New Semisolid Agar for the Detection of Motile Salmonellae

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A semisolid selective motility agar based on selenite broth and designated semisolid selenite fecal (SSF) agar was developed for recovery of motile salmonellae from large numbers of rodent fecal specimens. The medium is easily prepared and inoculated; results are readily interpreted. When combined with selenite or gram-negative broth enrichment, SSF agar yielded significantly better *Salmonella* isolation from experimentally infected rodents than a standard battery of media. Only 14 non-*Salmonella* isolates from 1,002 *Salmonella*-free rodent fecal specimens migrated on SSF agar.

The ideal selective primary isolation medium for enteric pathogens supports rapid and distinct growth of pathogenic species while suppressing the growth of nonpathogenic flora. Despite developments in isolation procedures spanning some 80 years, isolation of salmonellae from feces remains labor intensive and requires numerous media.

Since over 90% of all *Salmonella* spp. are motile (3), several isolation procedures have been based on motility. U-tubes have been used to physically separate *Salmonella* spp. from *Pseudomonas* spp. (6). These have been modified with side arms (9) and filled with enrichment broth (7) to facilitate the recovery of salmonellae.

A semisolid enrichment medium with indicator was described to isolate motile salmonellae from feces (2). A reduced pH of 6.0 minimized migration of most motile enteric bacteria except *Salmonella* species, *Enterobacter cloacae*, and *Citrobacter freundii*. Growth of the latter two was inhibited by addition of novobiocin and incubation at 42°C. Use of these media yielded an increased isolation rate over a standard isolation procedure (2).

Goossens et al. (4) reported the use of a modified Rappaport enrichment medium (magnesium chloride-malachite green broth) with 0.32% (wt/vol) agar as a semisolid medium (RAPPS) for primary isolation of motile salmonellae from human feces. Use of RAPPS over a 7-year period increased the isolation rate of salmonellae from human specimens by 22.5%. This medium was therefore evaluated by using pure cultures of motile rodent salmonellae and other enteric bacteria. Although RAPPS generally performed as described (4), it was found to be unsuitable for the following reasons: the fragile agar surface was easily broken, inverted plates often collapsed, large migration zones prohibited multiple-site inoculation, and condensates formed during incubation and refrigeration constituted a potential laboratory health hazard.

Due to these shortcomings, a new medium designated semisolid selenite fecal (SSF) agar was formulated and evaluated. The use of this medium increased recovery rates of rodent salmonellae compared with standard isolation procedures with MacConkey and brilliant green agars.

MATERIALS AND METHODS

Bacterial strains. Species commonly found in rodent feces and included in the development and testing of SSF were members of the genera *Citrobacter*, *Escherichia*, *Enterobacter*, *Proteus*, *Pseudomonas*, and *Salmonella*. Identification was based on standard biochemical test media, serological results, and API 20E test strips (Analytab Products, Inc. Plainview, N.Y.).

Formulation of SSF agar. Selenite broth (Difco Laboratories, Detroit, Mich.) with various concentrations of agar was tested to determine migration of enteric species. In preliminary experiments at pH 7.0, selenite broth was supplemented with 0.32% (wt/vol) agar (Difco), and the effects of increased agar concentrations (0.37, 0.42, 0.44, 0.46, 0.48, 0.50, and 0.60% [wt/vol] agar) on migration zone diameters were examined. In subsequent experiments, the effects of reducing the pH from 7.0 to 6.0, 6.2, and 6.4 while also varying the agar concentration (0.37, 0.42, and 0.47%) were evaluated; agars from several commercial sources were tested. The combination of pH, agar concentration, and agar source which consistently supported migration of the majority of salmonellae within 24 h while minimizing migration of other enteric strains was used in the following formulation: selenite broth (Difco), 23.0 g; agar (Oxoid Purified, code L28, lot 336 10819; Oxoid Ltd., Basingstokes, England), 3.7 g; distilled water, 1 liter; pH 6.2. The solution is heated with constant stirring just until boiling begins. Overheating imparts a red hue to selenite broth and adversely affects performance. The pH of the hot medium is adjusted to 6.2 with 4 M HCl. It is allowed to cool and poured into standard sterile petri dishes. Uninoculated SSF agar is clear with a slight straw yellow color. Areas of bacterial growth are characterized by a brick red precipitate formed by the reduction of the selenite salts.

Performance of SSF agar with rodent fecal specimens. Rodent fecal samples shipped to the Research Animal Diagnostic and Investigative Laboratory in gram-negative (GN) broth (BBL Microbiology Systems, Cockeysville, Md.) were determined to be free of salmonellae by inoculation onto MacConkey (Difco), brilliant green (BBL), and Hektoen (Difco) agars and enrichment in selenite broth. Each sample was inoculated onto SSF agar and incubated overnight at 35°C; bacterial growth from the edge of migration zones was isolated and identified by standard methods.

Experimentally infected rodents. Female C57BL/6 mice,

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BALB/c mice, and Wistar/Furth rats (Charles River Breeding Laboratories, Kingston, N.Y.), 10 weeks of age and free of murine pathogens, were individually identified and housed in nonsterile, sanitary, polycarbonate cages with filter bonnets. Food and water were provided ad libitum.

The following *Salmonella* strains were grown on 5% sheep blood agar (BBL) overnight at 35°C in 7% carbon dioxide: *S. cerro* 2112-83, *S. infantis* 1915-80, *S. oranienburg* 2322-80, and *S. typhimurium* ATCC 15277. These strains were chosen based on the range of migration zone diameters on SSF agar. Except for ATCC 15277, all isolates were murine pathogens recovered between 1980 and 1983, lyophilized in skim milk, and stored at room temperature. Suspensions of each strain were prepared in a buffered gavage solution (1) and enumerated by spreading 0.1 ml of appropriate dilutions onto MacConkey agar. Counts ranged from 2.5×10^8 to 2.2×10^9 per ml (mean, 9.4×10^8 per ml).

Rats received 0.2 ml of gavage suspension by a feeding needle (Popper and Sons, Inc., New Hyde Park, N.Y.). Mice received a 0.1-ml inoculum. Control rodents were administered sterile gavage solution.

At 3, 5, and 7 days after inoculation, one fecal pellet from each rat and two from each mouse were collected. Each fecal specimen was homogenized and divided in half; one half was added to 0.5 ml of GN broth and incubated overnight at 35°C, and the other half was emulsified in 0.2 ml of sterile saline and used to inoculate SSF agar (primary SSF culture), MacConkey agar and brilliant green agar (standard primary culture), and selenite broth. Overnight growth from GN broth was inoculated onto SSF agar (GN-enriched SSF) and MacConkey agar and brilliant green agar (standard GN enrichment). Overnight growth from selenite broth was inoculated onto Hektoen agar (standard selenite enrichment) and SSF agar (GN-enriched SSF).

SSF plates were examined for organism migration after 24 h of incubation. Growth from the edge of migration zones was inoculated onto MacConkey agar to determine purity. SSF plates without evidence of migration were incubated for an additional 18 h before being discarded. Isolates were identified by standard methods.

On day 7, all mice and rats were sacrificed with carbon dioxide after collection of fecal pellets. Cecal contents were plated directly onto SSF, MacConkey, brilliant green, and Hektoen agars.

Statistical analysis. Chi-square values were calculated from two-row by two-row column tables (8).

RESULTS

Formulation of SSF agar. Selenite broth prepared as described by the manufacturer has a pH of 7.0 ± 0.2 . When supplemented with 0.32% agar, all strains of *Enterobacter agglomerans*, *Citrobacter freundii*, and *Escherichia coli* tested migrated as well as the *Salmonella* strains did. At this pH, most gram-negative organisms also migrated at agar concentrations up to 0.60%. In preliminary experiments to inhibit migration of non-*Salmonella* strains, various agar concentrations and pHs were tested in selenite broth (see Materials and Methods). Based on the results of selective motility of salmonellae and inhibition of other enteric bacteria, subsequent experiments were performed with agar concentrations of 0.37, 0.42, and 0.47% at pHs of 6.0, 6.2, and 6.4.

In the course of these tests, it was noted that a change in agar lot number markedly affected the performance of SSF agar. Therefore, two lots each of Difco Bacterial Technical,

Agar Products (Van Nuys, Calif.), and Oxoid Purified agars were tested as described above. Migration zone diameters with the Oxoid Purified agars (lots 336 10819 and 213 13193) were the most consistent (data not shown). However, many of the *Salmonella* strains did not migrate. A trial with the above Oxoid agar lots at concentrations of 0.37, 0.42, and 0.47% and pHs of 5.8, 6.0, and 6.2 was performed. An agar concentration of 0.37% at pH 6.2 was found to be optimal.

The migration characteristics of 43 strains of enteric bacteria are shown in Table 1. Thirty of 31 *Salmonella* strains migrated on SSF agar after overnight incubation. *S. newport* ATCC 6962 migrated after 36 h of incubation. Only 1 of 12 other bacterial strains migrated.

Performance of SSF agar with rodent fecal specimens. Migration on SSF agar was observed in only 14 of 1,002 (1.4%) *Salmonella*-free rodent fecal specimens. Migrating

TABLE 1. Bacterial migration zone diameters on SSF agar after 24 h

Strain	Source	Migration zone diam (mm)
<i>Salmonella agona</i> 2112-83	Mouse	15
<i>Salmonella amsterdam</i> 2141-82	Rat	3
<i>Salmonella amsterdam</i> 2217-84	Rat	13
<i>Salmonella amsterdam</i> 2356-84	Mouse	11
<i>Salmonella anatum</i> 1395-78	Mouse	13
<i>Salmonella anatum</i> 2556-79	Mouse	7
<i>Salmonella anatum</i> 2160-82	Mouse	4
<i>Salmonella cerro</i> 2112-83	Mouse	9
<i>Salmonella cerro</i> 2415-83	Mouse	7
<i>Salmonella enteritidis</i> 1279-78	Mouse	19
<i>Salmonella enteritidis</i> 1283-78	Mouse	24
<i>Salmonella havana</i> 2189-80	Mouse	8
<i>Salmonella infantis</i> 1915-80	Mouse	8
<i>Salmonella infantis</i> 2182-82	Mouse	13
<i>Salmonella</i> sp. (monophasic)	Unknown	14
<i>Salmonella montevideo</i> 1602-80	Mouse	18
<i>Salmonella newport</i> ATCC 6962	Human	6 ^a
<i>Salmonella oranienburg</i> 2322-80	Mouse	16
<i>Salmonella rubislaw</i> 10717	Unknown	8
<i>Salmonella typhimurium</i> 1531-76	Guinea pig	15
<i>Salmonella typhimurium</i> 2145-83	Mouse	18
<i>Salmonella typhimurium</i> 2437-84	Guinea pig	8
<i>Salmonella typhimurium</i> 3212-81	Mouse	16
<i>Salmonella typhimurium</i> ATCC 15277	Unknown	5
<i>Salmonella typhimurium</i> MC1750	Human	7
<i>Salmonella typhimurium</i> MC2009	Human	17
<i>Salmonella typhimurium</i> MC2241	Human	24
<i>Salmonella typhimurium</i> MC2364	Human	18
<i>Salmonella typhimurium</i> MC2490	Human	5
<i>Salmonella typhimurium</i> MC2717	Human	16
<i>Salmonella worthington</i> ATCC 9607	Unknown	4
<i>Citrobacter freundii</i> 2713-83	Mouse	NM ^b
<i>Citrobacter freundii</i> KPHD	Mouse	NM
<i>Enterobacter cloacae</i> 3085-85	Mouse	NM
<i>Enterobacter cloacae</i> 2714-85	Mouse	NM
<i>Enterobacter cloacae</i> 2883-85	Unknown	5
<i>Escherichia coli</i> 3657-86	Mouse	NM
<i>Escherichia coli</i> ATCC 25922	Human	NM
<i>Proteus mirabilis</i> 1517-85	Goat	NM
<i>Proteus mirabilis</i> 2487-85	Rat	NM
<i>Proteus mirabilis</i> 2511-85	Rat	NM
<i>Proteus mirabilis</i> 3059-85	Unknown	NM
<i>Pseudomonas aeruginosa</i> ATCC 27853	Human	NM

^a Zone diameter after 36 h of incubation.

^b NM, No migration.

TABLE 2. Recovery of four *Salmonella* strains from experimentally infected BALB/c and C57BL/6 mice

Culture method ^a /sample	No. positive for strain/no. tested				Total positive/total tested (% positive)	<i>P</i> ^b
	<i>S. cerro</i> 2112-83	<i>S. infantis</i> 1915-80	<i>S. oranienburg</i> 2322-80	<i>S. typhimurium</i> ATCC 15277		
Primary MAC + BG/fecal	10/33	8/33	6/33	3/27	27/126 (21)	>0.10
Primary SSF/fecal	8/33	4/33	2/33	4/27	18/126 (14)	
Selenite broth-enriched MAC + BG/fecal	20/22	10/22	12/22	11/18	53/84 (63)	<0.005
Selenite broth-enriched SSF/fecal	22/22	18/22	18/22	16/18	74/84 (88)	
GN broth-enriched MAC + BG/fecal	20/33	13/33	14/33	7/27	54/126 (43)	<0.005
GN broth-enriched SSF/fecal	31/33	23/33	22/33	23/27	99/126 (79)	
Primary MAC + BG/cecal	1/11	0/11	0/11	6/9	7/42 (17)	>0.30
Primary SSF/cecal	0/11	0/11	0/11	6/9	6/42 (14)	

^a See Materials and Methods for description of culture techniques. Abbreviations: MAC, MacConkey agar; BG, brilliant green agar.

^b *P* versus corresponding non-SSF culture method.

enteric bacteria included seven strains of *E. cloacae*, three strains of *P. mirabilis*, and one strain each of *E. aerogenes*, *E. coli*, *Kluyvera* species, and *Serratia liquefaciens*. All strains were recovered in pure culture on MacConkey agar inoculated with growth from the edge of migration zones.

Experimentally infected rodents. Feces collected from rodents before gavage with *Salmonella* strains contained the following enteric bacteria: *C. freundii*, *E. aerogenes*, *E. cloacae*, *E. coli*, and *P. mirabilis*. Salmonellae were not isolated.

The results of the recovery of four *Salmonella* strains from experimentally infected BALB/c and C57BL/6 mice are shown in Table 2. The highest isolation rate (88%) was achieved on SSF agar inoculated with selenite broth-enriched specimens, compared with a 63% isolation rate achieved by inoculating MacConkey and brilliant green agars with selenite broth-enriched specimens (*P* < 0.005). The performance of SSF agar compared with that of MacConkey and brilliant green agars after GN enrichment of fecal specimens was also significantly better (*P* < 0.005). Subcultures made from the edge of migratory growth on SSF agar yielded pure cultures of *Salmonella* spp. in all cases.

The results for *Salmonella* recovery from Wistar/Furth rats are shown in Table 3. Recovery rates from rats were lower than those from mice; no culture technique yielded better results than the 50% fecal isolation rate achieved with selenite broth-enriched SSF agar. This rate was not significantly better than that achieved by using a standard battery of media after selenite broth enrichment. Only pure cultures of *Salmonella* spp. were recovered from the edge of migration zones.

DISCUSSION

SSF agar is a simple modification of a proven enrichment broth. It is easily prepared and requires no unusual or expensive ingredients or glassware. Because the agar concentration in SSF agar is relatively high, standard-sized petri dishes can be inverted during incubation and four sites may be inoculated on a single plate without confluent growth. Condensate, which can lead to cross-contamination of multiply-inoculated plates, excessive migration of bacteria, and potential exposure of laboratory personnel to pathogenic enteric bacteria, does not form on SSF agar during either incubation or refrigerated storage.

Migration on SSF agar is readily apparent, usually within 24 h of incubation. Moreover, all four strains of *Salmonella* inoculated into rodents and recovered from feces migrated as pure cultures. Chau and Garnier (2) reported that reducing the pH retarded the migration of most enteric strains, including *P. mirabilis* and *E. coli*. Harper (5) observed that the spread of organisms through a semisolid medium varied with the concentration of agar. The combination of reduced pH and increased agar concentration in SSF agar generally inhibited migration of non-*Salmonella* strains. The critical nature of the source and type of the agar in SSF agar necessitates careful quality assurance testing with bacterial strains with known migration characteristics. Therefore, the migration zone diameters of a number of readily available strains are reported in Table 1. Under the conditions described, motile salmonellae migrate on SSF agar within 24 to 36 h of incubation, while strains of other enteric genera generally do not migrate.

TABLE 3. Recovery of four *Salmonella* strains from experimentally infected Wistar/Furth rats^a

Culture method/sample	No. positive for strain/no. tested				Total positive/total tested (% positive)	<i>P</i>
	<i>S. cerro</i> 2112-83	<i>S. infantis</i> 1915-80	<i>S. oranienburg</i> 2322-80	<i>S. typhimurium</i> ATCC 15277		
Primary MAC + BG/fecal	0/15	0/15	0/15	0/15	0/60 (0)	>0.30
Primary SSF/fecal	1/15	0/15	0/15	0/15	1/60 (2)	
Selenite broth-enriched MAC + BG/fecal	9/10	3/10	1/10	2/10	15/40 (38)	>0.30
Selenite broth-enriched SSF/fecal	9/10	7/10	2/10	2/10	20/40 (50)	
GN broth-enriched MAC + BG/fecal	2/15	5/15	1/15	0/15	8/60 (13)	<0.05
GN broth-enriched SSF/fecal	7/15	8/15	1/15	1/15	17/60 (28)	
Primary MAC + BG/cecal	0/5	0/5	0/5	0/5	0/20 (0)	Undefined
Primary SSF/cecal	0/5	0/5	0/5	0/5	0/20 (0)	

^a See Table 2, footnotes *a* and *b*.

Selenite and GN enrichment of mouse feces markedly increased isolation rates of *Salmonella* spp. on SSF agar and afforded a significant improvement over enriched standard plating media. *Salmonella* isolation rates from rat feces were also significantly improved by the use of enrichment broths but did not achieve the levels for recovery from mice.

The ease of preparation and use, high isolation rates of salmonellae from experimentally infected rodents, and low incidence of migration of non-*Salmonella* strains on SSF agar suggest that this medium would be useful in laboratories screening large numbers of rodent fecal samples for salmonellae. Preliminary results with motile human *Salmonella* strains indicate that SSF agar could also be used for screening human fecal specimens. There is also a potential application for using SSF agar to quickly and easily screen large numbers of selenite broth-enriched food homogenates, milk, and water specimens for motile salmonellae.

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