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GELRITE as an Agar Substitute in Bacteriological Media

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GELRITE gellan gum (formerly known as PS-60 and S-60) is a new naturally derived, highly purified polysaccharide which displays several interesting properties, including selfgelling. The suitability of GELRITE as an agar substitute was tested by evaluating the performance of several media selected from among those most commonly used in the isolation, identification, and enumeration of microorganisms in clinical laboratories. Fifty different bacterial species previously implicated in human infections served as test strains. On the basis of the various parameters considered, namely, colony characteristics, biochemical reactions, hemolytic patterns, and plating efficiency, media gelled by agar and by GELRITE compared quite favorably.

GELRITE gellan gum (formerly known as PS-60 and S-60) is a new naturally derived (a fermentation product of a *Pseudomonas* species), highly purified heterosaccharide biopolymer discovered by Kelco Div., Merck & Co., Inc., San Diego, Calif. Its most prominent characteristics include ability to gel in the presence of a cation, agar-like rigidity, compatibility with nutrient additives, thermal stability, and optical clarity (1, 2). The present study was undertaken with a twofold objective in mind: to determine the effect of GELRITE in comparison with agar on bacterial growth as judged by plating efficiency and to evaluate the performance of this gellan gum as an agar substitute in several media commonly used for routine isolation and identification of bacterial species from clinical specimens.

MATERIALS AND METHODS

Bacterial species. Fifty different bacterial species representing a total of 26 genera were tested (see Tables 3 and 4). These cultures were chosen from a collection of approximately 2,600 clinical isolates of human origin acquired within the past 2 years from hospitals across the United States and kept frozen at −70°C. Before use, the test strains were regrown on a blood-based medium to check for purity.

Media. Culture media and ingredients used in this study are identified in Table 1. Freshly prepared plates of tryptic soy agar and tryptic soy GELRITE containing 5% sheep blood were provided by GIBCO Diagnostics, Madison, Wis. The following dehydrated media without the gelling agents were custom-made for Kelco by the indicated manufacturers: bismuth sulfite (BBL Microbiology Systems, Cockeysville, Md.), brilliant green (BBL), eosin methylene blue (Acumedia, Baltimore, Md.), MacConkey (BBL), salmonella-shigella (Acumedia), and triple sugar iron (Acumedia). All the other media and ingredients were taken from our own laboratory stocks as purchased from regular suppliers.

Media containing agar or GELRITE as the solidifying agents were freshly prepared in accordance with both published (Kelco Commercial Development bulletins CD-26 and CD-27) and unpublished information provided by Kelco (Table 1). An important consideration in the formulation of these guidelines was the achievement of comparable gel strengths (grams per square centimeter) between agar- and GELRITE-containing media. The basic procedure for the preparation of the various media entailed the following: (i) dissolution of specific quantities of dehydrated medium, $MgCl_2 \cdot 6H_2O$, and GELRITE or agar in distilled water; (ii) autoclaving the dissolved mixture of ingredients for 15 min at 121°C and 15 lb/in^2 in a small bench-type agar sterilizer (New Brunswick Scientific Co., Edison, N.J.); (iii) cooling the medium down to a holding temperature of 60°C (GELRITE) or 50°C (agar) after a gentle swirl; and (iv) dispensing the medium into either standard 15-mm petri dishes at 20 ml per plate or screw-capped tubes at 7 ml per tube (for triple sugar iron). The preparation of both salmonella-shigella and bismuth sulfite media did not include autoclaving. In each case, the medium mixtures were simply boiled (100°C) for approximately 1 min and dispensed after cooling to holding temperatures. Heat-labile supplements such as blood, egg yolk emulsion, and IsoVitaleX (BBL) were prewarmed briefly and added to appropriate media after each medium had been autoclaved and allowed to cool to the stated holding temperatures. For making chocolate medium, tryptic soy agar base was used; after being autoclaved, the mixture was enriched with an equal volume of prewarmed (60°C) 2% hemoglobin, plus 1% IsoVitaleX (vol/vol). The same procedure was used for making egg yolk medium, except that the mixture was enriched with prewarmed (37°C) 10% egg yolk emulsion. Phenylethyl alcohol medium consisted of tryptic soy agar base, phenylethyl alcohol (2.5 g/liter), 5% sheep blood, and the solidifying agents.

TABLE 1. Media and other ingredients

Media and ingredients[a]	Source	Lot	Amt (g/liter) of:			
			Base	Agar	GELRITE	Magnesium
BS	BBL	1123X	32	20	10	0.75
BHI	Difco Laboratories, Detroit, Mich.	577772	37	15	7.5	
BG	BBL	1122X	38	20	9	0.75
Chocolate			27	13	8	0.75
EY			27	13	8	0.75
EMB	Acumedia	8110-103	22.5	13.5	7.5	0.75
MacConkey	BBL	1121X	36.5	13.5	7	0.75
N	Difco	495555	8	15	8	0.75
PEA			27	13	8	0.75
SS	Acumedia	8110-100	46.5	13.5	5.5	
TSI	Acumedia	8110-101	46.5	13.5	6	0.75
TSB	BBL	JODG2M	27	15	7.5	0.75
TS agar with 5% sheep blood	GIBCO	1731241				
TS GELRITE with 5% sheep blood	GIBCO	1731247				
TS agar base	GIBCO	180388				
YE	Difco	647268				
PEA	ICN Pharmaceuticals Inc., Irvine, Calif.	27411				
EY emulsion	Oxoid Ltd., Basingstoke, England	50122				
IsoVitaleX	BBL	A4HDCC				
Agar	Difco	683803				
GELRITE (S-60)	Kelco	K1A93-63002A				
$MgCl_2 \cdot 6\ H_2O$	Fisher Scientific Co., Pittsburgh, Pa.	706396				
Defibrinated sheep blood	BBL	292				
Bovine hemoglobin solution	BBL	B3ESWL				

[a] BS, Bismuth sulfite; BHI, brain heart infusion; BG, brilliant green; EY, egg yolk; EMB, eosin methylene blue; N, nutrient broth; PEA, phenyl ethyl alcohol; SS, salmonella-shigella; TSI, triple sugar iron; TSB, Trypticase soy broth; TS, tryptic soy; YE, yeast extract.

Viable cell counts. Each of three replicate broth tubes (10 ml per tube) was inoculated with a loopful of culture from each of the test organisms. Trypticase soy broth (BBL) was used with all cultures except *Streptococcus pneumoniae*, the anaerobes (*Clostridium perfringens*, *Peptostreptococcus anaerobius*, and *Bacteroides fragilis*), and *Campylobacter fetus* subsp. *jejuni*. These were grown in brain heart infusion broth. When fastidious organisms were tested, the media were appropriately enriched as shown in Table 2. Except for the *Campylobacter* culture, which was incubated at 42°C, all the other broth cultures were

TABLE 2. Compositions of media used in quantitation of fastidious microorganisms

Microorganism	Composition[a] of:	
	Liquid medium	Plating medium with agar or GELRITE
Haemophilus influenzae CL1826	TSB + Iso + XV	TSB + 10% LHB + Iso
Haemophilus parainfluenzae CL1824	TSB + Iso + XV	TSB + 10% LHB + Iso
Streptococcus pyogenes CL1925	TSB + 10% HS	TSB + 10% HS
Streptococcus agalactiae CL 1342	TSB	TSB + 10% HS
Streptococcus faecalis CL1776	TSB	TSB + 10% HS
Group C *Streptococcus* species CL909	TSB	TSB + 10% HS
Vibrio cholerae CL2020	TSB	TSB + 10% HS
Group G *Streptococcus* species CL1929	TSB + 10% HS	TSB + 5% DSB
Moraxella species CL387	TSB + 10% HS	TSB + 5% DSB
Streptococcus pneumoniae CL1842	BHI + 5% DSB	BHI + 5% DSB
Campylobacter fetus subsp. *jejuni* CL2312	BHI + 5% DSB	TSB + 5% DSB
Clostridium perfringens CLA144	BHI + 5% DSB	BHI + 0.5% YE + He + Me
Peptostreptococcus anaerobius CLA107	BHI + 5% DSB	BHI + 0.5% YE + He + Me + 5% DSB
Bacteroides fragilis CLA177	BHI + 5% DSB	BHI + 0.5% YE + He + Me

[a] TSB, Trypticase soy broth; Iso, 1% IsoVitaleX; XV, Taxo X and V Strips (BBL); LHB, lysed horse blood; HS, horse serum; DSB, defibrinated sheep blood; BHI, brain heart infusion; YE, yeast extract; He, hemin (5 μg/ml); Me, menadione (0.5 μg/ml).

incubated at 35°C. The incubation period required to achieve maximum growth varied from 18 to 24 h for most of the test organisms to 48 and 72 h for *P. anaerobius* and *C. fetus* subsp. *jejuni*, respectively. The cultures were grown undisturbed except for the two strict aerobes, *Moraxella* and *Pseudomonas* species, which were grown on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 250 rpm. Anaerobes were incubated in an anaerobic chamber (Forma Scientific, Marietta, Ohio) in an atmosphere of 10% hydrogen–8% carbon dioxide–82% nitrogen. *C. fetus* subsp. *jejuni* cultures were incubated in a microaerophilic environment with GasPak jars and CampyPak II envelopes (BBL).

After incubation, the broth cultures were diluted serially with Trypticase soy or brain heart infusion broth. Portions (0.1 ml) of suitable dilutions were spread with a sterile glass rod over the surfaces of plating media containing agar or GELRITE. The plating was done in duplicate or, with the more fastidious organisms, in triplicate. The basic plating media to which agar or GELRITE were added varied according to the growth requirements of the test organisms. Except where indicated (Table 2), Trypticase soy broth with or without the additives was used throughout. Nutrient broth was used with *Proteus vulgaris, P. mirabilis*, and *Morganella morganii* to prevent swarming typically observed with these organisms.

Plated cultures were incubated under the same conditions as those described for liquid cultures. Colonies were counted with the aid of a Biotran II automated colony counter (New Brunswick Scientific Co.). Visual or manual counting was used only in those instances in which defibrinated sheep blood or lysed horse blood was incorporated into the media.

Colony morphology and biochemical reactions. In the portion of this study which involved a comparative evaluation of colony characteristics, hemolytic patterns, and biochemical reactions, the freshly prepared plates of media were inoculated by streaking for isolation, and the only tubed medium used (triple sugar iron) was inoculated by the usual stabbing of the butt and streaking of the slant. All cultures were incubated at 37°C for 24 h, except for the anaerobes and the *Campylobacter* isolate, which were incubated for 48 h at 37 and 42°C, respectively.

RESULTS AND DISCUSSION

Quantitative determination of viable cells. Duplicate or triplicate viable cell counts were made on each of the three subcultures of each of the 50 clinical isolates tested in this study, and the data shown in Tables 3 and 4 represent, for each test strain, the mean of six or nine individual counts ± standard deviation. For the 36 nonfastidious bacterial species grown on unsupplemented media (Table 3), it can be seen that the mean counts on media containing GELRITE were higher than those on agar-containing media in 29 instances. However, the analysis of variance (4) based on individual counts within each paired set of data showed the higher GELRITE mean to be statistically significant for only 8 of the 29 cultures, namely, *Citrobacter freundii, Enterobacter aerogenes, Klebsiella oxytoca, Pseudomonas maltophilia, Shigella dysenteriae, S. flexneri, Staphylococcus aureus*, and *Yersinia enterocolitica*.

The differences were not statistically significant for the seven remaining cultures (*E. agglomerans, P. mirabilis, M. morganii, Salmonella enteritidis, S. typhi, P. aeruginosa*, and *Arizona hinshawii*), for which lower means were recorded for GELRITE.

As for the 14 fastidious cultures (Table 4), a comparison of the mean counts for each test culture between GELRITE and agar showed 10 of the 14 cultures to have slightly higher values on agar than on GELRITE. For the remaining four cultures, which included *Streptococcus agalactiae, S. pneumoniae*, group G *Streptococcus* species, and *Peptostreptococcus anaerobius*, the mean counts were higher on GELRITE. A statistical evaluation of these data by analysis of variance showed the above-mentioned differences to be insignificant.

Overall, it is obvious that GELRITE compared favorably to agar in its ability to serve as a gelling agent in some of the basic and nonselective bacteriological media used in laboratories for quantitating bacterial species with different nutritional requirements. Because in most instances (33 of 50 isolates) viable bacterial counts were higher on GELRITE-containing media, it would appear that the new product is less inhibitory than agar to bacterial growth under the conditions of these experiments. Blood GELRITE medium gelled at approximately 46 to 48°C. A low holding temperature (50°C) caused the medium to solidify during pouring, whereas a high holding temperature (60°C) caused substantial hemolysis of the sheep blood. This problem was somewhat overcome by using small batches (250 ml) of the basal medium at 60°C and adding prewarmed (37 to 40°C) blood supplements immediately before pouring. The relatively short exposure time of the sheep blood to the high temperature prevented or greatly minimized the degree of hemolysis and allowed pouring of satisfactory blood plates.

Colony characteristics and biochemical reactions. Blood agar and blood GELRITE plates were used to gather information on the size, color, and surface properties of representative colonies as well as on the pattern and the size of the zones of hemolysis for each of the 50 bacterial species tested. Data on the sizes of the colonies and of the zones of hemolysis were expressed as the average diameters of five independent determinations. For approximately 90% of the isolates, the colony sizes on GELRITE-containing media differed from those on agar-containing media by ≤0.5 mm, as exemplified by *S. aureus* (Fig. 1). Differences of ≤1.5 mm were

TABLE 3. Viable cell counts: agar versus GELRITE

Microorganism	Mean[a] ± SD for:	
	Agar	GELRITE
Acinetobacter calcoaceticus CL1658	57.8 ± 9.2	60.3 ± 14.1
Arizona hinshawii CL2075	138.2 ± 18.6	131.0 ± 20.3
Citrobacter diversus CL1519	60.5 ± 14.6	64.5 ± 12.3
Citrobacter freundii CL1663	63.3 ± 11.5	85.3 ± 11.6
Edwardsiella tarda CL2076	26.7 ± 7.9	27.3 ± 10.5
Enterobacter aerogenes CL1548	61.3 ± 16.4	86.3 ± 17.9
Enterobacter agglomerans CL1387	76.7 ± 22.2	74.0 ± 28.1
Enterobacter cloacae CL1779	89.8 ± 15.6	100.7 ± 22.4
Escherichia coli CL1552	61.2 ± 10.4	65.3 ± 9.3
Flavobacterium odoratum CL1435	45.2 ± 12.0	56.8 ± 14.3
Hafnia alvei CL136	104.0 ± 26.2	108.7 ± 14.5
Klebsiella oxytoca CL1060	42.7 ± 9.9	52.0 ± 7.7
Klebsiella ozaenae CL904	55.0 ± 7.2	60.5 ± 16.1
Klebsiella pneumoniae CL1697	46.8 ± 12.7	54.2 ± 8.7
Morganella morganii CL1555	121.8 ± 28.7	127.0 ± 33.4
Proteus mirabilis CL1772	152.8 ± 14.7	153.0 ± 16.6
Proteus vulgaris CL1190	119.5 ± 26.9	107.8 ± 19.9
Providencia alcalifaciens CL787	141.3 ± 28.5	159.8 ± 18.3
Providencia rettgeri CL1192	122.8 ± 22.0	126.7 ± 17.1
Providencia stuartii CL1443	99.5 ± 16.8	103.7 ± 22.2
Pseudomonas aeruginosa CL1560	80.8 ± 17.6	68.7 ± 18.7
Pseudomonas fluorescens CL1542	61.7 ± 16.3	62.0 ± 24.9
Pseudomonas maltophilia CL2016	118.7 ± 14.4	172.8 ± 23.9
Salmonella enteritidis CL1362	88.8 ± 25.7	87.3 ± 15.7
Salmonella typhi CL1935	50.7 ± 11.6	48.2 ± 6.3
Salmonella typhimurium CL1866	100.5 ± 15.6	111.3 ± 20.5
Serratia liquefaciens CL1977	70.3 ± 13.4	80.7 ± 15.1
Serratia marcescens CL1520	116.5 ± 10.9	121.7 ± 24.2
Shigella boydii CL1727	24.3 ± 7.6	38.3 ± 4.1
Shigella dysenteriae CL1726	44.2 ± 15.6	81.2 ± 15.8
Shigella flexneri CL1719	25.8 ± 8.4	47.2 ± 14.6
Shigella sonnei CL1705	160.7 ± 55.6	212.2 ± 86.8
Staphylococcus aureus CL1500	90.3 ± 7.0	114.3 ± 9.8
Staphylococcus epidermidis CL1803	120.2 ± 78.0	132.8 ± 78.3
Staphylococcus saprophyticus CL1941	28.7 ± 5.8	32.2 ± 16.3
Yersinia enterocolitica CL1626	38.3 ± 8.3	59.2 ± 2.6

[a] Mean of six values. The counts shown were derived from the 10^{-6} dilution for all cultures except *S. sonnei* and *K. ozaenae* (10^{-5}) and *P. aeruginosa*, *P. fluorescens*, and *P. maltophilia* (10^{-7}).

noted between the two gelling agents with *C. perfringens* (1 mm), *B. fragilis* (1 mm), *E. cloacae* (1 mm), *Hafnia alvei* (1.5 mm), and *A. hinshawii* (1.5 mm).

The larger colonies were almost invariably seen on agar-containing media, with the exception of *Moraxella* species, *P. mirabilis*, *S. dysenteriae*, and *S. pneumoniae*, which showed slightly larger colonies on GELRITE-containing media. The *C. fetus* subsp. *jejuni* strain tested showed a difference of 4.2 mm in the average size of the colonies between the two products. It was subsequently noted that *C. fetus* subsp. *jejuni* cultures spread on blood agar plates purchased from GIBCO and Scott Laboratories, Inc., Fiskerville, R.I., but not on those purchased from BBL. In fact, the average size of colonies on blood agar plates prepared by BBL was nearly equal to that recorded for blood GELRITE plates provided by GIBCO. The reason for this difference is not known at this time.

Two cultures (*S. faecalis* and *S. pneumoniae*) produced alpha-hemolytic patterns, seven cultures (*Vibrio cholerae*, *C. perfringens*, *P. aeruginosa*, *S. pyogenes*, *S. agalactiae*, group C *Streptococcus* species, and group G *Streptococcus* species) produced beta-hemolytic patterns, and the rest of the test cultures produced gamma-hemolytic patterns on both agar-containing and GELRITE-containing blood plates. The size of beta-hemolytic zones ranged from 0.7 mm (*S. agalactiae*) to 9 mm (*C. perfringens*) on both agar and GELRITE plates. The only significant difference in zone sizes of beta-hemolysis observed between the two gelling agents was seen with *S. pyogenes* (1.8 mm versus 1.0 mm), the larger zone diameter being recorded for blood agar.

Further evaluation of GELRITE by comparison with agar was carried out with a selected panel of media commonly used in the isolation and

TABLE 4. Viable cell counts of fastidious strains for agar versus GELRITE

Microorganism	Mean[a] ± SD for:	
	Agar	GELRITE
Bacteroides fragilis CLA177	72.0 ± 8.6	69.4 ± 22.1
Campylobacter fetus subsp. *jejuni* CL2312	49.1 ± 41.0	46.3 ± 34.6
Clostridium perfringens CLA144	69.4 ± 21.6	67.3 ± 17.0
Haemophilus influenzae CL1826	58.0 ± 16.2	53.3 ± 8.9
Haemophilus parainfluenzae CL1824	42.4 ± 6.6	37.9 ± 14.3
Moraxella species CL387	198.5 ± 54.8	181.7 ± 46.2
Peptostreptococcus anaerobius CLA107	83.7 ± 29.6	84.8 ± 34.1
Streptococcus agalactiae CL1342	142.1 ± 37.4	158.1 ± 27.1
Streptococcus faecalis CL1776	194.0 ± 58.7	185.9 ± 48.5
Group C *Streptococcus* species CL909	226.7 ± 48.2	202.6 ± 44.7
Group G *Streptococcus* species CL1929	96.7 ± 71.2	113.7 ± 70.9
Streptococcus pneumoniae CL1842	74.7 ± 34.6	87.7 ± 36.7
Streptococcus pyogenes CL1925	56.2 ± 43.2	49.8 ± 23.1
Vibrio cholerae CL2020	80.0 ± 67.9	79.7 ± 49.6

[a] Mean of nine values except for *S. pneumoniae*, group G *Streptococcus* species, *Moraxella* species, and *V. cholerae* (mean of six values). The counts shown were derived from the 10^{-6} dilution for all cultures except *S. pneumoniae*, group C *Streptococcus* species, group G *Streptococcus* species, and *C. fetus* subsp. *jejuni* (10^{-5}), *V. cholerae* (10^{-4}), and *B. fragilis* (10^{-7}).

biochemical characterization of clinical isolates, namely, MacConkey, triple sugar iron, brilliant green, bismuth sulfite, eosin methylene blue, salmonella-shigella, egg yolk, and phenylethyl alcohol. Virtually all of the biochemical reactions and colony characteristics on all of the test media listed above were the same for agar and GELRITE (Fig. 2), with the only significant exception being lack of swarming by *P. vulgaris* and *P. mirabilis* on MacConkey GELRITE medium. In an earlier experiment in which 0.2% NaCl was used to promote solidification of a GELRITE-nutrient broth mixture, the degree of swarming of *P. mirabilis* and *P. vulgaris* on GELRITE was equivalent to that seen on nutrient agar with 0.2% NaCl added. The substitution of NaCl with 0.05% $MgCl_2$ caused gelling of GELRITE while minimizing swarming of these *Proteus* species. As it is known that electrolyte-deficient media normally do not support swarming of *Proteus* species (3, 5), it is possible that substitution of the salts sufficiently lowered the total electrolyte content of the medium. However, the addition of GELRITE and $MgCl_2$ to MacConkey medium containing 0.5% NaCl prevented swarming of both *Proteus* species, which normally spread on MacConkey agar. The addition of $MgCl_2$ was probably the primary reason for the observed lack of swarming, and it may have acted in concert with the bile salts component of this medium to prevent the swarming phenomenon.

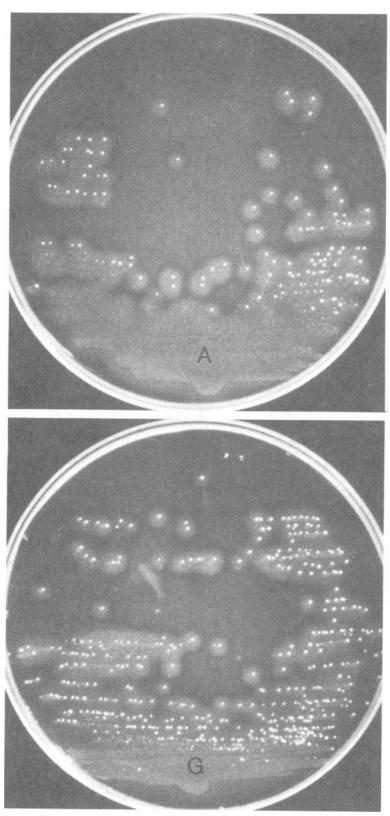

FIG. 1. *S. aureus* colonies on tryptic soy agar (A) and tryptic soy GELRITE (G) with 5% sheep blood. Plates were incubated at 37°C for 24 h.

As was the case with blood plates, bacterial colonies were somewhat smaller on the differential and selective media when GELRITE was used as the solidifying agent. From a technical standpoint, some difficulty was experienced in streaking the relatively softer surface of blood GELRITE media, and we were not properly equipped to rule out a possible difference in gel strengths between agar-containing and GEL-

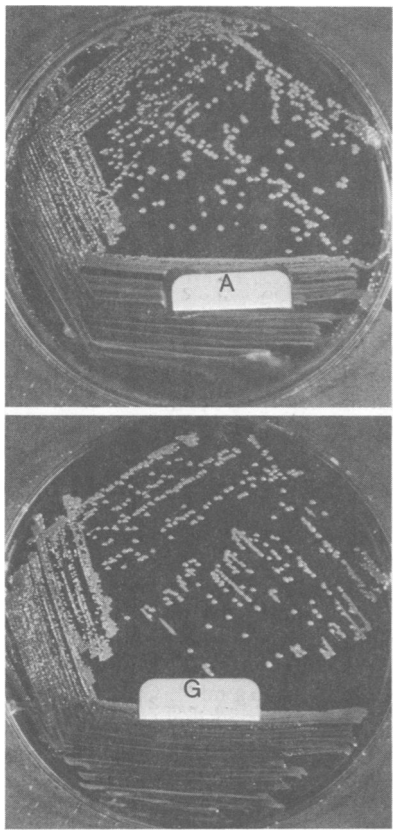

FIG. 2. *S. enteritidis* cultures (24 h) growing in brilliant green medium with agar (A) and GELRITE (G) as the gelling agents.

RITE-containing blood media. The higher gelling point and quicker setting properties of GELRITE as compared with those of agar should be advantageous in that more plates can be poured within a limited work space and stored away without the worry of disturbing the gel. In general, media gelled by agar and GELRITE appeared opaque and clear, respectively (Fig. 2). In this respect, we observed that in most instances, it was easier to evaluate colony characterisitcs and biochemical reactions on GELRITE-containing media.

In summary, it can be stated that, based on the results presented here, GELRITE can be substituted for agar in many routine medium applications and, in some cases, GELRITE-solidified media may actually give higher viable cell recoveries than similar media solidified with agar. It is hoped that a few apparently manageable technical problems encountered during the course of this study, namely, hemolysis of blood at the elevated GELRITE setting temperature and slight difficulties in the streaking of certain media (blood and chocolate), will eventually be resolved.

It should be noted that this study dealt with possible applications of GELRITE primarily in the field of bacteriology, especially in clinical laboratory settings. Information on plating efficiencies of this gelling agent for other microorganisms (e.g., algae and fungi) is very limited (1) or still lacking.

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