DIAGNOSTIC MICROBIOLOGY

A comparison of the isolation rates of *Salmonella* and thermophilic *Campylobacter* species after direct inoculation of media with a dilute faecal suspension and undiluted faecal material

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Regardless of media used, dilution of faecal samples before direct plating may improve isolation rates and reduce subcultures by freeing organisms from the faecal mass and diminishing competing flora. Despite the routine use of dilution in many laboratories, it has never been established properly whether direct or dilute inocula should be used in primary plating of faeces. A total of 3764 faecal samples was examined in four laboratories with a standardised methodology. The isolation rates, competing flora and confirmatory work performed for *Salmonella* spp. and *Campylobacter* spp. from primary plating media with a dilute faecal inoculum were compared with those after direct inoculation of faecal material. Inoculum effects on the isolation of *Shigella* spp. could not be assessed as only one isolate occurred during the study period. The overall isolation rates of both major enteric pathogens were unaffected by the inoculum. However, significantly fewer wasted subcultures were recorded with a dilute inoculum for *Campylobacter* spp., and competing flora was reduced in all cases without diluting out small numbers of the pathogen.

Introduction

Several authors [1–3] have identified the requirement for a standardised faecal inoculum when using enrichment media for salmonellae, usually a suspension in quarter-strength Ringer's solution. However, whether a similar dilution step is required for the optimal inoculation of primary plating media has never been established. Many laboratories currently use a dilute inoculum because organisms are released from the faecal mass and competing flora are reduced, allowing better colony separation and facilitating identification. Lynton-Moll and Johnson [3] mentioned that dilution gave a particular advantage over direct plating when low numbers of shigellae are present, but gave no supporting data. If isolation rates from primary plating can be improved by this method, clinicians may receive positive results earlier and laboratory costs may be lowered by a reduction in the number of subcultures.

The use of direct and dilute faecal inocula for primary plating was evaluated in four laboratories with a standardised protocol. Data were collected to determine isolation rates of *Salmonella* spp., *Shigella* spp. and thermophilic *Campylobacter* spp., to assess the levels of competing flora present and to gauge the relative amounts of confirmatory work generated.

Materials and methods

Media

Xylose lysine desoxycholate agar (XLD) (CM469; Oxoid) was prepared according to the manufacturer's recommendations. *Campylobacter* selective agar (CAMP) (CM739; Oxoid) was used as a base, and supplemented with cefaperazone 32 mg/L and amphotericin 10 mg/L (SR155H; Oxoid). Both media were produced at two sites from the same batches to avoid inter-laboratory variations. All media were prepared according to standard protocols and subjected to full quality control procedures before distribution. All plates were used within 14 days of preparation.

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Samples

Routine faecal samples, received from both hospital and external sources, were included early in the study. Samples that were insufficient for duplicate inoculation, and those submitted for limited examination such as salmonella screening, were excluded. The appearance of samples was recorded as either 'formed' or 'liquid'. For formed samples, a pea-sized portion of faecal material (c. 1 g) was emulsified in 3 ml of maximal recovery diluent (MRD) (CM733; Oxoid), i.e., a 1 in 4 dilution. For liquid samples, a 1 in 4 v/v dilution was made in MRD.

Media inoculation and incubation

Direct inoculum. A single, sterile, cotton swab was coated in faecal material and a portion was inoculated on to a third of the total surface area of each plate. The inoculum was streaked, with a new sterile loop, to give individual colonies.

Dilute inoculum. A 3-ml pastette was used to inoculate one drop (c. 45 µl) of faecal suspension on to XLD and two drops on to CAMP, and streaked for single colonies as before.

XLD plates were incubated in air at 36°C ± 1°C for 16–24 h. CAMP plates were incubated micro-aerophilically at 36°C ± 1°C for 40–48 h.

Plate reading

Plates inoculated directly and with diluted faecal suspension were examined independently by different members of staff. One example of each morphological type of suspect colony was subcultured per plate. Data on bacterial growth and potential pathogens subcultured were recorded at the time of reading and verified daily by a separate, senior member staff. Bacterial growth was recorded semi-quantitatively: 1–10 colonies, light growth (+); 11–50 colonies, moderate growth (++); and > 50 colonies, heavy growth (+++). An assessment of competing flora was also made on this basis.

Identification

Suspected *Campylobacter* spp., with the typical morph-

ology of flat, translucent grey colonies, were confirmed by their appearance on Gram's staining as S-shaped gram-negative bacilli and a positive oxidase test. Possible salmonellae, appearing as red colonies with a black centre on XLD, and shigellae appearing as red colonies, were first screened for urease production. Urease-negative isolates were then identified by the API 10S system (bioMérieux). Those confirmed as *Salmonella* or *Shigella* spp. were further identified by standard serological testing. All isolates were referred to the Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London, for confirmation.

Statistical methods

To detect small differences between the methods, the proposed sample size was 1000 per laboratory. The final sample size was just below this with an average of 941 samples per laboratory. The data were entered into an Epi Info Version 6.0 database and analysed in Epi Info [4]. Isolation rates were compared between laboratories and by sample type (liquid/formed) by χ^2 tests. The methods (dilute/direct) were compared by an exact binomial test on the discrepancies.

Results

During July–Sept. 1998, 3764 faecal samples were examined by the four laboratories (792, 838, 1000 and 1134, respectively). Only one *Shigella* sp. was recovered during the study period, rendering statistical analysis for this organism impossible. Results for *Salmonella* spp. and thermophilic *Campylobacter* spp. are summarised in Tables 1 and 2.

For *Salmonella* spp., no overall difference in isolation rates could be demonstrated between direct and dilute inocula (difference = 0.05%; 95%CI, -0.14–0.22%). A significant inter-laboratory variation in isolation rates was seen, from 0.8% to 2.63% ($p = 0.007$); however, this was explained by local variation in food-poisoning rates over the study period, from 30.1/100 000 population to 95/100 000 population in the corresponding laboratories, compared with a regional mean of 55.7/100 000 (unpublished data, Communicable Diseases Surveillance Centre, Midlands).

Table 1. Effect of inoculum on isolation rates of *Salmonella* and *Campylobacter* spp.

Micro-organism	Number (%) of cultures positive					
	Overall (n = 3764)		Liquid samples (n = 1112)		Formed samples (n = 2649)	
	Direct	Dilute	Direct	Dilute	Direct	Dilute
<i>Salmonella</i> spp.	59 (1.57%)	61 (1.62%)	26 (2.3%)	31 (2.8%)	33 (1.2%)	30 (1.1%)
<i>Campylobacter</i> spp.	262 (6.96%)	261 (6.93%)	132 (11.9%)	130 (11.7%)	130 (4.9%)	131 (4.9%)

Table 2. Comparison of semi-quantitative growth estimates for *Salmonella* and *Campylobacter* spp. by inoculum

Micro-organism	Growth estimate: direct inoculum	Growth estimate: dilute inoculum			
		0	+	++	+++
<i>Salmonella</i> spp.	0	3698	3	1	3
	+	3	2	4	3
	++	0	1	8	1
	+++	2	3	2	30
<i>Campylobacter</i> spp.	0	3483	7	6	6
	+	7	6	8	7
	++	6	7	11	11
	+++	7	14	22	156

No significant difference in numbers of colonies of salmonellae isolated from positive samples, estimated semi-quantitatively, was found. The direct inocula gave more colonies than dilute inocula for 11 samples, dilute inocula more than direct inocula for 15 ($p = 0.55$). Also there was no evidence that low numbers of salmonellae had been lost by the dilution step (Table 2). Liquid samples were more likely to be positive than formed samples (2.8% positive for liquid *versus* 1.1% for formed, $p = 0.002$) and this effect was most apparent in laboratory C where 11.7% of liquid samples were positive compared with 1.2% of formed samples.

More competing flora were apparent with the direct inoculum than after dilution; 67.4% of directly inoculated samples had a heavy growth compared with 59% of dilute inocula ($p < 0.001$). For salmonella-positive samples alone there was also a difference (50% direct *versus* 33% dilute, $p = 0.035$). A difference in both the number and positivity rates of confirmatory tests required was seen in only one laboratory. However, in this laboratory, a small set of liquid samples with a high positivity rate was received and, for only the directly inoculated plates, more than one subculture was made with the second subculture never proving to be a *Salmonella* spp. (Table 3).

For *Campylobacter* spp., no overall difference in

isolation rates was seen between direct and dilute inocula and, again, liquid samples were more frequently positive ($p < 0.001$). Of 309 subcultures made from plates inoculated directly, 84.8% were positive, compared with 284 subcultures of which 91.9% were positive from dilute inocula ($p = 0.007$).

Significantly fewer competing flora were detected from dilute inocula than direct (17.4% had heavy growth from dilute samples *versus* 26.5% for direct plating, $p < 0.001$). This effect was evident to a lesser extent in campylobacter-positive samples, but the difference was not statistically significant (10.7% *versus* 13.6%, $p = 0.22$). Estimated semi-quantitatively, there was some evidence, although not statistically significant, that numbers of colonies of *Campylobacter* spp. isolated were lower with dilution (direct inocula gave more than dilute inocula on 62 samples, dilute more than direct on 46, $p = 0.15$) (Table 2). There was no evidence, overall, that low numbers were diluted out.

Examining discrepant results, where *Campylobacter* spp. were isolated by only one of the methods, competing flora were consistently greater on the plate which failed to isolate the organism, the effect being larger for direct inocula (direct > dilute = 12:1) than dilute (dilute > direct = 4:2). For *Campylobacter* spp. the pattern of discrepancies between direct and dilute inocula significantly differed between laboratories ($p = 0.006$); this remains unexplained. Two laboratories showed marginal evidence in favour of direct inoculation. One laboratory had better results with dilute inocula and the fourth laboratory showed no difference between the two methods.

Discussion

Lynton-Moll and Johnson [3] found that one drop of an *c.* 25% suspension of faeces in Ringer's solution applied to DCA plates by rotary plating gave good colony separation which facilitated the identification of salmonellae and, more particularly, low numbers of shigellae from known positive faecal samples. However, their data were not well documented and dilution

Table 3. Percentage of urease and API tests indicating a salmonella by laboratory and inoculum

Laboratory	Inoculum	Urease tests		API tests	
		performed (n)	negative (%)	performed (n)	salmonella confirmed (%)
A	Direct	127	20.5	56	46.4
	Dilute	114	20.2	49	46.9
B	Direct	77	11.6	24	37.5
	Dilute	81	11.1	24	37.5
C	Direct	181	9.4	79	21.5
	Dilute	58	36.2	33	63.6
D	Direct	94	7.4	16	43.8
	Dilute	87	9.2	20	40.0

was not thought to be so effective for other media, such as XLD, bismuth sulphite and brilliant green agar. Moreover, their prime objective was to establish a standardised inoculum for salmonella enrichment media.

In the present study, a 1 in 4 dilution of inoculum had no effect on the overall isolation rates for salmonellae, although as dilution appeared to have little effect on numbers of both competing flora and salmonellae, a higher dilution may give better results. However, for *Campylobacter* spp. a higher dilution would risk the loss of low numbers of organisms. The differing effects for salmonellae and *Campylobacter* spp. may be due to the relative selectivity of the media used and incubation conditions, i.e., CAMP media is more inhibitory to enteric micro-organisms and is incubated micro-aerophilically. There was no evidence that direct counts of *Campylobacter* spp. were generally lower than those of salmonellae and, therefore, more susceptible to dilution effects.

Overall, no disadvantages to the use of a dilute inoculum were seen. The principal advantages of a standardised inoculum and a decreased workload due to fewer wasted subcultures for *Campylobacter* spp. may partially be offset by the increased work of preparing the inoculum. However, as a faecal suspension is pre-

ferable for inoculation of enrichment broths [1, 2], this should not pose a problem.

In conclusion, this large multi-centre study showed that the use of a dilute faecal inoculum resulted in significantly fewer wasted subcultures for *Campylobacter* spp., but had no effect on overall isolation rates of *Salmonella* spp. or *Campylobacter* spp. Furthermore, dilution provides a standardised inoculum and an appropriate preparation for inoculation of enrichment broth. Therefore, a faecal suspension of c. 25% in MRD is recommended for direct inoculation of solid media for the isolation of salmonellae and thermophilic *Campylobacter* spp.

References

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