Unusual *Enterobacteriaceae*: Lactose-Positive *Salmonella typhimurium* Which is Endemic in São Paulo, Brazil

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Received for publication 19 May 1975

Since 1971 a lactose-positive (lac^+) *Salmonella typhimurium* variety Copenhagen has been endemic in the city of São Paulo. The strain is a strong lactose fermenter and resembles *Escherichia coli* on primary plating media and in triple sugar iron agar. Although most isolates of the strain have uniform properties, some have slightly different antigens, antibiograms, phage types, or fermentation patterns. Most isolates have come from stools of infants under 1 year of age and are probably hospital acquired; however, other isolates are probably community acquired. Eighteen other lac^+ *Salmonella* isolated in the United States were also studied. Most of these strains resembled *E. coli* on primary plates and triple sugar iron agar; thus their identification would pose a problem for most clinical laboratories. A simple procedure for detecting lac^+ *Salmonella* mixed with lac^+ *E. coli* consists of touching 12 colonies in succession with a straight wire and then inoculating a peptone iron agar tube. H_2S production is apparent from lac^+ *Salmonella* even if 11 *E. coli* and one *Salmonella* colony are picked. If a positive peptone iron agar tube is observed, then individual colonies are tested to rule out other strong H_2S producers. The true incidence of lac^+ *Salmonella* is unknown because they are not isolated and identified in most laboratories.

Lactose-positive (lac^+) *Salmonella* have been known since 1905 (2, 3, 8, 12), but their incidence in clinical specimens is unknown (4, 7, 9, 11). Ewing (6) reported that lac^+ *Salmonella* comprised less than 1% of all *Salmonella* examined at the Center for Disease Control (CDC). However, the actual incidence may be higher because most laboratories use only enteric plating media which contain lactose and lac^+ *Salmonella* are indistinguishable from *Escherichia coli* on these media. lac^+ colonies would not be picked for further study. Table 1 shows that lactose is present in most media commonly used for isolating enteric pathogens.

The general concept that lac^+ *Salmonella* are rare may be correct; however, these strains can become important in certain ecological niches or localized geographical areas. Recently Blackburn and Ellis (2) reported 86 (15.6%) lac^+ isolates among the 552 *Salmonella* cultured from dried milk products and milk-drying plants. Lactose in these milk products probably selects strains which can use this disaccharide as a source of energy. In 1972 Pessoa (G. V. A. Pessoa, Thesis, Univ. de São Paulo, São Paulo, Brazil, 1972) reported that a lac^+ *Salmonella typhimurium* was endemic in São Paulo, Brazil, during 1971-72. The purposes of this article are to further describe this unusual lac^+

strain and to extend these observations to the isolation and characterization of lac^+ *Salmonella* in general.

MATERIALS AND METHODS

Media. Triple sugar iron agar (TSI), peptone iron agar (PIA), lysine iron agar (LIA), MacConkey agar (MAC), Mueller Hinton agar, and xylose lysine deoxycholate agar were all from commercial sources and prepared according to the manufacturer's instructions. Andrade broth base with 1% filter-sterilized lactose and a Durham tube was used to determine acid and gas from lactose fermentation. Phage agar contained nutrient broth, 20 g; NaCl, 8.5 g; agar-

TABLE 1. Lactose in enteric plating media

Media with lactose	Medium without lactose
Eosin methylene blue	Bismuth sulfite
MacConkey	
<i>Salmonella-Shigella</i>	
Brilliant green	
Hektoen enteric	
Deoxycholate	
Deoxycholate citrate	
Xylose lysine deoxycholate	
Endo agar	
Tergitol 7 agar	
Violet red bile agar	

agar, 20 g; and distilled water, 1,000 ml. All cultures were incubated at $36 \pm 1^\circ\text{C}$.

Lactose fermentation. We define two types of lactose fermentation: strong lactose fermentation (lac^+ strong) and weak lactose fermentation (lac^+ weak). Strains which produced red colonies on MacConkey agar after 24 h of incubation were defined as lac^+ strong. These colonies were indistinguishable visually from typical lactose-fermenting colonies of *E. coli*. Colonies which were colorless or only slightly pink on MAC plates after 24 h but produced acid in lactose broth (Andrade's indicator) within 48 h were defined to be lac^+ weak and thus were similar to colonies of the *Klebsiella-Enterobacter* group in this regard.

Bacterial strains. In 1971 10 lac^+ *Salmonella* from São Paulo, Brazil, were received from Gil Pessoa (Pessoa, 1972). During 1973, 205 *Salmonella* (116 lac^+ , 89 lac^-) were isolated by Trabulsi from inpatients and outpatients at three hospitals in the city of São Paulo. Two lac^+ *Salmonella* were isolated by A. C. Montelli in Botucatu, Brazil, a city about 70 miles from São Paulo. One lac^+ *Salmonella* was isolated by I. Suassuna in Rio de Janeiro, which is 270 miles from São Paulo. For comparison, 18 different lac^+ *Salmonella* sent to CDC from a wide variety of sources were also studied.

Epidemiological information. Information such as clinical source, patient's age, hospital or outpatient, and date was available with the isolates from Trabulsi's clinic (see Table 3). Epidemiological information on the cultures submitted by Pessoa was found in his thesis (1972). No definite epidemiological conclusions about the origin and spread of the lac^+ *Salmonella* in São Paulo could be made with the existing data. The workers of São Paulo concluded that some of the infections were community acquired and others hospital acquired. For these reasons we called the strain "one which is endemic in the São Paulo area."

Typing and antibiograms. Serotyping was done at CDC and São Paulo (6). Phage typing was done with the 13 phages of Callow (3) and with 34 additional phages in our laboratory. All of the isolates were nontypable; however, two cultures, no. 34 and 84, gave very weak reactions with the phages. Antibiograms were done by the method described by Bauer et al. (1) from a single colony and also from some whole cultures.

Dissociation. Cultures were streaked on MAC and lac^+ colonies were picked to stock cultures. After storage on TSI Slants for 10 to 14 days the cultures were restreaked and the number of lac^+ and lac^- colonies was counted. Antibiograms were also done on some of these single colonies.

RESULTS

lac^+ *Salmonella* in São Paulo. The endemic strain was first detected in 1971 at the Instituto Adolfo Lutz, the public health laboratory which serves the state of São Paulo (Pessoa, Thesis, 1972). Before this time, lac^+ strains were only rarely seen. From 1971 to the present, lac^+

Salmonella have been frequently isolated in the São Paulo area (Table 2) and most isolates have been from infants under 1 year of age. The sources of the cultures isolated in Trabulsi's laboratory are shown in Table 3. Many of these isolates were from hospitalized infants and probably represented nosocomial infection; however, community-acquired infections were also apparent.

Characterizations of the São Paulo isolates. The strain had two markers which made it easy to recognize. It was lac^+ strong and had an unusual antibiogram. Colonies on MAC were dark red with bile salts precipitated around them and they were indistinguishable microscopically from typical lac^+ colonies of *E. coli*. Figure 1 shows the antibiogram of the São Paulo strain compared with a wild-type or sensitive *S. typhimurium* isolated from a different source. The São Paulo strain was resistant to penicillin, ampicillin, carbenicillin, cephalothin, streptomycin, kanamycin, chloramphenicol, sulfadiazine, nalidixic acid, and tetracycline; and sensitive to colistin and gentamycin. All isolates had this pattern except as discussed under the section on genetic variation. Biochemically, the strain was a typical *Salmonella* except for lactose fermentation.

Subspeciation. Serological typing at CDC and Brazil indicated that the strain had the following antigens: O: 1,4,12; H(phase 1): i; and H(phase 2): 1,2. Since the strain was resistant to all of the phages in the original and ex-

TABLE 2. Isolation of lac^+ *Salmonella* in two different laboratories in São Paulo

Laboratory	Dates	Total <i>Salmonella</i> isolated	No. of lac^+ <i>Salmonella</i>
Pessoa	May 1971–April 1972	984	313
Trabulsi	Jan 1972–June 1974	239	130

TABLE 3. Distribution of the lac^+ strain^a by source and specimen

Source	Specimen			
	Feces	Urine	Blood	Other, not known
Hospital no. 1	78	10	1	3
Hospital no. 2	12	1	0	0
Outpatients, other hospitals	9	0	0	8

^a All of these isolates were from Trabulsi's laboratory 1972–1974.

FIG. 1. Antimicrobial susceptibility of the Sao Paulo strain (A) compared with a wild-type strain (B).

panded Felix-Callow system, it was nontypable. Thus, the complete designation of the strain would be *lac*⁺ *Salmonella enteritidis* serotype *Typhimurium* var. Copenhagen; phage type: nontypable; resistance pattern: chloramphenicol, sulfadiazine, nalidixic acid, tetracycline, penicillin, ampicillin, carbenicillin, cephalothin, streptomycin, and kanamycin (variety Copenhagen lacks "O antigen" 5, whereas typical *S. typhimurium* have this antigen).

Genetic variation. Although we could easily recognize the epidemic strain because of its combination of unusual markers, there were some isolates which differed slightly from the typical pattern (Table 4). The antibiograms in Table 4 were based on single colonies; however, when whole culture was repeated, the typical pattern was observed. Thus the reported differences (Pessao, 1972) in antimicrobial susceptibility may be due to R-factor segregation in single colonies.

***lac*⁺ *S. typhimurium* var. Copenhagen in Rio de Janeiro and Botucatu.** One of the two Botucatu isolates and the one isolate from Rio were identical to the outbreak strain from São Paulo in antibiogram, serology, and phage type. The other Botucatu isolate was resistant to nalidixic acid, chloramphenicol, and kanamycin (an unusual pattern) which makes us suspect it is also the outbreak strain in which the R factor may have segregated due to single colony picks. Residents of Botucatu frequently

visit São Paulo, but the patients from Rio de Janeiro had no known contact with São Paulo, so we wonder if the strain may also be endemic in other parts of Brazil. This point requires further study.

Isolation and characterization of *lac*⁺ *Salmonella* in general. We studied 18 *lac*⁺ strains of various serotypes which were isolated in the United States. Table 5 shows that 14 of these strains were *lac*⁺ strong and resembled *E. coli*

TABLE 4. Genetic variation in the São Paulo strain (120 isolates studied)

Type of variation	Description of variation	No. of isolates
Serological	Rough, no O antigens	1
	Nonmotile, no H antigens	2
Bacteriophage	Weak reaction with phages	2
Antibiogram ^a	Usual pattern except	
	C ^b	18
	Te	2
	C, Te, K	1
	SD, CF	1
Fermentation	<i>lac</i> ⁻ mixed with <i>lac</i> ⁺ colonies	40

^a Based on single colonies.

^b Abbreviations: C, chloramphenicol; Te, tetracycline; K, kanamycin; SD, sulfadiazine; CF, cephalothin.

TABLE 5. H_2S Production on various media by 14 lac^+ strong and 4 lac^+ weak *Salmonella* isolated from varied sources in the United States

Type of lactose fermentation	<i>Salmonella</i> (%) which produced H_2S on:			
	LIA	TSI agar	Kligler iron agar	PIA
lac^+ strong	100	21	14	100
lac^+ weak	100	100	100	100

on xylose lysine deoxycholate and MAC agar. This type of colony would not normally be picked in screening stool specimens for enteric pathogens because it is indistinguishable from a typical lac^+ *E. coli*. Only four strains were lac^+ weak and produced colonies which look lac^- at 24 h on primary plates. These colonies would probably be regarded as "suspicious" and studied further. Strong lac^+ *Salmonella* produce H_2S abundantly on PIA and LIA; however, on TSI and Kligler iron agar, H_2S production was not visible or seen as only a "trace." On xylose lysine deoxycholate agar, H_2S production was apparent only where the colonies were isolated. Only 21% of the lac^+ strong isolates produced H_2S on TSI (Table 5). Thus most lactose-fermenting *Salmonella* mimic *E. coli* on primary plates containing lactose and on TSI; however, on LIA and PIA, H_2S production is easily detected.

Stool cultures that yield only lactose-positive colonies. We have found a simple screening method to determine if these contain lac^+ *Salmonella*. About 12 lac^+ colonies are touched in succession with a straight wire which is then used to stab the butt then streak the slant of a PIA tube (LIA works also, but H_2S production is not as abundant or clear). After incubation for 24 h, the tube is examined for any blackening. Even if one lac^+ *Salmonella* and 11 lac^+ *E. coli* are added to the same tube, there is still a definite black color. Thus, *Salmonella* produce abundant H_2S in the presence of many more *E. coli*. Blackening in the PIA tube could also be due to *Citrobacter freundii* (or rarely to an H_2S^+ *E. coli* or lac^+ *Proteus*); however, it is a useful screening method to detect lac^+ *Salmonella*. Since about 61% of *Arizona hinshawii* cultures are also lac^+ on primary plates (5), this screening method would also be useful in isolating this pathogen. If blackening is observed in the PIA tube, then additional tests can be done on individual colonies to determine if they are *Salmonella* or *Arizona*. Most of the H_2S^+ , lac^+ isolates that we have detected so far by this method, however, have been *C. freundii*.

DISCUSSION

Since the late 1890's, lactose fermentation has played an important part in the differentiation of enteric pathogen from *E. coli* and other nonpathogens normally found in the human gut. When it was recognized that the typhoid and dysentery bacilli were lac^- , lactose became a standard ingredient in primary plating media. Among the common plating media for enteric bacteria, only bismuth sulfite medium does not contain lactose. Little attention has been paid to lac^+ colonies on primary plates unless the specimen is from a sick infant. In this case lac^+ colonies are usually screened to determine if their O and K antigens are the same as those associated with enteropathogenic *E. coli*. Few microbiologists even consider the possibility of lac^+ *Salmonella* or *Arizona* in their search of primary plates for enteric pathogens.

Colonies of strong lac^+ *Salmonella* even mimic *E. coli* on TSI agar. They have an acid slant and butt with abundant gas; however, 79% of the strains have no blackening due to H_2S production. Since H_2S production is abundant on PIA, acid produced from lactose fermentation probably dissolves the iron sulfide formed in the TSI (13). The " lac^+ weak" *Salmonella* produced abundant H_2S on TSI which is consistent with the above hypothesis.

Although lac^+ *Salmonella* may be rare in general, we have shown that a lac^+ strain can become endemic in a given geographic area. Since May 1971, a lac^+ *S. typhimurium* var. Copenhagen has caused diarrhea, septicemia, and meningitis in infants of Sao Paulo (Pessao, 1972). Between 30 to 50% of all *Salmonella* isolates have been this endemic lac^+ strain. Although certain isolates of the edemic strains have changed in one or more characteristics, the strain is still easy to recognize because of its overall properties (serological, biochemical, antibiogram, and bacteriophage typing). Antibiotic susceptibility patterns are most likely to change because this marker is under great selective pressure when treatment with antibiotics is begun. Our strain is unusual because there were changes in each of the epidemiological markers that we used. This was due to the fact that single colonies were picked from plates, and by chance a variant colony was picked from the population. This type of error is magnified each time single colonies are picked. The results of single colonies were quite different from those based on the whole culture (Table 4).

There are many instances when a diarrheal stool yields no bacteria pathogens. Although there are many possible causes for diarrhea.

lac⁺ *Salmonella* should be considered as one of these. The true incidence of these lac⁺ strains is unknown because most screening methods simply would not detect them. Bismuth sulfite is the only primary plating medium which does not contain lactose, and we suspect that few clinical laboratories use this medium (10). We have described a simple method of screening for lac⁺ *Salmonella* which is successful even if *E. coli* outnumber *Salmonella* 10 to 1. Another useful method is the use of bismuth sulfite agar and LIA. Blackburn and Ellis (2) found this combination particularly useful for detecting lac⁺ *Salmonella* and it would be useful for *Arizona* also.

Information on the true incidence of lac⁺ *Salmonella* is needed. These data should come from many sources widely distributed geographically. We wonder if there are other areas that have an endemic lac⁺ *Salmonella* which is as yet undetected, since at least one potential reservoir has been shown (2). In São Paulo the endemic lac⁺ strain has accounted for almost 50% of all *Salmonella* isolates, thus failure to recognize it would have resulted in considerable error.

ACKNOWLEDGMENTS

We thank I. Saussuna, Gil Pessoa, and A. C. Montelli for their kind gifts of cultures. D. P. Falcão's stay at CDC was funded by Fellowship Medicas 73/803 from Fundacao de Amparo a Pesquisa do Estado de São Paulo.

ADDENDUM IN PROOF

Recently, Le Minor, Coynault, and Pessoa (Ann. Inst. Pasteur 125A:261-285, 1974) described the genetics of the unusual lac⁺ *S. typhimurium* which caused the Brazil outbreak.

LITERATURE CITED

1. Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turk. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45:493-496.
2. Blackburn, B. O., and E. M. Ellis. 1973. Lactose-fermenting *Salmonella* from dried milk and milk-drying plants. *Appl. Microbiol.* 26:672-674.
3. Callow, B. R. 1959. A new phage-typing scheme for *Salmonella typhimurium*. *J. Hyg.* 57:346-359.
4. Easterling, S. B., E. M. Johnson, J. A. Wohlhieter, and L. S. Baron. 1969. Nature of lactose-fermenting *Salmonella* strains obtained from clinical sources. *J. Bacteriol.* 100:35-41.
5. Edwards, P. R., and M. A. Fife. 1961. Lysine-iron agar in the detection of *Arizona* cultures. *Appl. Microbiol.* 9:478-480.
6. Ewing, W. H. 1972. Isolation and Identification of *Salmonella* and *Shigella*. Center for Disease Control, Atlanta, Ga.
7. Gonzales, A. B. 1966. Lactose-fermenting *Salmonella*. *J. Bacteriol.* 91:1661-1662.
8. Kauffman, F. 1966. The bacteriology of Enterobacteriaceae. Williams and Wilkins. Baltimore.
9. Kunz, L. J., and W. H. Ewing. 1965. Laboratory infection with lactose-fermenting strain of *Salmonella typhi*. *J. Bacteriol.* 89:1629.
10. McCoy, J. H., and G. E. Spain. 1969. Bismuth Sulphite media in the isolation of salmonellae, p. 17-27. In D. A. Shapton and G. W. Gould (ed.), Isolation methods for microbiologists. Academic Press Inc. New York.
11. Saphra, I., and E. Seligman. 1947. Coliforms with complete *Salmonella* antigens or lactose-fermenting salmonellae. *J. Bacteriol.* 54:270-271.
12. Twort, F. W. 1907. The fermentation of glucosides by bacteria of the typhoid coli group and the acquisition of new fermenting powers by *Bacillus dysenteriae* and other micro-organisms. *Roy. Soc. London Proc.* 79B:329-336.
13. Veron, M., and F. Gasser. 1963. Sur la detection de l'hydrogene sulfure produit par certaines Enterobacteriacees dans les milieux dits de diagnostic paride. *Ann. Inst. Pasteur* 105:524-534.