J. Hyg., Camb. (1978), **80**, 385
Printed in Great Britain

Counts on viable *Bacteroides fragilis*: a modification of the microdroplet technique

By M. J. KELLY
Section of Microbiology, Department of Pathology, Tennis Court Road, Cambridge

(*Received* 7 *October* 1977)

SUMMARY

A modification of Sharpe's microdroplet counting technique is presented which permits viable counting of *Bacteroides fragilis*. Lysed horse blood was added to 0·1 ml soy digest agar droplets and 2% was found to be the optimal concentration which combined adequate intradroplet colony size with acceptable visibility on the counting screen of the Colworth Droplette machine.

INTRODUCTION

Recent reports have strongly suggested that anaerobes play an important part in human clinical wound infections (Nobles, 1973; Leigh, 1974; Lari, Kirk & Howden, 1976). Most of the classic studies on clinical and experimental wound infection have concerned *Staphylococcus aureus* which is a robust, stable, aerobic organism that is not fastidious in its culture requirements (Edlich *et al.* 1969). Little similar quantitative experimental work has been carried out on fastidious anaerobes, such as *Bacteroides fragilis*, partly because of the tedium and great expense involved in the use of anaerobic pour-plate techniques in colony counting. The microdroplet method (Sharpe & Kilsby, 1971; Sharpe, Dyett, Jackson & Kilsby, 1972), originally designed for the estimation of undemanding saprophytes in meat and dairy produce, provides a reliable, cheap solution to the counting problem. This paper describes a relatively simple modification which allows the method to be used for *B. fragilis*.

MATERIALS AND METHODS

Freeze-dried *B. fragilis*, NCTC 9343, was used. A 48 h broth culture was incubated at 37 °C. using a 'Gas Pak' anaerobic system (BBL) and 0·1 ml samples were freeze-dried and held as reference stock. The remainder of the culture was dispensed in 0·5 ml portions in screw-cap teflon vials. They were rapidly frozen and stored at −70 °C. For use, a vial was thawed by immersion in a 37 °C water bath and its contents diluted serially in digest broth previously boiled for 5 min to decrease its oxygen content.

In this laboratory a standard blood agar plate contains a 12·5 ml layer of Hartley's digest agar with a 12·5 ml overlayer of similar agar enriched with 5% horse blood. To compare the effect of blood concentration on size and number of colonies, 0·1 ml volumes of *B. fragilis* suspensions were used to inoculate blood

agar plates made up to contain differing amounts of horse blood. Concentrations of 7%, 5%, 2%, 1% and nil were used. Another sample of horse blood was lysed in its own volume of sterile distilled water and used to enrich a further series of plates in a similar manner. The 0·1 ml inoculum of *B. fragilis* was distributed on each plate with a glass spreader and incubated anaerobically at 37 °C for 48 h.

Counts were performed by the microdroplet technique (Sharpe *et al.* 1972) using a Colworth Droplette machine, model BA 6013 (A. J. Seward & Co., 3 Cavendish Road, Bury St Edmunds, Suffolk) for making dilutions and counts in 0·1 ml agar droplets.

Universal bottles containing 19 ml of heat-sterilized soy broth with 0·9% agar were placed in boiling water until the agar melted. The bottles were transferred to a 42 °C water bath. Horse blood was added to samples of this molten soy agar to produce final concentrations in the dispensed droplets of 7%, 5%, 2% and 1%. To achieve this, the actual concentration of blood in each of the above was increased by a factor of 10/9. Similar tubes were set up which contained the same concentrations of horse blood previously lysed in its own volume of sterile distilled water. The Colworth Droplette was used to dispense 1·8 ml samples of these mixtures to which were added 0·2 ml portions of the *B. fragilis* dilution known to contain 20–30 colony-forming units per 0·1 ml droplet. As the *Bacteroides* culture had previously been diluted in simple broth, 0·2 ml diluted the soy digest blood agar by a factor of 9/10, cancelling out the correcting factor applied earlier. Ten droplets were dispensed for each dilution of blood and the petri dish was incubated anaerobically in a Gas Pak jar for 48 h.

The higher the concentration of blood in the agar of the droplet, the more opaque it became and the more difficult were the microcolonies to discern. Once the optimal concentration of added blood had been determined, it merely needed to be added to the molten soy agar at 42 °C and this mixture was used as the diluent to perform counts on the *B. fragilis* suspension. These determinations were then performed with the same ease and speed that Sharpe and his colleagues had described (1972) in their account of the droplet assay of *Staph. aureus*.

RESULTS

Anaerobic incubation of standard blood agar plates spread with 0·1 ml of seria tenfold dilutions of thawed vials of *B. fragilis* suspension showed that there were approximately 10^9 organisms per ml of suspension. It was therefore possible to select a dilution that would yield 20–30 colonies in each 0·1 ml droplet. Triplicate samples of plates containing blood agar at different concentrations were spread with 0·1 ml samples of this dilution of *B. fragilis*. A similar series of plates containing lysed horse blood agar was also inoculated. After 48 h anaerobic incubation at 37 °C it was found that whereas the number of colonies per plate remained constant (mean 25, S.D. 5), the average colony size slightly decreased (see Table 1). There was no growth at all on plain agar (i.e. blood concentration 0%) or on the plates incubated aerobically. The colonies on the lysed blood plates were not

Table 1. *Effects of added blood on the size of* B. fragilis *colonies*

	Average colony diameter (mm)				
	7% blood	5% blood	2% blood	1% blood	No blood
Whole blood	2·5	2·3	2·0	2·0	—
Lysed blood	2·5	2·0	1·8	1·7	—

Each numerical result represents the average colonial diameter after 48 h incubation of petri dishes at 37 °C. When no blood was added to the agar, no growth occurred.

Table 2. *The effects of added blood on the counting screen visibility of microcolonies of* B. fragilis *in agar droplets*

	7% blood	5% blood	2½% blood	2% blood	1% blood	No blood
Whole blood	×	+	+ +	+ + +	+ + + +	–
Lysed blood	×	+ +	+ + + + +	+ + + + +	+ + + + +	–

These assessments were made on the same droplets as those in Table 3.

×, Microcolonies that could only be discerned on the counting screen in the dark room.

+ to + + + + + represents an arbitrary scale of visibility of microcolonies on the Droplette counting screen from poor to excellent.

–, Colonies almost impossible to count because of their small size.

significantly larger or smaller than those found on the unlysed blood plates. Thus it was demonstrated that *B. fragilis* would grow quite satisfactorily on plates containing the lower concentrations of either whole or lysed blood.

The experiment was repeated using 0·1 ml droplets containing similar concentrations of lysed or unlysed horse blood. Table 2 reveals the extent to which the microcolonies seen on the Droplette counting screen could be distinguished from the rather granular, orange background of the soy blood agar. Visibility was uniformly poor at blood concentrations of 7% as the background obscured the margins of the microcolonies and, even in the dark-room, they could only be seen with difficulty. As the concentration of blood decreased, it became easier to distinguish the bacterial colonies from their background, but colonial size was diminished (see Tables 2 and 3). The microcolonies in the agar droplets were not significantly larger or smaller when lysed or whole blood had been added. Table 2 shows that visibility was consistently better when lysed blood was added than with whole blood. Table 3 shows the average length of the image of a microcolony measured on the Droplette counting screen. The diameters decreased as the concentration of added blood was reduced and, when digest agar was used, the diameters were so small that counting became inaccurate. At a concentration of 1% blood the colonies were rather small at 48 h, but after 72 h incubation they were larger and visibility was improved.

Samples of the *B. fragilis* dilution that gave 20–30 colonies per 0·1 ml of blood agar were held at 42 °C and assayed at 5 min intervals. No loss or increase of colony count was observed in periods up to 30 min.

A concentration of 2% lysed blood was chosen as giving the best visibility

Table 3. *The effects of added blood on the counting screen images of microcolonies of* B. fragilis *in agar droplets*

	Lengths of microcolony images (mm)					
	7% blood	5% blood	2½% blood	2% blood	1% blood	No blood
Whole blood	4·5	3·5	3	2·5	2	0·05
Lysed blood	4·5	5	5	5	3	0·05

Each numerical result represents the average of the longest dimensions on the counting screen of images of at least five microcolonies, measured to the nearest 0·5 mm.

The droplets were incubated anaerobically for 48 h. After 72 h incubation the results for 1% added blood rose from 2 and 3 mm to 4 and 3·5 mm.

against the background with reasonable colony size after 48 h incubation. When this concentration was incorporated in the soy agar droplets, counts were performed on the *B. fragilis* suspension with great ease and could be read off the Droplette screen in daylight.

DISCUSSION

The growth of *B. fragilis* on the agar plates containing different concentrations of blood indicated that, although there was a loss of colony size, there was no change in colony count as the concentration of blood decreased down to 1%. However, some blood seemed to be necessary, as there was no growth visible when it was absent.

When the concentration of blood present in a standard blood agar plate, 5%, was added to the soy agar droplets, the background of the droplet became so red and opaque that the tiny bacterial colonies could not be seen inside it. As the amount of added blood decreased, these microcolonies became more visible but decreased in size, so that at 1% blood they were so small that 72 h incubation was needed for them to reach an easily countable size. It is suggested that the reason why agar enriched by whole blood gave uniformly poorer visibility than agar incorporating lysed blood was that the intact red blood corpuscles in the former diffracted the light and thus detracted from the clarity of outline of any object seen through such a suspension.

The observation that there was no change in the count of *B. fragilis* when held in soy blood agar at 42 °C. for up to 30 min suggested that no errors would be introduced by holding this bacterium in this way for short periods during the dilution procedure which forms part of the droplet viable count test.

The optimal concentration of lysed blood in the agar droplets appeared to be 2%, and this is now used by the author for counting suspensions of anaerobic bacteria used in experimental wound infections (Kelly, 1977).

The author gratefully acknowledges that he was supported by a Smith and Nephew Research Fellowship and his experimental expenses were paid by a grant from the NHS Locally Organized Research Scheme of the East Anglian Regional

Health Authority. The work was carried out under the general direction of Dr R. J. Olds as part of the requirement of the degree of M.Chir. of the University of Cambridge.

REFERENCES

EDLICH, R. F., ROGERS, W., KASPAR, G., KAUFMAN, D., TSUNG, M. S. & WANGENSTEIN, O. H. (1969). Studies in the management of the contaminated wound. *American Journal of Surgery* **117**, 323–9.

KELLY, M. J. (1977). Mixtures of aerobic and anaerobic human pathogens: a 4-plate counting technique. *British Journal of Experimental Pathology* **58**, 478–83.

LARI, J., KIRK, D. & HOWDEN, R. (1976). Bacteriological survey of acute appendicitis in children. *British Journal of Surgery* **63**, 643–6.

LEIGH, D. A. (1974). Clinical importance of infections due to *Bacteroides fragilis* and the role of antibiotic therapy. *British Medical Journal* iii, 225–8.

NOBLES, E. R. (1973). Bacteroides infections. *Annals of Surgery* **177**, 601–6.

SHARPE, A. N. & KILSBY, D. C. (1971). A rapid inexpensive bacterial count technique using agar droplets. *Journal of Applied Bacteriology* **34**, 435–40.

SHARPE, A. N., DYETT, E. J., JACKSON, A. K. & KILSBY, D. C. (1972). Technique and apparatus for rapid and inexpensive enumeration of bacteria. *Applied Microbiology* **24**, 4–7.