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Biochemically Aberrant *Salmonella enteritidis ser. newington* from Human Sources in Connecticut

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Three isolates of a lactose-fermenting, xylose-negative variety of *Salmonella enteritidis ser. newington*, identical in biochemical and serological reactions and in the antibiogram, were recovered from three patients in different areas of Connecticut in January 1974. Hydrogen sulfide production was not visible in Salmonella-Shigella agar, in triple sugar iron agar, and in Kligler iron agar but was noticed in lysine iron agar and on XLD agar, among others. The amount of fermentable carbohydrates present was found to correlate with failure to show hydrogen sulfide production (pH effect). In contrast to lactose-fermenting Salmonella strains reported by other authors, we could not elicit a direct transfer of the lac$^+$ character at frequencies above 10^{-6}. An epidemiological follow-up remained unsuccessful. Recommendations for the recognition of similar strains are presented.

Salmonella enteritidis ser. newington (henceforth called *S. newington*) was first isolated by Leo F. Rettger from ducks in Newington, Connecticut in 1937 (1). It belongs to group E_2 of the Kauffmann-White scheme (2). In various series, *S. newington* strains comprised only 0.3 (3), 0.4 (4), 2.1 (5), and 6.9% (6) of human Salmonella strains isolated. The 6.9% figure (37 of 532 strains for the period between 1934 and 1941) includes 31 carrier strains, which also figure large in other series (4, 7, 8). Reports of human disease due to *S. newington* have been infrequent (9, 10); two institutional outbreaks of gastroenteritis have been described (11, 12). In the last quarter of 1973, only eight *S. newington* isolates (of a total of 7686 Salmonella isolates) were reported by the Center for Disease Control for the United States, two of them from Connecticut (13).

In their main biochemical characteristics, *S. newington* strains resemble the majority of *S. enteritidis* serotypes (1, 2). In particular, they fail to ferment lactose and sucrose, ferment xylose, and produce hydrogen sulfide (H_2S) in iron chloride gelatin, Kligler iron agar (KIA), and triple sugar iron agar (TSIA).

Three biochemically and serologically identical isolates of *S. newington* that ferment lactose (lac$^+$) but fail to ferment xylose and to produce H_2S in some common laboratory media were observed in January 1974 in three different Connecticut hospitals. Similar human strains have only been reported from one case each of meningitis (10) and gastroenteritis (9); more often, they have been found in dried milk (14). A lac$^+$, H_2S-producing strain originating from dried milk was responsible for one institutional outbreak (12). Most lac$^+$ Salmonella strains have been able to transfer the lac$^+$ character to strains of *Escherichia coli* and/or Salmonella species as part of an extrachromosomal sex factor called F_0-lac (15), either directly (15, 16) or after mobilization by another plasmid (17).

We report on clinical, biochemical and genetic studies of the three lac$^+$ *S. newington* isolates.

MATERIALS AND METHODS

Clinical data. The first isolate originated from the appendiceal pus of a 25-year-old Stratford woman who was admitted to Bridgeport Hospital for appendicitis. An identical isolate was recovered from her stool a few days postoperatively and 2 weeks after discharge. The second isolate originated from the cerebrospinal fluid of a 14-day-old male infant from Willimantic who had been transferred to Hartford Hospital because of meningitis. The isolation was repeated twice during his illness which lasted for 3 weeks; identical isolates were recovered from the patient's feces four times and from his blood once. The third isolate originated from the stool of a 19-year-old New Haven man who had been admitted to Yale–New Haven Hospital for septic arthritis of a finger and who developed diarrhea with fever one day after admission. Salmonella was not isolated from the joint. All primary isolations were made within a period of one week.

Isolation and Identification. The appendiceal and spinal fluid strains were selected on MacConkey agar,[1] the stool isolates on XLD agar[1] and on Salmonella–Shigella (SS) agar[1] at 37°C. Further biochemical work-up followed methods described elsewhere (18). Production of H_2S was investigated in a variety of media. Lysine iron agar (LIA) was adjusted to several pH values before inoculation and was also supplemented with 0.5% glucose, 1.0% glucose, 1.0% sucrose, and 1.0% lactose, respectively.

Antimicrobial sensitivity was determined by the Kirby–Bauer disk method (19).

Genetic transfer studies. One of us (W. D. R.) investigated a possible transfer of lac$^+$ to the F$^-$lac$^-$ *E. coli* K 12 strain KL 320 (20) which is resistant to streptomycin and nalidixic acid (Str^r Nal^r). The method of Falkow and Baron (15) was followed by adding 1 ml of the *S. newington* culture to 9 ml of the *E. coli* culture, each culture containing $2 \times 10^8 - 4 \times 10^8$ cells/ml. After 2 hr incubation at 37°C, the mixture was plated on minimal medium (21) plates containing proline, methionine, histidine, tryptophane (required by *E. coli* K 12 KL 320), streptomycin (200 μg/ml) and nalidixic acid (50 μg/ml), and scored for the lac$^+$ Str Nal phenotype. All three *S. newington* isolates were checked separately. The F′ lac$^+$ *E. coli* strain Q served as a control donor strain, the recipient being again *E. coli* K 12 KL 320.

RESULTS

Epidemiology. The three patients recovered but were lost to follow-up. A search for a common source proved futile as a retrospective investigation was started only after it had become known that the three isolates had been sent to the Connecticut State Department of Health. The patient from Bridgeport Hospital had used powdered milk before admission; it was cultured and found negative for Salmonella. A check of 30 employees of Hartford Hospital failed to turn up any fecal Salmonella from personnel in contact with the infant in January 1974. No further cases of lac$^+$ *S. newington* have since been reported to the Connecticut State Department of Health. No contact between the patients could be elicited.

Cultural characteristics. On MacConkey agar, deeply pink, flat colonies with an entire margin grew in 24 hr. The 24-hour growth on XLD agar showed, beside

[1]BBL, Cockeysville, MD.

TABLE 1
Biochemical Reactions of Three Strains of *Salmonella enteritidis ser. newington* (24–48 hr at 37°C)[a]

Indole	–	Glucose acid	+
Urease	–	Glucose gas	+
Methyl red	+	Lactose	+
Voges-Proskauer	–	Sucrose	–
Citrate (Simmons)	+	Mannitol	+
Motility	+	Dulcitol	+
Gelatin (22C)	–	Salicin	–
Lysine decarboxylase	+	Adonitol	–
Arginine dihydrolase	+d	Inositol	–
Ornithine decarboxylase	+	Sorbitol	+
Phenylalanine deaminase	–	Arabinose	+
Malonate	–	Raffinose	–
Deoxyribonuclease	–	Trehalose	+
Beta galactosidase	+	Rhamnose	+
		Xylose	–

[a]+, positive; –, negative; d, delayed (more than 48 hr); w, weak reaction after 48 hr.

typical coliform colonies, those with black centers on a yellow base. On SS agar, lac$^+$ colonies without blackening were seen after 48 hr of growth; some colonies, however, showed an intensely pink color.

Colonies from MacConkey agar as well as the unusual lac$^+$ colonies from SS agar and the H_2S-positive ones from XLD agar were studied further (Table 1). The negative indole test coupled with H_2S production and decarboxylation in LIA suggested the presence of either Arizona or Salmonella sp. The three isolates gave identical reactions and agglutinated in Salmonella group E antiserum.[2] Antigenic

TABLE 2
Visible Production of Hydrogen Sulfide by Three Strains of *S. newington* in Different Media (24–48 hr at 37°C)[a]

Tubed media		Plated media	
Lead acetate (strip (19))	+	Salmonella-Shigella agar	–
Triple sugar iron agar	–	Bismuth sulfite agar	+
Kligler iron agar	–	XLD agar	+
SIM medium	+	Hektoen enteric agar	+w
Neutral Red-lysine-Iron-cystine broth (26)	+	Sulfite-dulcitol agar (15)	+

Tubed media	Initial pH	Final pH	
Lysine iron agar with adjusted initial pH	4.0	4.0	–
	5.2	7.4	+
	5.8	7.4	+
	6.0	7.4	+
	6.3	7.4	+
	6.4	7.4	+
	6.7	7.4	+
Lysine iron agar			
+ 0.5% glucose +	6.1	6.4	–
+ 1.0% glucose –	6.1	6.1	–
+ 1.0% lactose –	6.1	6.1	–
+ 1.0% sucrose	6.1	7.4	+

[a]Source for media or constitutents (25, 26): BBL, Cockeysville, MD. See Table 1 footnote.

analysis yielded the formula (3, 15): e,h:1,6[2] and thus established the isolates as *S. newington*. Atypical were the reactions for lactose and xylose fermentation while H_2S production varied considerably between different media (Table 2). The TSIA and KIA butts and slants were acid; there was also gas production.

Antimicrobial sensitivity. All three isolates were fully sensitive to ampicillin, carbenicillin, cephalothin, chloramphenicol, colistin, gentamicin, kanamycin, nalidixic acid, nitrofurantoin, and tetracycline.

Genetic transfer. No recombinants between each of the *S. newington* strains and *E. coli* K 12 KL 320 were found at frequencies higher than 10^{-6}. In the control experiment with *E. coli* K 12 Q as the donor, the transfer frequency to *E. coli* K 12 KL 320 was greater than 1 after mating for only 1 hr.

DISCUSSION

Lac$^+$ strains of Salmonella are very rare (0.8% of all Salmonella strains (18)) and have been observed mainly in the *S. enteritidis* serotypes *S. tennessee* (14, 18, 22), *S. anatum* (14), *S. newington* (9, 10, 12, 14), and, recently, *S. typhimurium* and *S. oranienburg* (17). Of 552 Salmonella strains from dried milk and milk-drying plants, however, 86 (15.6%) were found to be lac$^+$ (14). Failure to ferment xylose is commonly observed only in the *S. enteritidis* bioserotypes *S. paratyphi A* and *S. sendai* (18). One of the lac$^+$ *S. newington* strains that was described earlier (10) was also xylose-negative.

Lac$^+$ Salmonella strains often fail to show H_2S production in some media (17), notably on SS agar (10) and in TSIA (10, 14, 18, 22). We did not observe H_2S^+ variants on SS agar (10, 22), but after several subcultures on Trypticase Soy Agar[1] a lac$^-$ H_2S^+ variant was recovered from the first isolate, similar to findings of earlier authors (9, 22).

A similar failure to show H_2S production in TSIA has been reported for sucrose-positive strains of *Citrobacter freundii* and *Proteus vulgaris* and was thought to be due to products of fermentable carbohydrates (23). Also, lac$^+$ *C. freundii* and *Arizona hinshawii* strains, while showing blackening in glucose (0.1%)–lactose (1.0%)–H_2S medium, failed to blacken the medium when 0.9% glucose was added; however, this medium with 1.0% glucose (and 1.0% lactose) showed blackening upon secondary alcalinization (24). A plausible explanation was that, although H_2S was actually formed, an iron sulfide precipitate did not show at a low pH since the solubility of FeS increases with decreasing pH (molar concentration of Fe^{2+} at pH 5 is 3.7×10^{-6}; at pH 7, it is 3.7×10^{-10}) (24).

Taking a different approach, we tried to find out whether a similar pH drop is responsible for the absence of FeS precipitation in some identification media inoculated with our *S. newington* strains. The amount of substrates from which H_2S could be produced is higher in TSIA and in KIA than in LIA, and higher in SS agar than in XLD agar or in the sulfite-dulcitol agar of Padron and Dockstader (25) (Table 3). Thus, no correlation could be found between substrate quantity and visible FeS production. The total amount of carbohydrates is higher in TSIA and KIA than in LIA or in SIM medium;[1] on the other hand, it is higher in XLD agar, which showed FeS production, than in SS agar, which failed to show it. The amount of lactose is highest in the media in which visible FeS was not formed but there is no evidence that lac$^-$ Salmonella strains form H_2S less often in these media than in

[2]We thank the Laboratory Division of the Connecticut State Department of Health, Hartford, Connecticut, for the serological analysis and the confirmation of our biochemical results.

TABLE 3
Initial pH, Contents of Carbohydrates, and Contents of Substrates for H_2S Production in Various Media (in g/1000 ml)[a]

Ingredient	Triple sugar iron agar	Kligler iron agar	SIM medium	NR-lysine-iron-cystine broth (26)	Lysine iron agar	SS agar	Bismuth sulfite agar	XLD agar	Hekt enter agar	Sulfur dulc agar (25)
Initial pH	7.3	7.4	7.3	6.2	6.7	7.1	7.5	7.4	7.6	7.5
Glucose	1.0	1.0	–	1.0	1.0	–	5.0	–	–	1.5
Lactose	10.0	10.0	–	5.0	–	10.0	–	7.5	12.0	–
Sucrose	10.0	–	–	–	–	–	–	7.5	12.0	–
Salicin	–	–	–	1.0	–	–	–	–	2.0	–
Dulcitol	–	–	–	–	–	–	–	–	–	5.0
Kylose	–	–	–	–	–	–	–	3.5	–	–
Cystine and methionine	0.5	0.5	0.65	0.24	0.13	0.12	0.25	–	0.28	0.02
Ferrous (ammonium) sulfate	0.2	–	0.2	–	–	–	0.3	–	–	–
Sodium thiosulfate	0.2	0.5	0.2	0.1	0.04	8.5	–	6.8	5.0	–
Bismuth sulfite	–	–	–	–	–	–	8.0	–	–	–
Sodium sulfite	–	–	–	–	–	–	–	–	–	3.0
Bismuth citrate	–	–	–	–	–	–	–	–	–	0.4
Ferric (ammonium) citrate	–	0.5	–	0.5	0.5	1.0	–	0.8	1.5	–
Yeast extract[b] (or beef extract)	–	–	–	3.0	3.0	5.0	5.0	3.0	3.0	–

[a]Data from BBL Manual of Products and Laboratory Procedures, 5th edition, 1968; and from publications (25) and (26). The cystine and methionine contents were computed from the BBL Peptones table.

[b]Contains 16.6% carbohydrates and 3.6% cystine and methionine.

others. The *initial* pH of the H_2S-indicating media was, with two exceptions, above 7.0 (Table 3). The exceptions, LIA and neutral red–lysine–iron–cystine broth (26), contain 1% lysine which is decarboxylated by *S. newington* with a resulting alkaline pH. The sulfite-dulcitol medium of Padron and Dockstader (25), which contains no lysine (except for the amount present in the peptone) fails to exhibit FeS production in lac$^+$ and lac$^-$ Salmonella species if the initial pH is below 6.0 (25). Thus, there seems to be a correlation between visible FeS production and the *final* pH in the medium.

Our experiments with LIAs showed that, indeed, initial pH values above the lower limit for lysine decarboxylase activity (27) did not prevent blackening, and that under these circumstances the final pH (after decarboxylation) always rose to 7.4. However, if the final pH was at 6.4 or below, which happened only when fermentable sugars like glucose or lactose (but not sucrose) were added, FeS production was not visible. We conclude, therefore, that the amount of fermentable carbohydrates, whose catabolism resulted in a sustained (rather than in a mere initial) pH drop not overcome by decarboxylation or other countervailing processes, is of paramount importance for visible FeS production in our strains, which means H_2S production as well. We can, however, not explain why other lac$^+$ strains of Salmonella (14) and *C. freundii* do show FeS production in TSIA and KIA.

In previously reported lac$^+$ Salmonella strains, the transfer frequency of lac$^+$ ranged from 3×10^{-2} to 1×10^{-5} to *E. coli* (15) and from 2×10^{-3} to 6×10^{-5} to *S. typhi* (16). Only in one of seven strains of *S. anatum* was no transfer detected (16). Our strains behaved similar to this *S. anatum* strain. Two possibilities cannot be excluded: 1) that the lac$^+$ character is located on a plasmid or on a chromosome with a transfer frequency of less than 10^{-6}; and 2) that the lac$^+$ character can be transferred only after mobilization with other plasmid(s) (17). Experiments are in progress to study these possibilities.

Should the incidence of lac$^+$ Salmonella increase to a point where there is a reasonably high possibility of their being present in clinical specimens, particularly feces, the clinical laboratory would be faced with a serious diagnostic challenge. In order to detect such strains, we suggest 1) that stools be routinely plated on XLD agar which gives a high recovery rate for Salmonella and is superior to SS agar in the recovery of Shigella (28, and 2) that lac$^+$ indole-negative colonies be checked for H_2S formation on media with a low content of fermentable carbohydrates, such as SIM medium or LIA. The latter medium, however, is recommended for lysine decarboxylation only in Salmonella and Arizona (18). The use of lead acetate strips for routine diagnostic purposes is not advisable since they are overly sensitive (29).

Whether the intensity of the pink color displayed by our strains on MacConkey agar and SS agar had any diagnostic significance is unclear; further isolations of lac$^+$ Salmonella would have to bear this out.

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