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Novobiocin-Brilliant Green-Glucose Agar: New Medium for Isolation of Salmonellae

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A new medium, called novobiocin-brilliant green-glucose (NBG) agar, was developed for the isolation of *Salmonella* spp. and evaluated against other conventionally used media including bismuth sulfite, xylose-lysine decarboxylase, brilliant green-sulfa, hektoen enteric, and salmonella-shigella agars. NBG had recovery rates comparable to the other enteric media tested with pure cultures as well as with naturally contaminated amphibian and reptile waters and fecal specimens. However, NBG, hektoen enteric, and salmonella-shigella agars failed to differentiate *Salmonella typhi* from a fecal specimen even after enrichment in selenite F. Although *Citrobacter freundii* could grow and resembled salmonellae on NBG, at no time was the recovery of *Salmonella* spp. colonies jeopardized by the presence of *C. freundii* in either seeded or naturally contaminated samples. Confirmation rates of typical colonies from NBG agar also compared favorably to the other media tested; however, bismuth sulfite, although selective, was found to have varied differential characteristics for *Salmonella* spp. As a result, many more colonies had to be picked, which caused bismuth sulfite agar to have the lowest confirmation rate of the media tested. The distinct advantage that NBG agar offers over the conventional method tested, including bismuth sulfite, is the consistent differential reaction of all *Salmonella* subgroups including biochemically atypical strains. The medium is inexpensive, easy to prepare, and can be stored for at least 2 weeks at 4°C without loss of selective or differential properties.

The genus *Salmonella* has recently been divided into five subgroups based on biochemical characteristics and origins of isolation (7, 8). Members of *Salmonella* subgroup 1 are isolated mostly from human clinical specimens. Only 1% of these isolates are reported to be lactose fermenters. This low percentage of lactose-positive *salmonellae* isolated from clinical sources is in contrast to the 15.6% of *Salmonella* spp. isolations found fermenting this sugar from dried milk (2), a common constituent of many foodstuffs. In contrast to subgroup 1, where lactose fermentation is seldom reported, 60% of *Salmonella* subgroup 3 strains (*Arizona* spp.) ferment this carbohydrate (7, 8, 16). Strains belonging to subgroup 3 can be found from a variety of animal sources (5–8, 10), some of which are common foods for human consumption. Thus, it would be expected that isolates in this subgroup would account for a larger number of isolations from human disease. In fact, *Salmonella* subgroup 3 strains are reported infrequently from human clinical samples (7, 8). The isolation of both *Salmonella* subgroups 1 and 3 lactose fermenters from human clinical specimens is very important, since they have been the cause of severe infections and outbreaks of salmonellosis (5, 6, 9, 10, 13, 20, 21).

These inconsistencies in reported isolation of lactose-fermenting *Salmonella* spp. between human cases and environmental sources may be due to the isolation media used. Bacteriological media used routinely for isolation of salmonellae from clinical specimens such as xylose-lysine decarboxylase (XLD), salmonella-shigella (SS), brilliant green-sulfa (BGS), and hektoen enteric (HE) agars contain lactose. Fermentation of this carbohydrate by lactose-positive strains of salmonellae produces colonies which are indistinguishable from background coliforms and can thus be easily overlooked. This information has been known for some time (2, 5, 6, 8, 10, 20, 21), and Edwards et al. (5, 6) have questioned the wisdom of using media containing lactose for isolating salmonellae, but still the practice continues.

In addition, other media designed to isolate salmonellae have not taken into account the other carbohydrate-utilizing differences that occur between the *Salmonella* subgroups. For example, Hawa et al. (11) developed a differential selective medium containing dulcitol for the quantitative isolation of salmonellae from chicken carcasses. Although greater than 90% of salmonellae belonging to subgroups 1, 2, and 5 are able to utilize this carbohydrate, strains of subgroups 3 and 4 cannot and will be overlooked (7, 8).

Bismuth sulfite (BS) agar has been the only recommended medium to date which allows for detection of biochemically atypical salmonellae, especially the lactose-fermenting strains (1, 2, 13, 16, 20). However, the selectivity and differential properties of this medium can change dramatically as a result of preparation and storage conditions (3, 4). These properties make BS agar unpopular for routine diagnostic laboratory use.

The purpose of this paper was to develop and evaluate a new medium, called novobiocin-brilliant green-glucose (NBG) agar, for the isolation of a broader spectrum of salmonellae from pure cultures and naturally contaminated sources.

MATERIALS AND METHODS

Cultures. All cultures used in this study were obtained from the stock culture collection of the Enteric Bacteriology Reference Section, Central Public Health Laboratory, Ontario Ministry of Health, Toronto. They were maintained on tryptic soy-yeast extract agar slants until required for testing.

Medium preparation. For NBG agar, 40 g of tryptic soy agar, 1.5 g of ferric ammonium citrate, 5.0 g of sodium thiosulfate pentahydrate, and 80 mg of phenol red (sodium salt) were added to 1,000 ml of high-purity double-distilled

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water. This basal medium was boiled to dissolve the ingredients and autoclaved for 15 min at 121°C. After cooling to 50°C in a waterbath, the following stock solutions were added: 4.0 ml of glucose (0.25 g/ml), 1.0 ml of novobioin (10 mg/ml), and 1.0 ml of brilliant green (7 mg/ml). All stock solutions were prepared in high-purity double-distilled water and filter sterilized with a sterilization filter unit (Nalgene, Rochester, N.Y.) with a 0.2-μm membrane filter. The novobiocin and brilliant green solutions were stored at −20°C and 4°C, respectively, in 2.0-ml quantities in sterile corked tubes. Both solutions were brought to room temperature on the day of medium preparation, and any unused portions were discarded. The glucose stock solution was made in 100-ml quantities in a sterile 125-ml serum bottle. Once the necessary volume of glucose was added to the basal medium, this stock solution was held at 4°C for storage. The final NBG agar preparation was mixed gently and allowed to sit for 5 min in a 50°C water bath. The agar medium was then poured into sterile plastic petri plates and allowed to harden. The final pH was 7.3 ± 0.1. Other media used in this study were commercial products made by Difco Laboratories, Detroit, Mich. (BGS and HE agars), or Oxoid Ltd., London, England (XLD and BS [modified]), and prepared according to the manufacturers' instructions. BS was stored at 4°C for 2 to 4 days before use. SS agar was prepared in the laboratory using the formulation described in the Difco manual, except that sodium citrate was excluded because it inhibited the growth of *Shigella flexneri* and H_2S production by salmonellae (unpublished data).

Quantitative recovery. A total of 19 strains of *Salmonella* were tested for quantitative recovery (Table 1), including five *Arizona* spp., five lactose-positive strains, and nine biochemically typical serotypes of *Salmonella* spp. All organisms were streaked onto fresh blood agar plates from stock tryptic soy-yeast extract agar slants and incubated at 35°C for 20 h. Growth from the overnight blood agar plate for each organism was diluted to approximately 10^3 cells per ml in phosphate-buffered saline, and 100 μl was spread plated in triplicate onto surface-dried (inverted at 35°C for 20 min) NBG, BGS, and BS agar plates with a sterile glass hockey stick. Blood agar plates were also spread plated in triplicate and served as the nonselective control medium for comparative purposes. All agar plates were incubated for 24 h at 35°C, and colony counts and morphology were recorded.

Selectivity. A total of 22 non-*Salmonella* stock culture strains (Table 2) were inoculated from tryptic soy-yeast extract slants into 5.0 ml of brain heart infusion broth. The brain heart infusion broth cultures were incubated at 35°C for 20 h and streaked directly onto freshly prepared NBG agar plates with a 3-mm sterile inoculating loop. The NBG agar plates were incubated at 35°C for 24 h and observed for growth.

To further test the selectivity of NBG agar a lactose-positive *Salmonella* strain was seeded at 10^3 cells per g into the following salmonella-free foods: chicken salad, egg salad, bean salad, potato salad, cacciatore, ricotta cheese, raw Italian sausage, salami, spiced ham, and Black Forest ham. The foods were preenriched in 1% buffered peptone (35°C for 24 h) and selectively enriched in selenite-cystine (35°C for 24 h) and tetrathionate-brilliant green (43°C for 48 h) broths before streaking to NBG and BS agars. After 35°C incubation for 24 h, five typical salmonella-looking colonies from each medium were picked and biochemically confirmed as the seeded lactose-positive *Salmonella* spp. by using Kligler iron agar (acid slant-acid butt with gas, but no hydrogen sulfide production), lysine-iron agar (alkaline slant-alkaline butt with hydrogen sulfide production) and positive agglutination with *Salmonella* poly-O antisera.

Refrigerated storage of NBG agar. NBG agar was prepared as previously described, placed into plastic bags, and stored at 4°C. After 1, 3, 7 and 15 days' storage, a sufficient number of NBG agar plates were removed and surface dried, and quantitative recovery was done, as described above, with the following organisms: one *Arizona* sp., two lactose-positive *Salmonella* spp., and a biochemically typical *Salmonella* serotype. At the same storage times above, two *Escherichia coli* and two *Proteus mirabilis* strains were streaked directly onto NBG agar from overnight brain heart infusion broths as described above.

Isolation from amphibian and reptile waters. A total of 67 amphibian and reptile water samples were obtained through the Metroplitan Toronto Public Health Departments from pet stores in the area, as part of a survey for the presence of salmonellae in these waters. All water samples were filtered through sterile glass fiber filters (GF/F Whatman Ltd., Maidstone). The GF/F filters were preenriched in 50 ml of 1% buffered peptone. After overnight incubation at 35°C, 1.0 ml of the preenrichment broth was selectively enriched in 9.0 ml of tetrathionate-brilliant green broth for 48 h at 43°C. The tetrathionate-brilliant green broth cultures were then streaked onto NBG, BS, BGS and HE agars for well-isolated colonies, and the plates were incubated at 35°C for 24 h. Up to four typical-looking salmonella colonies from each medium were picked, placed onto Kligler iron agar and lysine-iron agar slants, and incubated at 35°C for 24 h. Isolates which biochemically resembled salmonellae were further screened with a slide agglutination test with *Salmonella* poly-O antisera. Confirmation of the isolates as being *Salmonella* spp. was performed by the Enteric Reference Section of the Ontario Ministry of Health Laboratories, Toronto.

Isolation from feces. A total of 20 fecal specimens were obtained from the Enteric Section of the Central Laboratory at the Ontario Ministry of Health. Each specimen was streaked directly onto SS, XLD, HE, BGS, BS, and NBG agars. In addition, 1 g of each fecal specimen was enriched in

TABLE 1. Quantitative recovery of 19 *Salmonella* strains on NBG, BGS, and BS agars[a]

Organism	Strain	% Recovery compared with blood agar		
		NBG	BGS	BS
Arizona sp.	1	88	105	116
	2	100	98	0
	3	87	98	99
	4	93	95	98
	5	96	91	110
Salmonella sp. (lactose positive)	1	108	36	91
	2	105	77	92
	3	100	59	98
	4	79	60	104
	5	81	104	125
S. brandenberg		101	100	94
S. enteritis		94	73	109
S. infantis		100	46	92
S. muenster		93	63	95
S. newport		106	90	99
S. thompson		88	112	87
S. ohio		95	89	105
S. typhimurium		73	110	106
S. typhi		32	63	2

[a] Overall recovery was 90, 83, and 91% for NBG, BGS, and BS, respectively.

TABLE 2. Selectivity of NBG agar for other gram-negative bacteria

Organism	No. of strains tested	No. of strains recovered
Citrobacter freundii	5	4
Aeromonas hydrophila	1	1
Edwardsiella tarda	1	1
Enterobacter aerogenes	1	1
Enterobacter cloacae	1	1
Escherichia coli	2	0
Hafniae alvei	1	0
Klebsiella pneumoniae	2	2
Proteus mirabilis	2	0
Proteus vulgaris	2	2 (pinpoint)
Providencia sp.	1	0
Pseudomonas aeruginosa	2	2
Pseudomonas putrefaciens	1	0

9 ml of selenite F broth (Difco) overnight at 35°C before streaking onto the above medium. Up to four typical-looking salmonella colonies were picked from each agar plate, and identification for salmonellae was performed as described above for the amphibian and reptile waters. Background bacterial growth was also recorded to compare the selectivities of the media.

RESULTS

The quantitative recovery of 5 *Arizona* spp. and 14 *Salmonella* spp. on NBG, BS an BGS agars with blood agar for comparison is shown in Table 1. The mean quantitative recovery for all 19 strains tested was 90% for NGB compared with 91 and 83% for BS and BGS, respectively.

Salmonella and *Arizona* colonies on BS agar varied in size from 0.8 to 1.8 mm in diameter, with an average of approximately 1.2 mm. All strains tested on this medium varied greatly in appearance of differentiating properties. The colonies were green to brown to dark brown or black with or without a metallic sheen zone around the colonies. Variation was also observed for the same organism between plates. On one plate of BS agar green colonies could predominate, whereas on another plate of the same BS agar batch the typical dark brown to black colonies with a metallic sheen zone would predominate. One strain of *Arizona* sp. failed to grow, and only 2% of *S. typhi* isolates were recovered after 48 h of incubation on the BS agar plates.

The morphological appearance of *Salmonella* colonies on BGS agar varied depending on the serotype tested. With the exception of *S. typhi*, biochemically typical serotypes of *Salmonella* spp. and *Arizona* spp. varied in size from 0.7 to 1.4 mm in diameter, with an average of 1.0 mm. Colonies were smooth and even, with the typical translucent pink-red color and red zones in the medium around the colonies. *S. typhi* colonies were similar in appearance, but a 48-h incubation was necessary for good colony development. The quantitative recovery for *S. typhi* with BGS was 63%. Lactose-positive *Salmonella* colonies on BGS varied from 1.7 to 2.9 mm in diameter, with an average size of approximately 2.1 mm. Colonies were smooth but with irregular margins and yellow in color, with yellow zones of coloration in the medium around the colonies.

With the exception of *S. typhi*, *Salmonella* and *Arizona* colonies on NBG were 2.0 to 2.9 mm in diameter, with an average size of 2.4 mm. They appeared smooth and entire with medium- to large-sized dark black nucleated centers (H_2S production) and thin outer translucent edges. In addition, reddening and a visible zone of clearing occurred in the medium around each colony. *S. typhi* colonies that grew on NBG agar required 48 h of incubation and were atypical in appearance. The colonies averaged 0.9 mm in diameter and were smooth and entire, with a light yellow-green color in the medium surrounding each colony, but negative for H_2S production.

The ability of 22 gram-negative bacterial isolates, representing 13 species, to grow on NGB agar is shown on Table 2. Strains of *E. coli*, *P. mirabilis*, *Providencia* sp., *Hafnia alvei*, and *Pseudomonas putrifaciens* were unable to grow on NGB. *Proteus vulgaris* grew very poorly, producing only minute opaque colonies in 24 h at 35°C. Other strains that grew on NGB included *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Edwardsiella tarda*, and *Aeromonas hydrophila*, but, due to their inability to produce hydrogen sulfide, they produced colonies distinctly different from those of *Salmonella* spp. and *Arizona* spp. Whereas one strain of hydrogen sulfide-producing *Citrobacter freundii* was not able to tolerate the selective conditions of NBG agar, four strains produced colonies identical to those of *Salmonella* spp. and *Arizona* spp.

The results of isolating a lactose-positive *Salmonella* sp. from seeded foods with NBG and BS agars are shown in Table 3. The seeded *Salmonella* strain was detected in only 8 of the 10 foods inoculated onto both the NBG and BS agars from the selenite cystine selective enrichment. Thirty-nine of 50 (78%) and 37 of 46 (80%) typical colonies picked from NBG and BS agars, respectively, were confirmed as the seeded lactose-positive strain. From the tetrathionate-brilliant green selective enrichment, the seeded lactose-positive *Salmonella* strain was detected from all 10 foods by both the NBG and BS agars. All 50 typical colonies (100%)

TABLE 3. Recovery of seeded lactose-positive salmonellae[a] from foods with NBG and BS agars streaked after selective enrichment

Selective enrichment	Plating medium	Total No. of foods inoculated	No. of foods from which salmonellae were isolated (% recovery[c])	No. of typical colonies picked	No. of colonies confirmed as lactose-positive salmonella spp., (% confirmation)[d]
Selenite-cystine[b]	NBG	10	8 (80)	50	39 (78)
	BS	10	8 (80)	46	37 (80)
Tetrathionate-brilliant green[e]	NBG	10	10 (100)	50	50 (100)
	BS	10	10 (100)	50	50 (100)

[a] Seeded at 10^3 bacteria per g of food.
[b] Incubated at 35°C for 24 h.
[c] Percent recovery was calculated as (number of foods from which salmonellae were isolated/total number of foods inoculated) × 100%.
[d] Percent confirmation was calculated as (number of colonies confirmed as lactose-positive *Salmonella* spp./number of typical colonies picked) × 100%.
[e] Incubated at 43°C for 48 h.

TABLE 4. Stability of recovery and selectivity characteristics of NBG agar after storage at 4°C

Test organism	Strain	% Recovery[a] after the following storage time (days)			
		1	3	7	15
Arizona sp.		105	99	83	116
Salmonella sp. (lactose positive)	1	85	92	64	89
	2	89	93	87	81
S. brandenburg		104	96	80	99

[a] Based on mean of triplicate plates compared with recovery on blood agar. Overall recovery was 96, 95, 79, and 94% after storage at 4°C in plastic bags for 1, 3, 7, and 15 days, respectively. Two isolates of *E. coli* and two isolates of *P. mirabilis* did not grow on streak plates.

picked from both media were identified as lactose-positive *Salmonella* sp.

The effect of refrigerated storage on the quantitative recovery and selectivity of NBG agar is shown in Table 4. There was no significant change in the overall quantitative recovery of *Arizona* or *Salmonella* strains tested with NBG agar after 15 days of storage in 4°C in plastic bags. In addition, two strains of *E. coli* and *P. mirabilis* failed to grow on NBG agar over the same time period.

The results of *Salmonella* spp. isolation from amphibian and reptile waters are shown in Table 5. A total of 67 water samples were analyzed, of which 22 (33%) were shown to contain *Salmonella* spp. A variety of serotypes were isolated, including *Salmonella poona* (six samples), *Arizona* spp. (five samples), *S. panama* (two samples), *S. blockley* (two samples), *S. stanley* (one sample), *S. derby* (one sample), *S. newport* (one sample), and *Salmonella* group B untypable (four samples). A 100% recovery rate [(Number of samples where *Salmonella* spp. were isolated with a test medium/total number of samples having salmonellae using all media) × 100%] was achieved with NBG, BS, and BGS agars. With HE agar, only 17 of 22 positive water samples (77%) were found to contain *Salmonella* spp. *Arizona* spp. were not detected on HE in five of the samples. The *Arizona* isolates found from these water samples were lactose fermenters (acid formation in lactose broth after 24 h at 35°C), and colonies on HE agar appeared atypically bright yellow, with some containing pinpoint black centers. Confirmation rates [(number of typical colonies confirmed as *Salmonella* spp./total number of typical colonies picked) × 100%] were 68, 67, 56, and 37% with BGS, HE, NBG, and BS agars, respectively. Because of the lack of differential properties of BS agar, a variety of colony types were picked (Table 6). Although all 22 typical dark brown to black colonies with metallic sheen zone were confirmed as *Salmonella* spp., 20 of 37 green (54%), 3 of 14 green-brown (21%), 40 of 129 brown (31%), and 1 of 32 black (3%) were also identified as *Salmonella* spp.

The results of salmonella isolation from feces by both direct streaking and streaking after overnight selective enrichment in selenite F are shown in Table 7. Nine of 20 samples (45%) analyzed were found to contain *Salmonella* spp. A variety of serotypes were isolated, including *S. typhimurium* (four samples), *S. heidelberg*, *S. hadar*, *S. cabana*, *S. paratyphi* B, and *S. typhi* (1 sample each). BS was the most selective medium in eliminating background colonies, but NBG was as effective as SS and BGS agars and better than HE or XLD agar, especially with direct streaking. With direct streaking, *Salmonella* spp. were detected in seven (BS), five (NBG), four (SS), three (BGS and HE), and two (XLD) of the nine positive samples. After enrichment in selenite F, only BS detected salmonellae in all nine positive samples. NBG, XLD, HE, SS, and BGS detected salmonellae in eight of the nine (89%) positive samples. In addition, BS agar was the only medium to isolate *S. typhi* both by direct streaking after selective enrichment in selenite F. *S. typhi* was isolated with BGS and XLD, but only after enrichment in selenite F. NBG, HE, and SS failed to differentiate this organism.

Confirmation percentages for typical colonies from fecal specimens were 100% (HE and SS), 89% (XLD), 88% (NBG), 75% (BGS), and 53% (BS) from direct streaking and 100% (HE), 94% (SS), 89% (XLD and NBG), 78% (BGS), and 49% (BS) from selective enrichment. A variety of colony types were again observed and picked from BS agar (Table 6). As with the isolates from amphibian and reptile waters, all 4 (100%) typical dark brown to black with metallic sheen zone colonies picked after direct streaking were confirmed as *Salmonella* spp.; however, 14 of 36 brown (39%) and 7 of 7 black (100%) colonies were also confirmed as *Salmonella* spp. Similarly, 29 of 30 typical dark brown to black colonies with metallic sheen zones (97%) and 6 of 22 brown (27%) colonies tested after selenite F enrichment were verified as *Salmonella* spp., but none of the 16 green or the 4 green-brown colonies was confirmed.

DISCUSSION

Media used to isolate salmonellae, such as XLD, HE, BGS, and SS agars, are designed to recover other disease-producing enteric bacteria as well. Much of their success has relied on the inclusion of certain carbohydrates such as sucrose, lactose, or salicin. Background coliforms which are able to utilize one or more of these sugars are differentiated from the enteric pathogens by the high acidity produced. Reliance on such differentiation can be a problem in detecting salmonellae under certain instances where (i) the high acidity produced by the background colonies can mask the differential reaction of *Salmonella* colonies (18), and (ii) certain strains of salmonellae are able to utilize these sugars and subsequently resemble the background coliform organisms (2, 10, 16, 18, 20). These conditions can mean missed opportunities for isolating members of this genus. BS agar is the only medium which allows for the isolation of these atypical strains (1, 2, 13, 16, 20). However, preparation, storage time, and temperature can affect the selective and

TABLE 5. Comparative performance of agars for the recovery of *Salmonella* spp. from 67 amphibian and reptile water samples[a]

Medium	No. of samples where *Salmonella* spp. were isolated (% recovery[b])	No. of typical colonies picked to KIA and LIA[c]	No. of colonies confirmed as *Salmonella* spp.	% Confirmation[d]
NBG	22 (100)	158	88	56
BGS	22 (100)	123	87	68
HE	17 (77)	98	66	67
BS	22 (100)	234[e]	86	37

[a] Of 67 amphibian and reptile samples analyzed, 22 were confirmed as having *Salmonella* spp.

[b] Percent recovery calculated as (number of samples where *salmonellae* were isolated by test medium/22) × 100%.

[c] KIA, Kligler iron agar; LIA, lysine-iron agar.

[d] Percent confirmation was calculated as (number of colonies confirmed as *Salmonella* spp./number of typical colonies picked) × 100.

[e] A variety of colony types were picked from BS agar. See Table 6. Data are inclusive for all colony types.

TABLE 6. Confirmation rates for various colony types picked from BS agar

Colony type	Sample source	Enrichment	No. of colonies picked to KIA and LIA[a]	No. of colonies confirmed as *Salmonella* spp.	% Confirmation[b]
Green	Amphibian and reptile water		37	20	54
	Feces		0	NA[c]	
	Feces	Selenite F[d]	16	0	0
Green-brown	Amphibian and reptile water		14	3	21
	Feces		0	NA	
	Feces	Selenite F	4	0	0
Brown	Amphibian and reptile water		129	40	31
	Feces		36	14	39
	Feces	Selenite F	22	6	27
Black	Amphibian and reptile water		32	1	3
	Feces		7	7	100
	Feces	Selenite F	0	NA	
Typical[e]	Amphibian and reptile water		22	22	100
	Feces		4	4	100
	Feces	Selenite F	30	29	97

[a] See footnote *c* of Table 5.
[b] See footnote *d* of Table 5.
[c] NA, Not applicable.
[d] Samples of 1 g of feces were enriched with 9 ml of selenite F overnight at 35°C before streaking.
[e] Dark brown to black with metallic sheen.

differential properties of BS agar, which can influence recovery of salmonellae (3, 4). Despite these criticisms, the use of BS agar has been recommended, along with other enteric media, for salmonella isolation (1).

NBG agar has been designed as a salmonella isolation medium which takes into account the reclassification of the *Salmonella* genus into five subgroups based on biochemical differences and a greater awareness of atypical strains. To avoid missing atypical strains, all carbohydrates except glucose were eliminated from NBG agar. Glucose is the only carbohydrate used by organisms of all *Salmonella* serotypes (7). Like Littell (16), we found that a 0.1% glucose concentration gave fast colonial growth and better differentiation in 24 h for salmonellae. The utilization of small amounts of glucose does not inhibit H_2S expression, due to the limited accumulation of acid end products. Hydrogen sulfide production has been used in the medium to detect salmonellae because it is the easiest and most reliable indicator system for this organism. Moats and Kinner (18) found that an H_2S indicator added to different brilliant green agar formations aided in the identification of *Salmonella* colonies, especially in mixtures with other bacteria. To eliminate background

TABLE 7. Relative performance of agars for isolation of *Salmonella* spp. from 20 fecal specimens

Agar	Enrichment	No. of samples with indicated growth[a]			No. of samples where *Salmonella* spp. were confirmed (% recovery[b])	No. of colonies picked to KIA, and LIA[c]	No. of colonies confirmed as *Salmonella* spp. (% confirmation[d])
		3+ to 4+	1+ to 3+	0 to few			
NBG		5	11	4	5[e] (56)	17	15 (88)
	Selenite F[f]	13	5	2	8[e] (89)	36	32 (89)
BS		5	9	6	7 (78)	47[g]	25 (53)
	Selenite F	10	8	2	9 (100)	72[g]	35 (49)
BGS		4	12	4	3[e] (33)	16	12 (75)
	Selenite F	11	6	3	8 (89)	40	31 (78)
HE		13	6	1	3[e] (33)	9	9 (100)
	Selenite F	13	7	0	8[e] (89)	31	31 (100)
XLD		13	6	1	2[e] (22)	9	8 (89)
	Selenite F	14	6	0	8 (89)	36	32 (89)
SS		7	10	3	4[e] (44)	14	14 (100)
	Selenite F	13	4	3	8[e] (89)	34	32 (94)

[a] Based on the quadrant streak method, where 1+ indicates growth in the first quadrant only, etc.
[b] Percent recovery was calculated as (number of samples for which salmonellae were isolated by test medium/9) × 100.
[c] See footnote *c* of Table 5.
[d] See footnote *d* of Table 5.
[e] *S. typhi* was not isolated.
[f] See footnote *d* of Table 6.
[g] A variety of colony types were picked from BS agar. See Table 6. Data are inclusive for all colony types.

organisms including other H_2S producers, the selective agents brilliant green and novobiocin were added. Brilliant green used at a concentration of 6 to 9 mg/liter has been shown to effectively inhibit coliforms and gram-positive organisms without affecting growth of salmonellae (14). Brilliant green was added after autoclaving to prevent any loss in selectivity (17, 19). Novobiocin was included to retard growth of coliforms and of *Proteus* sp. (11, 12), an organism which can look like salmonellae on enteric media. The selectivity of novobiocin was confirmed in this study, since *Proteus* sp. was never isolated on NBG agar.

The pure culture experiments showed that, although some species of gram-negative bacteria could grow well on NBG agar (Table 2), only H_2S-positive strains of *C. freundii* could resemble salmonellae on this medium. This was confirmed with the seeded food experiments (Table 3) as well as the naturally contaminated amphibian and reptile waters (Table 5) and fecal specimens (Table 7). All typical colonies from NGB agar which were not salmonellae proved to be *C. freundii* on further analysis (data not shown). Where present in large numbers, *C. freundii* organisms could jeopardize the recovery of salmonellae. However, that phenomenon was not observed in this study.

As shown in Table 3, regardless of the enrichment used, NBG agar performed as well as BS agar in both overall recovery and colony confirmation rates for the seeded lactose-positive *Salmonella* strains in the presence of competing background bacteria. In addition, NBG was shown to be as effective as BS and BGS and better than HE for the recovery of *Salmonella* strains from naturally contaminated amphibian and reptile waters. Although HE and BGS agars had higher colony confirmation rates than NBG, HE failed to recognize the five samples containing lactose-positive *Arizona* spp. Had other lactose-positive salmonellae been present, they would have been missed with BGS as well as HE. The lactose-positive *Arizona* colonies were not missed on BGS because, with their slower growth and lactose utilization in 24 h, they appeared small, clear, and neutral to pale pink in color and thus were sampled as typical *Salmonella* colonies. This phenomenon with lactose-positive *Arizona* spp. on BGS has also been observed elsewhere (10). Like NBG, BS agar had a 100% recovery rate from the amphibian and reptile waters, but the many different morphological colony types on BS had to be included to compare favorably with NBG. *Salmonella* colonies were shown to be green, brown, black, or the typical dark brown to black with a metallic sheen zone. Had only typical colonies been sampled, the recovery rate with BS would have been considerably lower. *Arizona* colonies on BS agar have been reported as looking like typical *Salmonella* spp. colonies (13, 16); however, like Greenfield et al. (10), we found *Arizona* colonies on BS to be coliform-like, being olive green with darker centers. In addition, one strain of *Arizona* sp. failed to grow on BS in the pure culture study (Table 1), indicating the possible toxicity of this medium for certain strains of this *Salmonella* subgroup. Toxicity and questionable recovery of other *Salmonella* serotypes with BS has been documented (3, 4, 11).

NBG was second only to BS agar in the recovery of *Salmonella* spp. from direct streaking of feces (Table 7). BS agar was the only medium to successfully isolate *S. typhi* from direct streaking. However, as was observed with the amphibian and reptile waters, *Salmonella* colonies on BS agar exhibited a variety of sizes and colors (Table 6). As a result many more colonies had to be picked from BS, and the colony confirmation rate was much lower than with the other media tested. After selenite F enrichment, colony confirmation rates did not change over direct streaking. BS still required the most work with the lowest rate of confirmation; however, recovery rates markedly improved for all media tested.

NBG, HE, and SS agars failed to differentiate *S. typhi* from the one fecal sample containing this organism. Although *S. typhi* was isolated after enrichment with BGS and XLD, these media failed to isolate a less fastidious, faster-growing *Salmonella* serotype. Although BS agar was the only medium to have 100% recovery rate and to isolate *S. typhi* from both direct streaking and overnight enrichment in selenite F broth, the *S. typhi* strain used in the pure culture recovery experiments (Table 1) would not grow on BS agar. This indicates once again the erratic nature of the media for both differentiation and selectivity for *Salmonella* spp. isolation. *S. typhi* poses particular problems for successful isolation compared with other salmonellae. Its inability to grow well on all selective media and its poor H_2S expression (7) make it a difficult organism to isolate consistently on many media designed for enteric *Salmonella* sp. isolation. Although *S. typhi* will grow on NBG agar, as evidenced in the pure culture experiments (Table 1), NBG would not be the medium of choice for this organism.

It must be emphasized that 5% of most serotypes of salmonellae isolated from clinical specimens are H_2S negative (7) and thus will not be detected with NBG agar. Consequently an enteric salmonella medium which does not incorporate an H_2S indicator, such as BGS agar, should be used with NBG so that H_2S-negative *Salmonella* strains are not overlooked.

NBG agar has been formulated to isolate *Salmonella* spp. from all subgroups. A full 22 to 24 h of incubation is necessary to allow sufficient time for H_2S production in some *Arizona* strains. In addition, some *C. freundii* strains resemble *Salmonella* colonies on NBG, but the use of lysine-iron agar slants for presumptive identification will effectively differentiate these two organisms. However, at no time in this study did *C. freundii* interfere with salmonella identification. The results show that if any salmonellae, with the possible exception of *S. typhi*, are present in a sample they will be detected with NBG agar.

The distinct advantage of NBG agar over other enteric media, especially BS, is the consistent differential colonial morphology. Regardless of the subgroup or of atypically reacting H_2S-positive strains in a subgroup, colonies of salmonellae on NBG agar are large with medium to large dark black centers and thin outer translucent zones. The medium is inexpensive, easy to prepare, and can be stored for at least 2 weeks at 4°C without loss of selective or differential properties. NBG could replace BS agar, especially in investigations where biochemically atypical salmonellae are suspected.

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