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Improved Staphylococcus Medium No. 110

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ABSTRACT

SMUCKLER, STAN A. (University of Southern California, Los Angeles), AND MILO D. APPLEMAN. Improved Staphylococcus Medium No. 110. Appl. Microbiol. **12**:355–359. 1964.—Most media have been unreliable when used to determine the number of coagulase-positive staphylococci in meat pot pies in the presence of overwhelming numbers of *Bacillus* cells. Various metabolic inhibitors were tested to determine whether they would suppress the growth of *Bacillus* cells without appreciably affecting the staphylococcal growth. The use of Staphylococcus Medium No. 110 modified by the addition of 0.75 mM sodium azide appears to be practical for the isolation of small numbers of coagulase-positive staphylococci from frozen meat pot pies.

The large number of selective and diagnostic media which have been described for the isolation of staphylococci clearly indicates that the perfect medium has not been developed. Some of the media most commonly employed for the isolation of *Staphylococcus* species are those described by Chapman (1945, 1946, 1948), Barber and Kuper (1951), Gillespie and Alder (1952), Zebovitz, Evans, and Niven (1955), Esber and Faulconer (1959), Innes (1960), Carter, Leininger, and Surkiewicz (1960), Hopton (1961), Finegold and Sweeney (1961), Raj and Liston (1961), and Baird-Parker (1962*a*). Crisley (1963) presented an excellent review on the media and method for the isolation and enumeration of staphylococci.

Most methods have been unreliable when used to determine, with some degree of accuracy, the number of coagulase-positive staphylococci in meat pot pies in the presence of overwhelming numbers of *Bacillus* cells. In studies utilizing locally purchased meat pot pies, staphylococci present at 10 to 100 per g could not be determined in a reproducible manner, by most of the available plating methods, in the presence of 50,000 to 100,000 *Bacillus* cells. This problem becomes increasingly important, because staphylococci should be absent from a food material or should be within prescribed limits to safeguard against human illness.

In this paper, we describe experiments in which various metabolic inhibitors were tested to determine whether they might suppress the growth of *Bacillus* without affecting the numbers of staphylococci.

MATERIALS AND METHODS

The metabolic inhibitors tested were hydroxylamine, 2,4-dinitrophenol, and the sodium salts of arsenate, arsenite, azide, fluoride, and iodoacetate. The staphylococci studied were coagulase-positive strains isolated from food poisoning outbreaks. In addition to the species of *Bacillus* studied, strains of *Aerobacter*, *Escherichia*, *Micrococcus*, *Salmonella*, *Streptococcus*, *Proteus*, and *Pseudomonas*, which are not infrequently encountered in food products, were also utilized. Cultures were carried on Trypticase Soy Agar (BBL), and transfers were made into Brain Heart Infusion (BBL) broth 20 hr before the actual test.

A method for the rapid screening of the inhibitors was used in which filter paper disks saturated with inhibitors at various concentrations were placed on seeded Trypticase Soy Agar plates. The width of the zones of inhibition gave a rough approximation of the effectiveness of the inhibitors, and quantitative studies were based upon this qualitative methodology.

Inhibition studies were also carried out on Trypticase Soy Agar and Staphylococcus Medium No. 110 modified by the addition of Seitz-sterilized increments of the inhibitor tested. Each inhibitor was tested over a concentration range from at least 0.1 to 100 mM. Plates were dried at 37 C for 24 hr before use.

Dilutions were made in phosphate buffer rather than phosphate-peptone water, even though early studies in this laboratory had shown that the latter resulted in more reproducible counts over long periods of time. It was thought that the peptone might become one more complicating factor in the inhibition studies.

A modification of the drop-plate technique of Mallman and Broitman (1956) was used in which dilutions of each test culture were made in 0.02 M phosphate buffer (pH 7.0) and 0.1-ml samples were placed on replicate quadrants of the test media. After seeding, the plates were incubated at 37 C and examined for growth after 24 and 48 hr. The number and size of the colonies on media containing inhibitors were compared with inocula made simultaneously on Trypticase Soy Agar or Staphylococcus Medium No. 110 as control media.

After the preliminary evaluations of the various metabolic inhibitors, additional studies were made on Staphylococcus Medium No. 110 into which sodium azide was incorporated. For these studies, in addition to pure cultures grown in laboratory medium, strains of *Bacillus* or *Staphylococcus* were inoculated into 20-g samples of chicken pot pie, previously autoclaved with 180 ml of water in a Waring Blendor cup. The inoculated pie samples were

comminuted for a total of 5 min: 2 min slow speed, and 3 min fast speed. Portions from the original samples and subsequent tenfold dilutions of each sample were drop-plated onto Trypticase Soy Agar and modified Staphylococcus Medium No. 110. Other studies were made upon meat pies purchased locally.

RESULTS

Many of the inhibitors could be used to separate one or two genera of bacteria from the other groups tested, but the clear repression of the *Bacillus* species without interference with *Staphylococcus* strains was limited to sodium azide. Consequently, it is not necessary, in this paper, to tabulate the mass of data obtained by both qualitative and quantitative experiments with the other inhibitors and organisms used.

The disc screening method indicated that sodium azide incorporated into Trypticase Soy Agar at a level of approximately 1.0 mM would inhibit *Bacillus* species but would not appreciably inhibit the *Staphylococcus* strains. Quantitative studies in which staphylococci and *Bacillus* strains were drop-plated on Trypticase Soy Agar containing azide at levels of 0.0 to 5.0 mM also indicated that sodium azide at levels above 1.0 mM might be utilized for the selective isolation of staphylococci. The *Staphylococcus* strains grew on 5.0 mM azide without significant loss of numbers or colony size as compared with the control medium. The *Bacillus* species, however, were inhibited by levels of azide above 1.0 mM.

Staphylococcus Medium No. 110 is a good medium, but the *Bacillus* in the meat pot pies under study overgrew the staphylococci. Consequently, trials were made to determine whether lower levels of sodium azide than were used in Trypticase Soy Agar would effectively inhibit the spore-former. Strains of staphylococci and *Bacillus* were streaked onto Staphylococcus Medium No. 110 containing azide at final concentrations of 0.0, 0.1, 0.5, 1.0, and 2.0 mM. The *Bacillus* strains were inhibited by 0.5 mM azide, but, although colony size decreased at concentrations higher than 1.0 mM, 2.0 mM sodium azide was required to inhibit the staphylococci.

A study was made to determine whether the nutritive constituents of meat pot pies would have any protective effect on the organisms. The results of one *Bacillus* species and one *Staphylococcus* strain individually tested in chicken pot pie against 0.0, 0.25, 0.5, 0.75, and 1.0 mM sodium azide are shown in Table 1. The results indicate that the growth of the *Bacillus* was inhibited by 0.5 mM azide at 24 hr of incubation, and those colonies on the plates containing 0.25 mM azide were distinctly smaller than colonies on the control plates. Colonies of the *Bacillus* were discernible at 48 hr on the medium containing 0.5 mM azide, but again the colonies were appreciably smaller than those on the control plates. The *Staphylococcus* was not inhibited by 0.75 mM azide, and growth was essentially complete after 24 hr. There were no appreciable losses in numbers of staphylococci as compared with control plates. The colonies were slightly smaller on the medium containing 1.0 mM azide, and the pigment was not quite as intense as that in the colonies on the control plates.

A comparison of several media (Table 2) indicated that the recovery of ten coagulase-positive strains of *S. aureus* was equally as good on Staphylococcus Medium No. 110 containing 0.75 mM sodium azide as on other media which permitted the growth of *Bacillus* spp. found in meat pies and was definitely superior in some cases to Tellurite Glycine Agar.

More than 300 frozen pot pies have been tested relative to the standard plate count and to the number of staphylococci present in each pie; those results will appear in a later article. Table 3, however, illustrates the efficiencies of five plating media for the recovery of staphylococci from frozen meat pot pies. Trypticase Soy Agar was used as the control medium in this series to determine the standard count by

TABLE 1. *Comparison of the growth of Bacillus and Staphylococcus strains from inoculated chicken pot pies, on Staphylococcus Medium No. 110 containing sodium azide*

Medium*	Sodium azide	*Bacillus*†		*Staphylococcus*‡	
		24 hr	48 hr	24 hr	48 hr
	mM				
TSA	0	20	20	89	89
S-110	0	13	16	81	81
S-110	0.25	5	15	87	87
S-110	0.5	0	16	86	86
S-110	0.75	0	0	83	83
S-110	1.0	0	0	84	84

* TSA, Trypticase Soy Agar; S-110, Staphylococcus Medium No. 110.
† Final dilution of $1:8 \times 10^5$.
‡ Final dilution of $1:2 \times 10^7$.

TABLE 2. *Comparison of several media for the isolation of coagulase-positive staphylococci based on 75% recovery**

Staphylococcus strain	Medium†				
	MSA	TGA	CMA	S-110	S-110 + azide
1	+	−	−	+	+
2	+	−	+	+	+
3	−	+	+	−	+
4	+	+	+	+	+
5	+	−	+	+	−
6	+	−	ND‡	−	−
7	−	−	ND	−	+
8	+	−	ND	+	+
9	−	−	ND	−	−
10	+	−	ND	+	+

* Trypticase Soy Agar was used as the control medium.
† MSA, Mannitol Salt Agar; TGA, Tellurite Glycine Agar; CMA, coagulase mannitol agar; S-110, Staphylococcus Medium No. 110; S-110 + azide, Staphylococcus Medium No. 110 containing 0.75 mM sodium azide.
‡ ND, not determined.

the drop plate method per gram of sample. Coagulase mannitol agar was included in the study at the request of the manufacturer. This is a good differential medium but, although it does inhibit some microorganisms, it is not sufficiently selective to permit the quantitation of staphylococci in the presence of a heterogeneous population of organisms. Mannitol Salt Agar was intermediate in its selectivity for staphylococci. The total number of colonies on Mannitol Salt was lower than that on the Trypticase Soy or coagulase mannitol agars. However, quantitation of staphylococci was not possible in most cases because of the presence of *Bacillus* and other miscellaneous microorganisms. Tellurite Glycine Agar inhibited all miscellaneous microorganisms, but this medium also appeared to have inhibited some strains of staphylococci, and the developing colonies were smaller than the staphylococcal colonies on the other media. Staphylococcus Medium No. 110 permitted satisfactory recovery of staphylococci, but in over 50% of the cases there were some contaminating organisms present. Only Staphylococcus Medium No. 110 containing

TABLE 3. *Comparison of media for the recovery of staphylococci from frozen meat pot pies*[a]

Brand	Type of pot pie	Medium[b]									
		TSA	CMA		MSA		TGA		S-110		S-110 + azide	
			Count[c]	MG[d]	Count	MG	Count	MG	Count	MG	Count	MG
I	Beef	97,000	46,000	+	18,000	+	1,900	0	3,300	0	3,200	0
		410,000	98,000	+	32,000	+	5,400	0	15,000	+	12,000	0
		160,000	47,000	+	7,600	+	2,700	0	6,200	+	5,000	0
		200,000	83,000	+	9,600	+	3,300	0	7,600	0	7,000	0
		210,000	68,000	+	18,000	+	6,800	0	6,600	0	7,300	0
		190,000	60,000	+	8,000	+	4,600	0	9,300	+	7,200	0
			23,000	+	10,000	+	6,200	0	7,900	+	6,300	0
	Chicken	700,000	98,000	+	Spread[e]	+	12,000	0	29,000	0	25,000	0
		950,000	180,000	+	Spread	+	30,000	0	28,000	+	30,000	0
		300,000	82,000	+	46,000	+	8,700	0	14,000	+	10,000	0
		640,000	160,000	+	56,000	+	14,000	0	18,000	0	20,000	0
		580,000	160,000	+	32,000	0	12,000	0	18,000	0	17,000	0
		460,000	160,000	+	18,000	0	9,800	0	16,000	0	15,000	0
			31,000	+	21,000	+	400	0	1,600	+	3,300	0
II	Beef	1,700,000	250,000	+	26,000	+	600	0	18,000	+	12,000	0
		470,000	57,000	+	6,500	+	4,300	0	5,800	+	5,200	0
	Chicken	4,400,000	1,100,000	+	110,000	+	120,000	0	Spread	+	50,000	0
		900,000	300,000	+	140,000	+	1,800	0	4,700	0	2,900	0
III	Beef	700,000	63,000	+	7,600	+	1,900	0	4,600	0	3,300	0
			50,000	+	5,000	+	300	0	2,600	+	2,800	0
			75,000	+	2,800	+	1,100	0	3,200	+	2,500	0
	Beef stew	120,000	47,000	+	4,500	0	1,300	0	6,700	+	4,600	0
		410,000	6,400	+	4,700	0	700	0	1,200	0	1,000	0
			3,400	+	1,300	0	300	0	900	0	600	0
	Chicken	400,000	65,000	+	5,700	0	3,900	0	4,500	0	3,000	0
		500,000	210,000	+	24,000	+	3,200	0	11,000	+	7,300	0
	Turkey	500,000	50,000	+	5,700	+	3,300	0	3,400	0	3,600	0
		300,000	50,000	+	4,200	+	2,900	0	3,300	+	2,300	0
		500,000	163,000	+	29,000	+	6,800	0	40,000	+	8,700	0
	Chicken and rice	800,000	500,000	+	150,000	0	24,000	0	30,000	+	25,000	0

[a] Values are recorded in numbers per gram of sample.

[b] TSA, Trypticase Soy Agar; CMA, coagulase mannitol agar; TGA, Tellurite Glycine Agar; S-110, Staphylococcus Medium No. 110; S-110 + azide, Staphylococcus Medium No. 110 containing 0.75 mM sodium azide.

[c] Count is the number of organisms per gram of sample.

[d] MG is "miscellaneous growth," or the presence of spreading or other contaminating microorganisms.

[e] Spread, is "spreaders," or the presence of spreading organisms on the plates from the highest dilution used, so that quantitative counts were not possible.

0.75 mM sodium azide was sufficiently selective to permit complete colonial development of the staphylococci and inhibit contaminating microorganisms.

DISCUSSION

The primary effect of sodium azide is thought to be the uncoupling of oxidative phosphorylation in the electron transport chain; that is, it prevents the formation of adenosine triphosphate but permits oxidation to proceed, even at an accelerated rate, with the generation of heat. Therefore, sodium azide would be expected to be relatively nontoxic to the fermentative staphylococci as compared with the more oxidative *Bacillus* spp.

Eddy and Ingram (1962) reported that Mannitol Salt Agar and Staphylococcus Medium No. 110, which gave satisfactory counts with coagulase-positive colonies, were unreliable with coagulase-negative staphylococci. Jay (1961) had previously reported that in the isolation of staphylococci from certain market meats only about 10% of the suspect colonies on Tellurite Glycine Agar were actually coagulase-positve. In a study of the efficiencies of six media in isolating coagulase-positive staphylococci from meats, Jay (1963) reported that the simultaneous u of mannitol-sorbic acid broth and Staphylococcus Medium No. 110 fortified with egg yolk gave the best results when compared with Mannitol Salt Agar, Tellurite Glycine Agar, Polymyxin agar, and Vogel and Johnson agar.

Since Gillespie and Alder (1952) observed that 80% of the coagulase-positive staphylococci examined had the capacity to produce an opaque zone when grown on egg-yolk media, numerous investigators have utilized this activity in developing selective media. However, studies by Colbeck et al. (1956) and Graber et al. (1958) indicated that there is no definite correlation between coagulase activity of *S. aureus* strains and ability to produce opacity on egg-yolk media. Some coagulase-positive staphylococci failed to give opacity, whereas some coagulase-negative strains were found to be egg-yolk-positive. These results do not agree with those of Gillespie and Alder (1952), who found that all of the 60 coagulase-negative staphylococci examined were egg-yolk-negative. Thus, although the egg-yolk reaction is correlated with the coagulase activity of staphylococci to some extent, it is not absolute.

Baird-Parker (1962*a*) recently developed an egg yolk, tellurite glycine, pyruvate agar (ETGPA) that is a modified form of Tellurite Glycine Agar (Zebovitz et al., 1955). On ETGPA, *S. aureus* appears to metabolize pyruvate, whereas many other organisms, such as micrococci, either metabolize it slowly or not at all. Using ETGPA, Baird-Parker (1962*b*) was able to confirm all suspect coagulase-positive colonies. Baird-Parker (1962*a*, *b*) summarized the drawbacks in the use of his medium. They include the complexity of preparation, lability in storage, the similar appearance of *Proteus vulgaris* and *S. aureus*, and the occasional occurrence of yeasts, *Bacillus* spp., and egg-yolk nonclearing strains of *S. aureus*.

There exists the possibility that pyruvate is actually removing the tellurite from the medium, and this apparently occurs under some circumstances. This tellurite may be precipitated as a white oxide or other compound rather than being reduced to the black tellurium metal. Thus, the concentration of tellurite necessary to inhibit coagulase-negative staphylococci might not be present. It is also possible that the occasional nonclearing coagulase-positive strains actually give only an apparent negative test in that the remaining cloudiness may be due to a tellurium oxide or other compound. This will be studied further in these laboratories and may explain the few discordant reports involving the medium of Baird-Parker (1962*a*).

Peterson, Black, and Gunderson (1962), in studies on the growth of naturally occurring mixed populations in chicken pot pies, found that it was not possible to promote the growth of appreciable numbers of staphylococci under any conditions of defrost. Apparently, the greater the saprophytic population, the greater is the protection against staphylococcal growth through antagonism, competition for nutrients, and modification of the environment to conditions less favorable to staphylococcal growth. Dack and Lippitz (1962) obtained essentially the same results with meat pot pies into which they had inoculated *Salmonella typhimurium*, *E. coli*, or a food poisoning strain of *Staphylococcus*. The natural flora exerted an inhibitory effect upon the growth of the added organisms after incubation at 35 C for 18 hr. However, if small numbers of staphylococci must be detected, direct plating media, which are either insufficiently selective or excessively inhibitory to staphylococci, would not be appropriate.

The Staphylococcus Medium No. 110 containing sodium azide described in this paper appears to be practical for the isolation of small numbers of staphylococci from meat pot pies. Further investigation into the effects of the variety of metabolic inhibitors available may lead to the development of media which will be selective for other potential pathogens.

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