SUGGESTED MODIFICATIONS OF THE CALCIUM ALGINATE SWAB TECHNIQUE

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(Received for publication October 25, 1963)

Summary

Modifications of the calcium alginate swab technique recommended as an alternative to cotton swabs in *Standard Methods for the Examination of Dairy Products*, American Public Health Association, 1960 are presented. The modifications are based on observed lysis of some gram negative bacteria during dissolution of the swab with sodium hexametaphosphate (HEX) and inhibition of some gram positive bacteria when the solvent is included in certain plating media. Since NaCl protects cells against lysis, Ringers' solution is suggested as the swab buffer for sample collecting. During dissolution of the swab HEX concentration should be 2% or less in order to minimize carry-over to the plating medium. Several plating media were studied and trypticase soy agar or its equivalent as determined with a sensitive gram positive organism such as *Sarcina lutea* is recommended. It is also recommended that contact time between cells and HEX be as short as possible and that 20 ml of plating medium be used for each ml of HEX-alginate-Ringers' sample.

Although considerable variation in results has been reported by many authors *(3)* substitution of calcium alginate for cotton in the surface swab technique has been recommended as an alternative method in "Standard Methods" *(1)* based primarily on the work of O'Neill and Reed *(2)*. The standard technique requires moistening the alginate swab with 5 ml of 0.0044% KH_2PO_4 buffer water, swabbing the surface and replacing the swab in the buffer. One ml of a 10% solution of sodium hexametaphosphate (HEX) is then added to dissolve the swab and a portion of sample is plated in nutrient agar. The variation in results reported by many authors and the fact that the solute is a chelating water softener suggested possible toxic effects to some bacteria. These toxic effects have been reported in an earlier paper *(3)*. During the course of this earlier work it was discovered that inhibition of sensitive bacteria could be eliminated by proper choice of buffer and plating medium. The purpose of the following report is to present evidence for modification of the existing method of the alginate swab technique.

Materials and Methods

Organisms

Wild populations were obtained from the activated sludge tank of a sewage treatment plant. *Sarcina lutea* was used in this study since it had been previously shown to be extremely sensitive to small amounts of HEX included in solid media and *Pseudomonas fluorescens* was used since it exhibited considerable lysis in the presence of HEX during dissolution of the swab. These pure cultures are maintained as stock in our laboratory.

Chemicals

Sodium hexametaphosphate (HEX) is a product of Fisher Scientific Co., New York, New York. Calcium alginate was obtained from Splain and Lloyd, Inc., Milford, Ohio.

Inhibition effects of HEX in solid media

To determine the most effective final plating medium, HEX was incorporated into commercially prepared media or combinations of the ingredients of commercial media before sterilization or sterilized separately and added to the medium at the time of pouring plates as is done in the "Standard Methods." In some experiments 50 mg of calcium alginate (the approximate weight of a swab) were dissolved in an amount of 2% HEX simulating that of "Standard Methods" and then incorporated into the medium. At later stages various sterile buffers were used, to which sterile calcium alginate and sterile HEX were added and portions of this added to the solid medium. Survival of known numbers of bacteria was determined by modification of the drop plate technique as reported in the previous work *(3)* and by standard pour plate technique. This step was considered important since many gram positive bacteria are inhibited in the final medium by the slight carry over of HEX. In most of these studies *S. lutea* was the test organism. Colonial size was also used as an indicator of inhibition in some experiments.

Dissolution of the swab

Also of considerable importance is the buffer to which the swab and HEX are added. *P. fluorescens* exhibits considerable lysis at this stage and was the primary test organism. Various buffers were used with known numbers of cells and variable times of exposure to the HEX. Determination of survival was by the drop plate technique and pour plate techniques using the most effective solid medium as determined in the preceding section. Appropriate controls were used in all experiments.

TABLE 1. INHIBITION OF ACTIVATED SLUDGE BACTERIA ON VARIOUS ENRICHMENT MEDIA CONTAINING HEX

Medium	% HEX in medium					
	0	0.01	0.05	0.1	0.5	1.0
TSA (BBL)	+	+	+	+	+	−
TGE (Difco)	+	+	−	−	−	−
BHIA (BBL)	+	+	+	+	−	−
BHIA (Difco)	+	+	+	+	−	−
NA (Difco)	+	−	−	−	−	−

+ = Normal growth; − = Inhibition (smaller number than control); TSA = trypticase soy agar; TGE = tryptone glucose yeast extract agar; BHIA = Brain heart infusion agar.

RESULTS

Solid media

Four enrichment media in addition to nutrient agar (NA) (Difco)-trypticase soy agar (TSA) (BBL), tryptone glucose yeast extract agar (TGE) (Difco), and two brainheart infusion agars (BHIA) (Difco and BBL) were studied for their capacity to counteract at various levels of HEX using wild populations and *S. lutea* as indices of inhibition. Results will be found in Tables 1, 2 and 3. When colony size is taken into account as well as number of cells recovered (Table 3), TSA appears to be slightly better than the other media. A further study of this medium is given in Tables 3 and 4. Table 3 indicates variation in colony size relative to HEX concentration and to number of cells recovered. Colony size appears to be directly related to HEX concentration while number of cells recovered is not so closely related. Table 4 illustrates that exposure time to HEX is not important while the plating medium is. These results were identical whether drop plate or pour plate techniques were used.

An attempt to improve recoverability of NA by including meat extract, calcium and magnesium salts, sodium chloride and a vitamin mixture containing pyridoxine, pyridoxamine, pyridoxal, calcium pantothenate, riboflavin, nicotinic acid, para aminobenzoic acid, and folic acid did little to improve the

TABLE 2. INHIBITION OF *Sarcina lutea* ON VARIOUS MEDIA CONTAINING HEX

Medium	% HEX in medium					
	0	0.01	0.05	0.1	0.2	0.5
NA	+	+	−	−	−	−
NA + 0.5% phytone	+	+	+	−	−	−
NA + 0.5% phytone + 0.5% NaCl	+	+	+	−	−	−
NA + 1.5% typticase	+	+	+	−	−	−
TSA Lot 90567	+	+	+	+	+	−
TSA Lot 211615	+	+	+	+	+	−
TSA made from ingredients	+	+	+	+	*	−

+ = Normal growth; − = Total inhibition; * = Partial inhibition; NA = nutrient agar (Difco); TSA = trypticase soy agar (BBL); Trypticase and phytone (BBL).

TABLE 3. NUMBER AND SIZE OF *Sarcina lutea* COLONIES WHEN GROWN ON THE SURFACE OF NA OR TSA CONTAINING HEX

Medium	Conditions[a]		% HEX in medium					
			0	0.01	0.05	0.1	0.2	0.5
NA	HEX alone	Colonies[b]	14	14	1.2	−[d]	−	−
		Diameter[c]	372	428	84			
	HEX + alginate	Colonies:	14	17	0.001	−	−	−
		Diameter:	372	359	64			
TSA	HEX alone	Colonies:	11	25	18	13	17	−
		Diameter:	386	414	372	263	166	−
	HEX + alginate	Colonies:	11	22	14	19	11	−
		Diameter:	386	469	331	276	152	

[a]Bacterial count determined by drop plate on plates with number of colonies between 15 and 150.

[b]Number per ml x 10^6.

[c]Diameter in microns; average of six representative colonies.

[d]No growth.

growth of S. *lutea*. The individual ingredients of TSA, however, added separately to NA did improve growth somewhat but not as much as the combination of TSA ingredients (Table 2). Only the inclusion of yeast extract (Difco) improved this medium. Table 5 shows that the concentration of yeast extract required for reversal is directly related to the HEX concentration.

Solute

P. fluorescens, which lyses readily on exposure to HEX during dissolution of the alginate swab has been previously shown *(3)* to be protected by the addition of NaCl or $MgSO_4$ but not KH_2PO_4 to the solution. Ringers' solution has been used by many investigators and since this contains 0.9% NaCl was studied as a replacement for the "Standard Methods" buffer. Results of this experiment are given in Table 6.

DISCUSSION AND CONCLUSIONS

HEX has been demonstrated to markedly inhibit many species of gram positive bacteria when included in media at concentration levels encountered in the recommended procedure *(1)*. The most sensitive organisms so far discovered to the small amounts of HEX carried over into the plating medium is S. *lutea (3)*. The reason for this inhibition is not completely clear but very likely involves magnesium metabolism and cell division since $MgSO_4$ can partially reverse the observed inhibition. Enriched media represented in this study by TSA eliminate inhibition at HEX levels normally encountered in the standard test and to a greater extent than $MgSO_4$ alone. This inhibition is also reversible by high concentrations of yeast extract in nutrient agar.

The reversal by yeast extract suggested the possibility that vitamins could be substituted; however, the inclusion of a vitamin mixture in nutrient agar had little or no reversal effect. The possibility still remains that the proper vitamins or the proper form of one of the vitamins was not used. Also of interest is the fact that the ingredients of TSA (phytone, trypticase, and sodium chloride) provided only partial reversal of S. *lutea* inhibition when used separately in nutrient agar, yet when together gave nearly the same results as commercial TSA. When measured in terms of recoverable numbers of cells, TSA and yeast extract show reversal of inhibition up to 0.2% HEX i.e., every cell grows. However, if one looks instead at the colony diameter at 24 hours as a measure of growth rate it is also clear that HEX affects the growth rate even when all cells are recovered. This effect is directly related to the HEX concentration and may support somewhat the inference that HEX interferes with cellular division of gram positive bacteria, possibly through the agency of magnesium.

Another reaction to HEX characteristic of some gram negative bacteria is given by *P. fluorescens* which promptly lyses on exposure to HEX during dissolution of the alginate swab. This effect no doubt has some relation to cell wall integrity but can be completely negated by inclusion of NaCl or $MgSO_4$ in the solute. Ringers' solution represents a useful buffer for use in this technique.

Ultimate success of the procedure lies in complete or consistent recovery of organisms from a wild population of cells. Wild populations, however, differ markedly in composition. Activated sludge for example has a high component of gram negative bacteria which appear to be a group showing considerable lysis during swab dissolution. The use of an adequate buffer i.e. high in NaCl is thus quite important with such populations. The earlier report of the authors indicates that such a population also contains a large number of organisms inhibited by the mere presence of HEX in the plating medium. Some of those are gram negative organisms.

TABLE 4. EFFECT OF *Sarcina lutea* EXPOSURE TO 2% HEX ON PLATE COUNT WITH NA AND TSA

Time of exposure to 2% HEX or water prior to planting	Colonies ml x 10^6		
	NA[a]	TSA	Water exposed control
15 min	1.5	18	22
3 hr	1.8	20	20
6 hr	1.7	18	20

[a]Colonies were considerably smaller than the control.

TABLE 5. REVERSAL OF HEX INHIBITION BY YEAST EXTRACT

	% HEX in the medium			
	0.01	0.05	0.1	0.2
% yeast extract giving uninhibited growth:	<0.01	1.0	5.0	>5.0

TABLE 6. EFFECT OF HEX ON *Pseudomonas fluorescens* WITH AND WITHOUT RINGERS' SOLUTION AS THE BUFFER

	15 minute exposure to:				
	2% HEX Plated on		2% HEX + alginate + Ringers' plated on		
	NA	TSA	NA	TSA	Control
Colonies bacteria per ml x 10^7:	17	22	56	66	70

On surfaces, presumably a large proportion of the bacterial population are gram positive bacteria which as a group are grossly inhibited by the presence of HEX in the plating medium. The choice of buffer for the solute and the medium for plating thus become extremely important. Tests on the wild population of activated sludge indicate that TSA gives virtually the same results with HEX present as TSA does without HEX. However, the technique modification proposed here presumes that the most sensitive organisms were used. If more sensitive strains are discovered a reevaluation of the technique will be warranted.

CONCLUSION

On the basis of the results presented in this report the following modifications of the alginate swab technique are suggested:

Buffer:

Ringers' solution- to 960 ml of 0.154 M NaCl solution add 20 ml 0.154 M KCL solution and 20 ml 0.11 M $CaCl_2$ solution.

HEX:

No more than a 2% solution during swab dissolution and should be added only at the time of plating. Reduction of HEX to less than 1%, while operationally possible incurs the risk of incomplete dissolution of a 50-mg swab (a common weight) and should be avoided. Exposure to HEX should be as short a time as practical for dissolution of the swab.

Medium:

Trypticase soy agar (BBL) or equivalent as determined by test against a sensitive organism such as *S. lutea*. The quantity of medium used to pour plates should be at least 20 ml per ml of sample to permit a final concentration of less than 0.1% HEX in the agar.

Procedure:

Ringers' solution is dispensed in 5-ml amounts in screw capped vials with 25-50-mg alginate swabs and sterilized. Swab is moistened with the solution, excess pressed out and the surface swabbed. The swab is returned to the vial and transported to the laboratory. Just prior to plating, 1 ml of a sterile 10% HEX solution is added to the vial and vigorously shaken until the swab is dissolved (this is 1.6% HEX in the vial). One-ml samples are withdrawn and placed in petri dishes. Twenty ml of TSA or equivalent are poured into each plate (this is 0.08% HEX in the medium) and incubated as usual.

REFERENCES

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