

about 10 µg, enough for more than 100 PCRs. No degradation was found after incubation at 37°C for one month (figure), indicating the long term stability of the sample at 4°C. The clotted blood before DNA extraction can be stored at -20°C in the tube used for serum separation for up to a month, and can be transferred on dry ice to other laboratories.

Discussion

The salt/chloroform method is better than the use of phenol, because it avoids having to use a dangerous solvent. However, unlike the procedure described before,⁴ neither absolute ethanol nor propanol are recommended for DNA precipitation because it also precipitates salts and blood pigments, which inhibit poly-

merases and restriction enzymes. This method is simple, rapid, economical, and within the scope of most clinical laboratories. It facilitates the collection of many samples for studies of genetic disorders because it uses normally discarded blood clots, and is widely applicable to medical, genetic, epidemiological, and forensic studies.

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Comparison of formalin and Bouin's reagent for fixation of coagulase negative staphylococcal biofilm

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Abstract

Methodological modifications, particularly the use of different fixatives, may account for discrepancies between studies of the relation between virulence and biofilm production *in vitro* by isolates of coagulase negative staphylococci. The efficacy of formalin and Bouin's reagent for fixing coagulase negative staphylococcal biofilms in a microtitre tray assay was compared. The optical density of stained adherent growth by three strains was reduced by an average of 20% following fixation with 10% formaldehyde compared with Bouin's reagent. This difference seemed to be mainly because of increased background staining and blackening of the biofilm when Bouin's reagent was used. Formalin fixation was also effective at identifying early and late biofilm production in adherence growth kinetic experiments with 10 coagulase negative staphylococcal clinical isolates.

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Coagulase negative staphylococci adhere to and accumulate on polymer surfaces by producing extracellular slime, forming biofilms. Although authors have frequently referred to the *in vitro* measurement of coagulase negative staphylococcal slime production, the most commonly used methods¹ primarily

examine surface accumulation of bacterial cells, rather than slime *per se*.² The term "biofilm" has been used here as a compromise, accepting that cell accumulation is the main parameter under investigation. Many groups have examined biofilm formation by coagulase negative staphylococci *in vitro* to determine whether clinical isolates associated with infection of medical devices are more adherent than control strains, with conflicting results.¹⁻⁸ Methodological differences in the commonly used microtitre tray assay of adherent growth are possible causes of some of these discrepant results.^{1-5,7,8} In particular, methods used to fix the coagulase negative staphylococcal biofilm to the bottom of the wells in the microtitre tray after washing may not be equally effective.⁹ The original report of the microtitre tray assay described the use of Bouin's reagent as a fixative.¹ This reagent, however, is potentially explosive, relatively expensive, and less readily available than alternatives such as formalin and glutaraldehyde, which have been used as fixatives in some cases.^{8,10} This study compares the efficacy of formalin and Bouin's reagent for the fixation of coagulase negative staphylococcal biofilms *in vitro*.

Method

Twelve coagulase negative staphylococcal clinical isolates were obtained from patients with peritonitis undergoing continuous

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ambulatory peritoneal dialysis (CAPD). These were identified by API Staph (API, Basingstoke, UK) and included 10 strains of *Staphylococcus epidermidis* (s1, s2, s3, s8, s31, s34, s49, s59, s900, and s902), one of *Staphylococcus warneri* (s79), and one of *Staphylococcus haemolyticus* (s907). A well described coagulase negative staphylococcal strain which is positive in the tube and microtitre tray adherence assays, *S. epidermidis* ATCC 35984, was used as a control.¹ Strains s1, s2, and s3 were chosen at random from a collection of CAPD isolates, and the remainder of the clinical isolates were known adherence positive strains, as described before.¹⁰ Coagulase negative staphylococcal were stored in horse serum at -70°C and were maintained by subculture on blood agar.

The microtitre tray adherence assay has been described previously.¹ Briefly, an inoculum equivalent to a final concentration of 10^6 colony forming units/ml was prepared from an overnight culture in tryptone soya broth (TSB; Oxoid, Hampshire, UK). Polystyrene microtitre trays with flat bottomed wells (M29A, Dynatech Laboratories, Sussex, UK) were used, with a final well volume of $250\ \mu\text{l}$. Following incubation in TSB in air at 37°C , well contents were removed and non-adherent bacteria displaced by washing three times with saline. Fixation consisted of adding $75\ \mu\text{l}$ of either Bouin's reagent (75 ml picric acid, 25 ml formalin, and 5 ml glacial acetic acid) or 10% v/v formaldehyde (25% v/v formalin; BDH, Poole, Dorset, UK) to each well for 30 minutes. Fixed biofilms were stained with crystal violet and, after further washing and air drying, the optical density of adherent bacterial growth was measured using a microtitre tray spectrophotometer (SLT 210, Kontron Analytical Instruments, Zurich, Switzerland) at an absorbance of 546 or 600 nm. Background staining was assessed in control wells inoculated with sterile TSB.

Biofilm formation by strains s1, s2, and s3, following incubation for 24 hours, was compared in the microtitre tray adherence assay following fixation with either Bouin's reagent

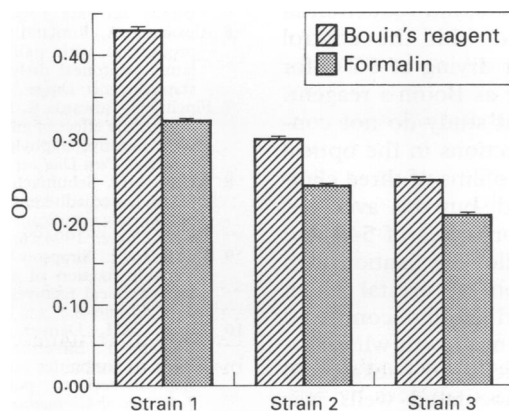


Figure 1 Comparison of biofilm formation by three *S. epidermidis* strains following fixation with Bouin's reagent or 10% formaldehyde, measured as the optical density (OD) of stained bacteria at an absorbance of 546 nm.

or 10% formaldehyde. For each method of fixation, strains were cultured in eight wells in each of three different microtitre trays, and mean and SEM values for the 24 separate readings calculated. In a separate series of experiments the kinetics of coagulase negative staphylococcal adherent growth by 10 strains was examined to determine whether fixation with 10% formaldehyde alone permitted identification of both early and late biofilm formation. In duplicate experiments strains were inoculated into eight wells in each of seven microtitre trays. Adherent growth was measured in each tray after incubation periods of two, four, six, eight, 18, 24, and 48 hours and mean optical densities were calculated.

Results

The inter- and intratray coefficients of variation of adherent growth for a control coagulase negative staphylococcal strain were both consistently less than 10%. Biofilms were successfully fixed by either Bouin's reagent or 10% formaldehyde (fig 1). Biofilm was macroscopically visible as black colonies following fixation with Bouin's reagent and as violet coloured adherent growth when 10% formaldehyde was used. The optical densities of stained biofilms were reduced by 25, 18, and 17% with strains s1, s2, and s3, respec-

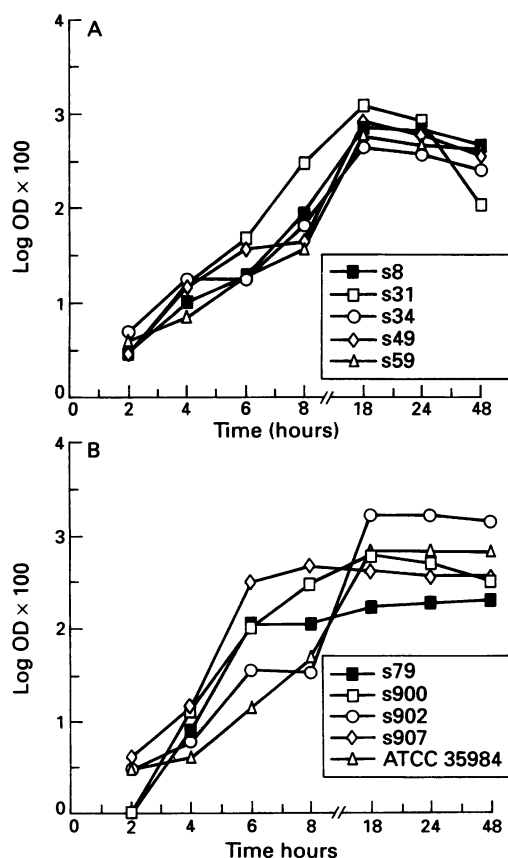


Figure 2 (A) Kinetics of biofilm formation by 10 coagulase negative staphylococci following fixation with 10% formaldehyde, measured as the optical density (OD) of stained bacteria at an absorbance of 546 nm. (B) Relatively early biofilm formation was observed for s79 and s907.

tively, following fixation with 10% formaldehyde compared with Bouin's reagent. Corresponding reductions in optical density at an absorbance of 600 nm were 14, 9, and 8%, respectively (data not shown). Optical densities of control wells, indicating the level of background staining, were 30% lower, on average (the mean reduction in optical density was 0.7 at 546 nm), following fixation with 10% formaldehyde compared with Bouin's reagent.

The kinetics of biofilm formation were identified clearly and noticeable interstrain variations observed following fixation with 10% formaldehyde (fig 2). The majority of strains achieved maximal biofilm formation after incubation for 18 hours (fig 2A); increases in optical density were not observed after 18 hours. Close to maximal adherent growth was observed with coagulase negative staphylococcal strains s79 and s907 after incubation for six hours (fig 2B). Viable counts of planktonic bacteria increased at least 10-fold after between eight and 18 hours of incubation (data not shown).

Discussion

The quantitative microtitre tray assay of coagulase negative staphylococcal biofilm formation is clearly superior in terms of discriminatory power and reproducibility to the qualitative tube test.¹⁴ There is disagreement in the literature over whether there is an association between the virulence of coagulase negative staphylococci and biofilm production in vitro. Substitution of Bouin's reagent with either formalin or other fixatives is a possible cause of such discrepant results in the microtitre tray assay. Conflicting results, however, have been obtained using both the tube test,^{4,6} which does not involve a stage to fix bacterial adherent growth to the polymer surface, and the microtitre tray assay.^{1,3-5,7,8}

Baldassarri *et al* compared 12 different methods of fixation of a bacterial biofilm using a single strain, a defined clone of the control *S epidermidis* strain ATCC 35984.⁹ They found that neither 2.5% nor 10% formaldehyde adequately fixed the staphylococcal biofilm, with a severalfold reduction in optical density compared with the control biofilm, and that only air drying at 60°C for one hour was as effective as Bouin's reagent. The results of the present study do not confirm these findings. Reductions in the optical densities of the stained biofilms of three clinical isolates were observed, but this averaged only 20 and 10% at absorbances of 546 and 600 nm, respectively. Following fixation with Bouin's reagent, addition of crystal violet results in a black stained biofilm compared with violet stained adherent growth when formalin fixation is used. Increased background staining of control uninoculated wells was

evident when Bouin's reagent was used. Furthermore, in contrast to the observations of Baldassarri *et al*, biofilm fixation with formalin enabled identification of early and late adherent growth for all 10 coagulase negative staphylococcal strains studied (including *S epidermidis* ATCC 35984).⁹ Biofilm formation by two strains was notably more rapid and the importance of these observations warrants further study. Clonal variation of *S epidermidis* ATCC 35984 may account for the different results obtained in the two studies. In the author's experience the washing procedure is the most difficult part of the assay to standardise, but this is rarely regarded as a source of error.

Formalin is widely available, inexpensive, and gives reproducible results when used as a fixative in the microtitre tray adherence assay. Air drying at 60°C has been suggested as an alternative method of fixation, but hot air ovens are not always available.⁹ If a modification of the microtitre tray adherence assay is used,^{7,10} consisting of silicone rubber discs fixed by grease to the bottom of the wells, prolonged drying leads to detachment of the discs and, therefore, to errors during measurement of the optical density of stained biofilm (data not shown). This modified method is useful for the study of biofilm formation on clinically relevant polymers such as silicone rubber, which is often very different compared with the polystyrene used in the manufacture of microtitre trays.^{8,10}

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