sterile MRD, with vortex mixing, to produce turbidity equivalent to that of a 0.5 McFarland standard. A suspension adjusted in this way contains approximately 1.0×10^8 colony-forming units (cfu) per ml.

3.4.4 In the case of *A. brasiliensis*, the plate surface was flooded with MRD and fungal hyphae were emulsified in the diluent, which was then pipetted from the plate into a sterile vial.

3.4.5 Each bacterial and fungal suspension was used to prepare serial decimal dilutions in MRD, to a final level of 10^{-7}.

3.4.6 For each bacterial or fungal strain, dilutions were applied to the surface of the appropriate agar plates (see 3.2) using spiral plating and conventional surface spread plating, as follows:

- Spiral plates were produced using the Whitley Automatic Spiral Plater (WASP 2). A 50 µl volume of each dilution 10^{-2}, 10^{-3} and 10^{-4} was applied to 90 mm plates of the appropriate agar.

- Conventional spread plates were produced by applying a 0.5 ml volume of each dilution 10^{-5}, 10^{-6} and 10^{-7} to 90 mm plates of the appropriate agar and distributing the inoculum with a sterile plastic "hockey stick" spreader.

3.4.7 Each prepared plate was incubated as described in 3.4.2 above, with the exception that *A. brasiliensis* plates were incubated for only 4 days to ensure the formation of discrete, countable colonies.

3.5 Procedure: selection of plates for colony counting

3.5.1 For each organism, a single incubated plate was selected from each series (spiral plate and spread plate) on the basis that discrete colonies were obtained. Thus, a single spiral plate and a single spread plate were read for each bacterial or fungal species.

3.6 Procedure: colony counting with ProtoCOL 3

3.6.1 Each selected plate was placed on the ProtoCOL 3 stage and the instrument was configured in accordance with the manufacturer's instructions. The following settings were used:

- When reading CBA plates (for *M. haemolytica* and *S. pneumoniae*), lower illumination was removed by inserting the black plate.
- Software settings were adjusted to count light colonies on a dark background or dark colonies on a light background, as appropriate.
- When reading spiral plates for E. coli and C. albicans, which produced relatively large, touching colonies, it was necessary to adjust the "sensitivity" slider control from "Automatic" to "99%". This was necessary to achieve accurate separation of touching colonies.
- Spiral plates were read using the spiral frame setting with two sectors.
- Conventional spread plates were read using the circular counting frame

3.7 Procedure: manual colony counting

3.7.1 Colonies on both spiral and spread plates were enumerated manually, using the on screen image of the plates produced by ProtoCOL 3.

4. RESULTS AND DISCUSSION

4.1 Colony count results achieved using ProtoCOL 3 and manual count methods are presented in Table 1

4.2 For each plate type (spiral and spread) the comparison between ProtoCOL 3 and manual counts was analysed using a two-tailed t-test for paired samples (Microsoft Excel 2010 software).

In the case of spiral plate data, the log cfu/ml figures were used for analysis. The logarithmic counts (*i.e.* transformed data) were used to ensure normally distributed data. Furthermore, the "raw" colony counts (count per sector) could not be used for analysis because, in some cases, the selected sector differed between ProtoCOL and manual counts.

In the case of conventional spread plate data, the count per frame was used for analysis. This ensured that the raw colony counts within the circular frame were compared.

4.3 Results obtained using the t-test were as follows:

Spiral plate data: $p = 0.105$. Thus, there was no difference between the ProtoCOL 3 colony counts and the corresponding manual colony counts, at the 95% confidence level.

Spread plate data: $p = 0.143$. Thus, there was no difference between the ProtoCOL 3 colony counts and the corresponding manual colony counts, at the 95% confidence level.

[for a significant difference to be identified, it would be necessary for "p" t be ≤ 0.05].

4.4 Based on spiral plate data and spread plate data obtained from the ten microbial species used in the present study, no statistically significant differences could be identified between the ProtoCOL 3 instrument and conventional manual colony counting, for the enumeration of colonies on agar plates.

Table 1 Comparison of ProtoCOL 3 with manual counting for enumeration of colonies on spiral plates and conventional surface spread plates

Organism name	Spiral Plates									Pour Plates (count per frame)	
	Dilution	ProtoCOL 3 Count				Manual Count				ProtoCOL 3 Count	Manual Count
		Sector	Colony Count	cfu/ml	log cfu/ml	Sector	Colony Count	cfu/ml	log cfu/ml		
Pseudomonas aeruginosa	10^{-4}	3a	79	3.0×10^{8}	8.5	3a	68	2.6×10^{8}	8.4	211	203
Escherichia coli	10^{-4}	3a	54	2.0×10^{8}	8.3	3a	50	1.9×10^{8}	8.3	42	42
Staphylococcus aureus	10^{-4}	3b	45	3.3×10^{8}	8.5	3b	46	3.4×10^{8}	8.5	100	97
Kocuria rhizophila	10^{-4}	4a	69	5.5×10^{7}	7.7	4b	42	5.6×10^{7}	7.7	220	203
Enterococcus faecalis	10^{-4}	4a	54	4.3×10^{7}	7.6	4a	50	4.0×10^{7}	7.6	82	76
Mannheimia haemolytica	10^{-4}	3a	83	3.1×10^{8}	8.5	3a	62	2.3×10^{8}	8.4	71	72
Bacillus subtilis	10^{-4}	4c	52	1.1×10^{8}	8.0	4c	51	1.1×10^{8}	8.0	60	62
Streptococcus pneumoniae	10^{-2}	3b	106	7.7×10^{6}	6.9	4c	100	7.3×10^{6}	6.9	43	43
Aspergillus niger	10^{-4}	Whole plate	37	7.4×10^{6}	6.9	Whole plate	41	8.2×10^{6}	6.9	29	29
Candida albicans	10^{-4}	4b	67	8.9×10^{7}	7.9	4b	52	6.9×10^{7}	7.8	124	125