APPLIED MICROBIOLOGY, July 1973, p. 126-127
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Vol. 26, No. 1
Printed in U.S.A.

Some Observations on the Incorporation of Novobiocin into Hektoen Enteric Agar for Improved *Salmonella* Isolation

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Received for publication 29 March 1973

Novobiocin was incorporated into Hektoen enteric agar as a means of suppressing those *Proteus* and *Citrobacter* organisms that produce colonies which are often mistaken for *Salmonella*.

It has already been established by Jeffries (2) and Hargrove et al. (1) that novobiocin is an effective agent for suppression of nonsalmonellae in enrichment broths. This preliminary study was conducted to determine whether the selectivity of Hektoen enteric agar (HEA) (Difco Laboratories) for *Salmonella* could be improved by the incorporation of novobiocin and to determine what concentration of antibiotic would be required to accomplish this.

HEA has been reported as a plating medium for *Salmonella* isolation by King and Metzger (3). It is routinely used for *Salmonella* isolation in this laboratory and in various other quality control laboratories. Although selective for *Salmonella*, HEA suffers the disadvantage of also being selective for certain other nonsalmonellae. Some *Citrobacter* and *Proteus* species produce colonies that appear similar to salmonellae (black-centered colonies) on HEA and that are sometimes identified as *Salmonella*.

For testing purposes, pure cultures of salmonellae and related organisms, which are often found in tetrathionate broth enrichments of feed materials, were employed in this study. Cultures were grown in lactose broth for 18 h at 37 C, chilled to stabilize their growth, diluted, and surface plated onto dry HEA plates containing novobiocin at 0, 2, 5, 10, 15, and 20 μg/ml (Upjohn Co.). These cultures included six serotypes of *Salmonella*, namely *S. cubana*, *S. seftenberg*, *S. typhimurium*, *S. urbana*, *S. montevideo*, *S. worthington*, and representative strains of *Citrobacter freundii*, *C. diversus*, *Escherichia coli*, *Proteus mirabilis*, and *P. morganii*. Various unidentified strains of *Citrobacter*, *E. coli*, *Proteus*, and *Salmonella*, which previously had been isolated from feed materials, chicken skins, frozen eggs, and sliced, prepared apples and identified by their biochemical reactions in differential media, were also employed. Broth cultures were surface plated in quadruplet at two different concentrations. HEA plates were incubated at 37 C for 18 to 20 h prior to counting. A reduction in bacterial colonies was considered an inhibitory result. Table 1 shows the relative degrees of growth of these enteric organisms with increasing concentrations of novobiocin in HEA.

When novobiocin at 5 or 10 μg/ml was incorporated into HEA, the *Proteus* and *Citrobacter* varieties employed in this study were inhibited. Two of the 10 *Citrobacter* and 12 of the 14 *Proteus* cultures produced black-centered colonies similar to salmonellae. A 50% reduction in *Citrobacter* growth was observed when novobiocin was added to HEA at 10 μg/ml of agar. No growth was observed with any of the *Proteus* strains employed in this study at this level. The *E. coli* varieties used in this study presented no problems, because they produced salmon-colored colonies; however, the growth of most strains tested appeared reduced at these concentrations of antibiotic. Ten serotypes of *Salmonella* grew without any apparent inhibitory effects when novobiocin was used at 2, 5, 10, and 15 μg/ml in the HEA. All of these serotypes produced black-centered colonies on HEA. At 20 μg of antibiotic per ml of agar, *Salmonella* grew, but at a much slower rate and without the production of the typical black-centered colonies.

Novobiocin was stable at concentrations up to 20 μg/ml in HEA. Agar plates containing novobiocin stored for several weeks at 22 C gave

TABLE 1. *Effect of various concentrations of novobiocin in Hektoen enteric agar on the growth of 46 strains of Enterobacteriaceae*[a]

Organism	No. of strains tested	No. of strains tested that produced black-centered colonies on HEA	Concn of novobiocin (μg/ml of agar)					
			0	2	5	10	15	20
Citrobacter	10	2	XXXX	XXXX	XXX	XX (50% Inh)	XX[b]	XX[b]
Escherichia coli	12[c]	0	XXXX	XXXX	XXXX	XXX (25% Inh)[d]	XXX[b, d]	XXX[b, d]
Proteus	14	12	XXXX	XX[b]	X[b]	O (100% Inh)	O	O
Salmonella	10	10	XXXX	XXXX	XXXX	XXXX	XXXX	XXX[b,e]

[a] There were 30 to 300 organisms per ml of broth culture. 4+ Growth = 4X; 2X = 50% of 4X = 50% inhibition (Inh); O = 100% inhibition = no growth.

[b] Colony morphology altered, variegated edges.

[c] Salmon-colored colonies on HEA.

[d] Several strains not inhibited.

[e] Poor growth, no black centers produced at 24 h of incubation at 37 C.

similar inhibitory results to freshly prepared plates with the nonsalmonellae.

A considerable difference was noted in the results obtained from HEA plates with and without novobiocin added when they were streaked with tetrathionate broth enrichments of feed materials. Twenty different *Salmonella*-positive feed materials were selected for this study. These, bone meal, fish meal, feather meal, and poultry-by-products meal, because of their content of *Citrobacter* and *Proteus* organisms, presented large numbers of colonies which could be mistaken for *Salmonella* on the HEA plates without novobiocin. HEA plates containing the antibiotic at 10 or 15 μg/ml consistently developed fewer *Proteus* and *Citrobacter* colonies, thus reducing the need for picking large numbers of presumptive *Salmonella* colonies for further biochemical characterization to establish the presence of salmonellae.

These findings suggest that the selectivity of HEA for *Salmonella* can be improved by the addition of novobiocin without adversely affecting *Salmonella* recovery. These results in HEA substantiate the findings in LICNR broth by Hargrove et al. (1) and in tetrathionate broth by Jeffries (2). The addition of novobiocin to other selective agars for improving their selectivity for *Salmonella* isolation is suggested.

Since only a relatively small number of salmonellae were tested, it is possible that some may not produce eugenic growth on HEA in the presence of novobiocin and/or their colonial morphology may be altered.

HEA is frequently used by diagnostic laboratories for the isolation of *Salmonella* and *Shigella*. Because the growth of *Shigella* on HEA in the presence of novobiocin has not been studied, this modified medium, for obvious reasons, should not be used for the isolation of enteric pathogens from clinical specimens.

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