# Variable Fixation of Staphylococcal Slime by Different Histochemical Fixatives

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**A variety of histochemical fixatives were used to compare the fixation of bacterial films produced by a standard slime-producing strain of *Staphylococcus epidermidis* on plastic tissue culture plates. Some reagents were completely ineffective in fixing the slime layer, whereas others gave variable results. The best alternative to the fixative of the reference method, the potentially explosive Bouin's reagent, was air drying.**

In the past ten years the coagulase-negative staphylococci have emerged as prominent nosocomial pathogens, particularly in the setting of indwelling medical devices (1). This predilection for producing biomaterial infections is not understood, but investigations have focused on the role of bacterial adherence, particularly slime production, in the pathogenesis of these infections (2–4). "Slime" refers to the thick tenacious film of bacteria coating inanimate objects immersed in vitro in a culture medium (5, 6) which appears morphologically indistinguishable from the in vivo biofilm that coats the surfaces of infected medical devices (6).

Most epidemiologic investigations have found an association between virulent strains of coagulase-negative staphylococci and the production of slime (3, 4, 7, 8). This phenomenon has also been suggested to have prognostic implications, as slime-producing coagulase-negative staphylococci tend to persist in spite of antimicrobial therapy or are more likely to lead to clinical complications (4, 7, 9). Nevertheless, some investigators do not concur with these findings (10–12). Technical factors are the major source of variance between these reports.

Most investigators assay slime production by a tube test, in which tubes containing an overnight culture of coagulase-negative staphylococci in trypticase soy broth are emptied and the residual bacterial film stained. Tubes are scored positive or negative based on the visual appreciation of the bacterial film (5). Subsequent investigations from this (13) and other laboratories (7, 14) revealed that this subjective assay may not be reliable, leading to the elaboration of a more reproducible measure of slime production. The assay developed used an automatic spectrophotometer to measure the optical density (OD) of fixed and stained bacterial films attached to the floors of tissue culture plate wells after overnight growth in trypticase soy broth (13).

Our original description of the plate test was based on Fletcher's fundamental investigations on the attachment of marine pseudomonads to petri dishes (15). Following the method of Fletcher, we used a picric acid-based reagent, Bouin's fixative, to fix the bacterial films. Picric acid, however, is potentially explosive. Perhaps for this reason, many investigators report slight modifications of fixation and staining when following this procedure (16–19). Since the measurement of slime production is of both clinical and research interest, we have systematically examined the influence of different fixatives on the effectiveness of the plate test.

***Materials and Methods.*** With the exception of the fixative used, the plate test was performed as described previously (13). Trypticase soy broth overnight cultures of H1, a defined clone of *Staphylococcus epidermidis* sensu stricto ATCC 35984, were diluted 1:2 in fresh trypticase soy broth, and 0.2 ml/well was distributed across a 96-well microtiter plate. After 24 h of incubation (or shorter for kinetic experiments), wells were emptied, washed with phosphate-buffered saline (pH 7.2), fixed by a variety of fixatives and stained with Hucker crystal violet. The OD of the plates was then determined by an automatic spectrophotometer (BioRad EIA reader, model 2550; BioRad Laboratories, USA) at a wavelength of 570 nm using a full scale (0.001–7.5).

For fixation we used reagents cited in the literature as fixatives for slime and others cited as standard histochemical fixatives. Our list included methanol, 10 % formalin, 25 % formalin (17), phosphate-buffered (PB) formalin, PB 2 % glutaraldehyde (16, 19), Carnoy's solution

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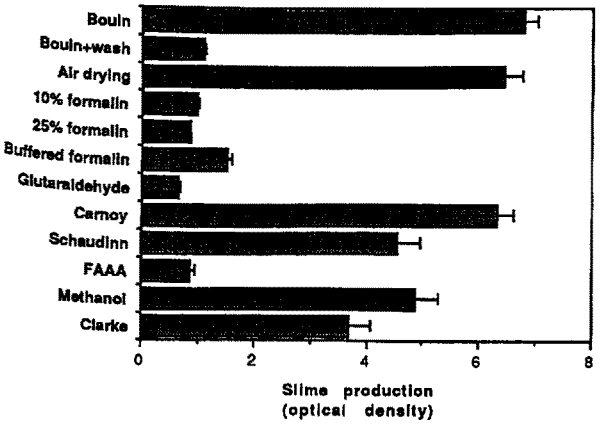


**Figure 1:** Effect of different fixatives on the quantitative measure of slime produced after 24 h by *Staphylococcus epidermidis* sensu stricto H1. Shaded bars represent the mean optical density obtained for each fixative ± SE.

(ethanol-chloroform-glacial acetic acid, 15:5:1) (19), Schaudinn's fixative (saturated aqueous $HgCl_2$ solution-ethanol, 1:5; glacial acetic acid added before use to make 2 % [v/v] solution), FAAA solution (formalin-ethanol 80 %-glacial acetic acid, 1:9:5) and Clarke's solution (ethanol-glacial acetic acid, 3:1). For a reference we fixed films with Bouin's fixative (saturated aqueous picric acid solution-formalin-glacial acetic acid, 15:5:1). We also tried a modification of fixation with Bouin's reagent in which we removed the picric acid by washing with 50 % ethanol. Finally, samples were fixed by drying for 1 h at 60 °C.

***Results and Discussion.*** The OD of bacterial films was greatly affected by the choice of fixative. In Figure 1 the mean values for three experiments employing 16 wells per fixative are displayed. Some of the reagents (10 % formalin, 25 % formalin, buffered formalin, glutaraldehyde and FAAA) were completely ineffective in stabilizing the bacterial film. Even fixation with Bouin's reagent was ineffective when followed by washing with alcohol, suggesting picric acid was the active agent. Methanol, Clarke's solution and Schaudinn's fixative appeared to be almost as effective as Bouin's reagent (Figure 1); these three reagents, however, exhibited considerable variation from well to well, with the ODs varying in the same experiments from 0.934 to 7.536 for a single fixative. Although Carnoy's reagent also appeared effective, this result was questionable since the chloroform it contains often destroyed the optical clarity of the plates by partially dissolving the surface of the plastic wells. Of the fixatives tested, only air drying and the reference procedure reliably and reproducibly demonstrated the full extent of the slime production phenomenon. These findings indicate that a source of the discordance in the literature regarding the production of slime by various organisms is the choice of fixatives.

It has recently become apparent that the colonization of inanimate surfaces by coagulase-negative staphylococci is a multistep process involving different adhesive mechanisms. Some components could be responsible for the early initial attachment (20) of bacteria to surfaces, whereas others could be involved in the late accumulation phase mediated by cell-to-cell attachment (21, 22). Since the capacity of a reagent to fix the bacterial film depends upon the chemical nature of the adhesive agents binding the bacterium to the surface, it is possible that different fixatives will be effective at different stages of bacterial colonization. For this reason we also examined the fixation of bacterial films as a function of time. In these experiments it was not possible to distinguish between early attachment and late accumulation with 10 % formalin, 25 % formalin, buffered formalin, glutaraldehyde and FAAA (data not shown). Only air drying and, partly, Carnoy's reagent were as effective as Bouin's fixative in stabilizing the bacterial film at different times. Roughly, fixatives could be subgrouped in three categories of effectiveness, an example of each being displayed in Figure 2. With 25 % formalin, strain H1 mimicked a non-slime producing

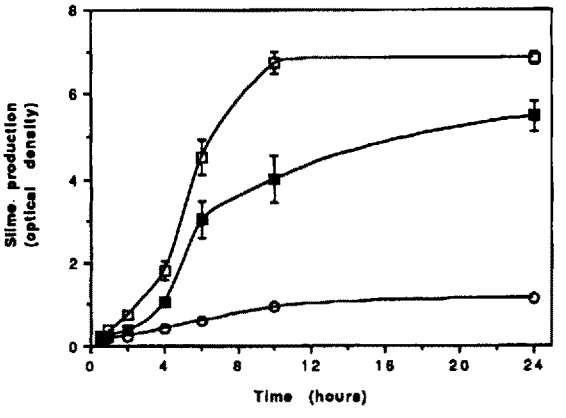


**Figure 2:** Slime production kinetic as revealed by Bouin's, Clarke's and 25 % formalin fixation. 25 % formalin (o) is completely ineffective in revealing the slime production phenomenon by *Staphylococcus epidermidis* sensu stricto H1, whereas fixation by Bouin's (□) and Clarke's (■) reagents evidences, even though to different extents, a multiphase colonization of the tissue culture plate. The data are presented as means of three experiments of 14 wells for each time and fixative ± SE.

strain, whereas when fixed with Clarke's reagent, the H1 strain showed a multiphase colonization of the tissue culture plate which was demonstrated to a greater extent by the reference fixative.

These studies indicate that air drying and fixation with Bouin's reagent are the only reliable methods to quantitatively demonstrate the slime production phenomenon. Schaudinn's reagent is a potentially toxic hazard and should be avoided, Carnoy's reagent is not compatible with the plastic plates, and the remaining agents either produced variable results or were ineffective. Because of the safety concerns regarding Bouin's fixative, air drying appears to be the method of choice that will produce results most comparable to the reference method. Measurement of slime should be standardized so that clinical and research studies from different centers can be compared.

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