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Occurrence of Gram-Positive Organisms Possessing Characteristics Similar to Those of *Salmonella* and the Practical Problem of Rapid and Definitive *Salmonella* Identification

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ABSTRACT

SILLIKER, J. H. (St. James Hospital, Chicago Heights, Ill.), R. H. DEIBEL, AND J. Y. CHIU. Occurrence of gram-positive organisms possessing characteristics similar to those of *Salmonella* and the practical problem of rapid and definitive *Salmonella* identification. Appl. Microbiol. **12**:395-399. 1964.—The occurrence and incidence of gram-positive organisms which possess some biochemical and serological characteristics in common with salmonellae are described. These organisms were encountered as contaminants in various food products as well as in feces. Their growth in various *Salmonella* enrichment media, typical *Salmonella*-like growth on differential selective media, biochemical similarities to *Salmonella*, and agglutination in *Salmonella* polyvalent O antiserum serve as a source of possible confusion in analyses for *Salmonella*. Although a definitive identification was not performed, preliminary results indicate that these bacteria closely resemble members of the genus *Brevibacterium*. No sanitary significance could be associated with these bacteria, because they were never found to coexist with *Salmonella*. The occurrence of these bacteria illustrates the pitfalls of a cursory examination for *Salmonella*. On the premise that ultimate identification of *Salmonella* rests upon serological procedures, a rapid and definitive identification of suspect *Salmonella* isolates is proposed, based upon reactions with polyvalent group O, group O, and Spicer-Edwards H antisera.

The detection of salmonellae generally involves preliminary culture in an enrichment broth, followed by subculture in selective or differential media, or in both media. The selective and differential nature of these media is based upon, and is a reflection of, the metabolic similarities among the various members of the genus *Salmonella*. Ordinarily, secondary biochemical screening of selected colony isolates is effected through subculture in one of a variety of multiple-sugar agar media, such as Triple Sugar Iron Agar (TSI). Subsequently, at the discretion of the analyst, further biochemical screening may be conducted. It should be emphasized, however, that, regardless of its apparent conformity with the biochemical characteristics of the genus *Salmonella*, the final identification of a suspected isolate rests upon antigenic analysis.

This study relates to the frequent occurrence of a group of gram-positive bacteria (tentatively designated as members of the genus *Brevibacterium*) which were isolated from a variety of sources in the course of routine analyses for *Salmonella*. These gram-positive bacteria were capable of growing in selenite broth, and produced colonies indistinguishable from those of typical *Salmonella* on a variety of differential media. The majority of these strains possessed physiological characteristics which were similar to those of typical salmonellae, and most strains agglutinated in *Salmonella* polyvalent O antisera. These peculiar and possibly confusing physiological and serological characteristics prompted a more extensive study of these microorganisms. The results of this investigation, coupled with the recognized necessity for rapid and definitive identification of salmonellae in food products, have given rise to a procedure which, in our hands, has allowed rapid and definitive identification of *Salmonella*.

MATERIALS AND METHODS

Enrichment and isolation procedures. The organisms were encountered in connection with routine examination of food and clinical specimens (feces) for salmonellae. Three different enrichment procedures were used for food samples: (i) direct inoculation of cysteine-selenite broth (North and Bartram, 1953), (ii) inoculation of lactose broth followed by subculture in cysteine-selenite broth (North, 1961), and (iii) direct enrichment in selenite broth containing 10% sterile feces (Silliker, Deibel, and Fagan, 1964). Samples (25 g) of food were inoculated into 650 ml of enrichment broth. Brilliant Green Agar (BGA) and Salmonella Shigella (SS) Agar were used as recovery media. *Salmonella*-like colonies developing on BGA or SS agar, or both, were subcultured on TSI agar slants. Specimens of feces were enriched in cysteine-selenite broth (10 ml per tube).

Serological identification. Salmonella O (Difco) and H (Spicer-Edwards) antisera were used. O agglutinations were carried out on saline suspensions prepared from TSI slants. H agglutinations were conducted with Brain Heart Infusion broth (BHI) subcultures from the TSI slants, with the use of equal volumes of 0.6% formalinized saline. The BHI cultures were incubated 4 to 5 hr prior to the addition of the formalin.

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Serotyping of *Salmonella* cultures isolated during the course of these studies was carried out by the Illinois Public Health Laboratories.

Antibody absorption studies. Heavy suspensions of cells from TSI slants were washed two times in physiological saline. The packed cells were suspended in polyvalent O antiserum and incubated for 2 hr at 37 C. The preparations were then centrifuged, and the clear supernatant serum was used for slide agglutination tests.

Physiological characteristics. These tests were performed according to the procedures described in the *Manual of Methods for Pure Culture Study of Bacteria* (1946). Unless otherwise indicated, all cultures were incubated at 37 C.

Antibiotic sensitivity studies. Sensitivities were determined with the use of Multidisks (Consolidated Laboratories, Inc., Chicago Heights, Ill.) on heavily streaked blood agar plates. The results were read after 18 hr of incubation.

Gram stain. The Hucker modification of the Gram stain (*Manual of Methods for Pure Culture Study of Bacteria*, 1946) was applied to heat-fixed films with the use of commercially prepared reagents (Hartman-Leddon Co., Philadelphia, Pa.).

RESULTS

The organisms described in this paper were encountered during the routine examination of food and clinical specimens. Each of the cultures grew on SS agar and BGA, producing small-to-moderate sized colorless colonies indistinguishable from those produced by salmonellae. Subsequently, the various strains were cultured in MacConkey, Desoxycholate, and Eosin-Methylene Blue agar media. Uniformly, the strains evidenced growth on these media which was indistinguishable from that of typical salmonellae. When subcultured onto TSI, the isolates characteristically produced alkaline slants and acid butts, with or without gas production, depending upon the individual strain.

Upon holding certain of these cultures at ambient room temperature for several days, it was noted that some of the strains formed a buff to orange pigment. Gram stains revealed that these organisms were not gram-negative rods but were, indeed, gram-positive, short pleomorphic rods.

Certain food samples showed a rather high incidence of these bacteria (Table 1). It should be emphasized that these organisms were encountered during the examination of relatively large samples of food, and their absolute number in the food samples was, in most cases, quite low. Although the majority of isolations were made from dried food products, the primary observation regarding the occurrence of these organisms was made in the examination of restaurant-prepared ham, tuna, egg, and chicken salads used to make sandwiches. Analysis of the ingredients of these salads showed that fresh celery was the source of the organisms. Subsequently, these gram-positive bacteria were detected in each of ten fresh celery samples obtained from a local supermarket. On four occasions, the organisms were detected in feces.

TABLE 1. *Incidence in foods of gram-positive organisms which may be confused with Salmonella*

Sample	No. of samples tested	Incidence of described gram-positive organisms
Animal feeds	8	6
Dried egg albumen	158	17
Dried egg yolk	190	9
Dried whole egg	161	38
Cream-filled pastries	48	8

In the examination of food products containing these bacteria, large numbers of colonies developed on SS agar and BGA. Growth studies indicated that these organisms actually grow in selenite broth. These organisms were never encountered in food samples from which salmonellae were likewise isolated.

Morphology. Gram stains of young (4 to 8 hr) BHI broth cultures revealed the presence of weakly gram-positive, rod-shaped bacteria; with longer incubation (1 to 6 days), the gram-positive nature of the cultures became more pronounced. TSI cultures (24 and 48 hr) are strongly gram-positive. The fully developed cultures, either from slants or from broth, show a heterogeneity of cell forms, with rod-shaped organisms predominating. However, within the same pure culture, coccoid forms do occur. Hanging drop preparations from broth cultures indicate that these organisms are actively motile and, in such preparations, the coccoid morphology is only rarely seen.

Serological reactions. These organisms almost invariably show a prompt agglutination in polyvalent O *Salmonella* antiserum, but fail to react with any of the group-specific O sera. None of the strains agglutinates in Spicer-Edwards H antisera.

Antibody absorption studies were carried out with five of the strains. Cultures 2, 3, 4, and 6 showed positive agglutination in a final dilution of 1:8 with polyvalent antiserum; culture 7 agglutinated at a 1:32 dilution. After homologous absorption, the serum no longer agglutinated with the absorbing strain. Tests carried out with heterologous strains indicated that strains 2, 3, and 4 were antigenically related, in that the serum absorbed with one of them no longer reacted with the other two strains. Strains 6 and 7 appeared to be antigenically unrelated to strains 2, 3, and 4. Absorption of the serum with strain 6 reduced its antibody titer for strain 7 from 1:32 to 1:8. Absorption of the serum with strain 7 reduced its antibody titer for strain 6 from 1:8 to 1:2. Thus, strains 6 and 7 are antigenically related but not identical.

The polyvalent antiserum reacted in titers from 1:16 to 1:64 against five known salmonellae. Sera absorbed with the gram-positive bacteria did not reduce the antibody titer of polyvalent serum for any of the five salmonellae. These studies indicate that the gram-positive

strains react with naturally occurring antibodies in polyvalent O antiserum. There is no indication that the gram-positive organisms share common antigens with the salmonellae.

Physiological characteristics. Seven of the strains isolated in this study were chosen for additional physiological characterization. These strains were all isolated from various dried egg products. Strains 2, 3, and 4 produced an increased quantity of CO_2 from the fermentation of glucose when tested by the modified Eldridge technique of Williams and Campbell (1951). However, in agar shake cultures, all strains evidenced abundant gas production. All strains produced an acid butt and an alkaline slant without H_2S formation; however, we recently isolated an H_2S-positive strain which, on the basis of its Gram reaction and growth characteristics, is similar to the organisms described in Table 2.

All the strains failed to grow at 45 C, and all grew at 12 C (Table 2). Four of the seven strains formed indole, and by this characteristic they could be differentiated biochemically from typical salmonellae. One of the strains (strain 3) possessed a unique set of characteristics, in that it failed to hydrolyze urea or produce indole, decarboxylated lysine, formed acid and gas in dulcitol broth, and reacted in TSI agar in a manner similar to a typical H_2S-negative *Salmonella*. Strain 3 also agglutinated in polyvalent O antiserum.

As a group, these organisms were salt-tolerant in that all grew in media containing 5% sodium chloride. Three of the strains tolerated 7% sodium chloride.

TABLE 2. *Physiological characteristics of organisms, encountered in food products, which may be confused with Salmonella*

Characteristic	Result (7 strains)
Catalase	7+
Growth relationship to oxygen	Facultative
Indole production	4+, 3−
Lysine decarboxylation	2+, 5−
Nitrate reduction	+
Motility	+
Pigment formation	3−, 4+ (pale yellow)
Hydrolysis	
Gelatin	3+, 4−
Urea	−
Growth	
12 C	+
45 C	−
5% NaCl	+
7% NaCl	3+, 4−
9% NaCl	−
Fermentation*	
Raffinose	2+, 5−
Salicin	3+, 4−
Glycerol	1+, 6−
Dulcitol	4+, 3−

* All strains fermented xylose, arabinose, fructose, galactose, lactose, maltose, mannitol, glucose, sorbitol, and trehalose. None fermented sucrose or melizitose.

The lactose fermentation was delayed; however, after 48 hr of incubation, definitive acid production was observed with all strains in lactose broth. Undoubtedly, the acid produced from this substrate when the organism is cultured in differential selective media is not sufficient to effect differentiation, even upon prolonged incubation. In addition, TSI cultures of the organisms fail to evidence an acid slant upon prolonged incubation.

The organisms showed a common pattern with respect to antimicrobials; most of the strains were sensitive to nitrofurazone, nitrofurantoin, chloramphenicol, furazolidone, polymyxin, neomycin, dihydrostreptomycin, triple sulfa, and tetracycline. They were resistant to penicillin, novobiocin, erythromycin, and oleandomycin.

Although sensitivity tests indicated that these bacteria were readily sensitive to triple sulfa, no indication of overt sensitivity to sulfadiazine was demonstrable when growth was tested on Brilliant Green-Sulfadiazine Agar (BBL). Certain of the strains did evidence a diminished growth response on this medium (as compared with growth on BGA); however, this response would only serve to confuse the analyst, in that the alkaline reaction of the medium would indicate the presence of suspicious colonies but their visual detection would be hampered. Between 24 and 48 hr of incubation, the strains developed typical *Salmonella*-like colonies on the sulfadiazine-containing BGA.

The strains could be divided into two groups of the basis of acid production in 1% glucose medium. One group (strains 1, 5, 6, and 7) fermented to a final pH value of 4.7 to 4.8. The other group (strains 2, 3, and 4) produced less acid as indicated by a higher final pH value (6.1 to 6.4). A correlation was noted in that strains producing more acid failed to produce indole or hydrolyze gelatin. In addition, these strains formed a pale-yellow pigment and were decidedly less salt-resistant. As previously indicated, our initial recognition of *Salmonella*-like, gram-positive organisms involved strains which produced a buff to orange pigment. We also encountered strains (from feces) which produce a red *Serratia*-like pigment.

Evidence of growth in enrichment media. Two of the strains (1 and 4) were grown in BHI broth. Dilutions of these cultures were inoculated into 250 ml of lactose broth, selenite broth, and selenite broth containing 10% sterile feces. A duplicate set of these enrichment media (except selenite broth) was supplemented with 10% dried egg yolk. After 24 hr of incubation, the selenite-feces cultures were streaked onto BGA, and the lactose pre-enriched cultures were subcultured in cysteine-selenite broth (10-ml inoculum into 100 ml). These selenite broth cultures were then streaked onto BGA after 24 hr of incubation. Lactose-nonfermenting organisms developing on BGA were subcultured in TSI agar. The numbers of lactose-nonfermenting organisms in the enrichment media were determined by quantitative surface plating on BGA.

The results (Table 3) clearly show that both strains multiply in the enrichment media. The TSI subcultures

uniformly showed alkaline slants and acid butts with gas production, and were each H_2S-negative. Saline suspensions of the growth on the slants reacted promptly with polyvalent O *Salmonella* antiserum, but they reacted negatively in the specific O group antisera and in Spicer-Edwards antisera. These results afford an explanation of the previously mentioned large numbers of these organisms occurring on SS and BGA plates streaked from enrichment cultures of foods containing them.

Discussion

The taxonomic position of these gram-positive, catalase-positive, motile organisms is uncertain. It would appear, however, that these bacteria correspond most closely to the genus *Brevibacterium* (Breed, Murray, and Smith, 1957). These bacteria have been reported in dairy products, soil, salt- and fresh-water, and in a great variety of various substances undergoing decomposition. This is not inconsistent with the sources of these organisms in the present study.

It seems unlikely that these bacteria have any sanitary significance. They were never found to coexist with salmonellae, and were isolated from egg samples containing <0.03 coliforms per gram.

Obviously, the growth patterns and biochemical reactions of these gram-positive organisms cause them to be easily confused with salmonellae. Although, generally speaking, individual strains will differ from typical salmonellae in one or another of the biochemical reactions usually employed, mention has previously been made of strain 3 which reacted as a typical *Salmonella* in the urea, indole, lysine, and dulcitol tests. Although biochemical tests provide a means of screening out certain organisms which are not salmonellae, one may question the practical value derived from exhaustive biochemical studies on individual isolates, since membership in the genus *Salmonella* is ultimately dependent upon antigenic analysis.

TABLE 3. *Growth of gram-positive Salmonella-like organisms in various Salmonella enrichment media*

Enrichment medium	Viable count per ml of enrichment broth[a]	
	Strain 1[b]	Strain 4[c]
LS[d]	52,600,000	1,200,000
LS[d] + egg yolk (10%)	Lost	8,000,000
SF[e]	1,400,000	1,000,000
SF[e] + egg yolk (10%)	17,000,000	15,000,000
S[f]	1,030,000	890,000

[a] Counts were determined by surface plating on BGA after 24-hr incubation of enrichment media.

[b] Initial viable count per ml was 55,000 organisms, except in S (250).

[c] Initial viable count per milliliter was 93,000 organisms, except in S (540).

[d] Culture pre-enriched 24 hr in lactose broth, followed by transfer of 10 ml into 100 ml of selenite-cysteine broth.

[e] Selenite-cysteine broth containing 10% sterile feces (Silliker et al., 1964).

[f] Selenite-cysteine broth.

Since most of these organisms agglutinate rapidly in *Salmonella* polyvalent O antiserum, it is apparent that reliance upon this reaction for identification is fraught with serious difficulties. Reliance upon group-specific O antisera may likewise lead to spurious conclusions. Cross agglutination with paracolon bacteria occurs (Edwards and Ewing, 1962), thus leading to false-positive results. On the other hand, we have frequently encountered salmonellae which did not agglutinate in commercially available group-specific O antisera.

Some time ago, this laboratory started using the commercially available Spicer-Edwards antisera for *Salmonella* H agglutination tests. These antisera have been used in conjunction with polyvalent O and group-specific O antisera. Since that time, many hundreds of clinical specimens and food samples have been analyzed for the presence of salmonellae. All isolates agglutinating in the Spicer-Edwards antisera have been submitted to the Illinois Public Health Laboratories for serotyping. To date, every strain that has reacted with Spicer-Edwards antiserum has ultimately been classified as a *Salmonella* by the State Laboratory. This includes a large number of isolates from foods and 25 isolates from feces. Among these cultures were several which did not agglutinate in group-specific O antisera (i.e., *S. cerro* and *S. sieburg*), but these organisms did react positively and specifically with the Spicer-Edwards sera. Further, a number of isolates which agglutinated in group-specific O antisera but not in the Spicer-Edwards H antisera were submitted to the State Laboratory, and these were uniformly reported as not members of the genus *Salmonella*.

TABLE 4. *Comparison of recommended serological procedures employed in various methods for identification of Salmonella in food products*

Source of method	Food product examined	Recommended serological procedure
Institute of American Poultry Industries (Anonymous, 1960)	Eggs	Polyvalent O
American Public Health Association (1958)	Foods, general	Polyvalent O Group O
Canadian Food and Drug Directorate*	Foods, general	Polyvalent O Group O
Central Institute for Nutrition and Food Research (Netherlands) (Mossel and Twart, 1960)	Foods, general	Polyvalent O
Bulletin Institute American Poultry Industry (Ballas, 1960)	Eggs	Polyvalent O Group O
U.S. Department of Agriculture (Banwart, 1960)	Eggs	Polyvalent O
U.S. Army Quartermaster Corps (1960)	Meringue powder	(Biochemical only)
U.S. Department of Health, Education and Welfare (Galton et al., 1964)	Foods, general	Polyvalent O Group O Spicer-Edwards H

* Thatcher, *personal communication*. Method based on procedure described by Montford and Thatcher (1961).

In the control laboratory, whether clinical or food, the rapid and definitive identification of salmonellae is of considerable urgency. In the clinical laboratory, the manner of reaching this decision is generally left to the discretion of the bacteriologist. With respect to food products, on the other hand, several "standard" or "recommended" procedures have been published. Table 4 summarizes the serological methods recommended for *Salmonella* identification in some of the more important standard texts. It will be noted that in five of the eight methods listed, only polyvalent O agglutination is specified, and in one procedure identification is based solely upon biochemical tests. Only in the recent publication of Galton, Boring, and Martin (1964) is the utility of H agglutination emphasized. Indeed, it is of interest to note that the description of *Salmonella* in *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957) states: "Any organism showing the above-mentioned characters [biochemical] should be verified as a member of the genus *Salmonella* by antigenic analysis. For most practical purposes the employment of polyvalent or group antisera will suffice. Exact antigenic characterization and bacteriophage typing for epidemiological purposes is the task of *Salmonella* centers." Since the errors associated with this proposition are on the side of "safety," it is easy to rationalize this approach to the problem, and thus reduce the manipulations associated with a more definitive identification.

It has been our experience that the errors inherent in reliance upon polyvalent and group-specific O agglutinations can be avoided by use of commercially available Spicer-Edwards H antiserum. In our laboratory, TSI slants inoculated the previous day from differential media (BGA and SS) are examined the following morning. Growth from cultures showing *Salmonella*-like reactions is suspended in physiological saline and tested in polyvalent and group-specific O antisera. Cultures agglutinating in polyvalent antiserum (whether or not positive in group) are inoculated into BHI broth. After 4 to 5 hr of incubation, the broth is sufficiently turbid to be used for H agglutinations, and it is diluted with an equal volume of formalinized saline. For control purposes, cultures agglutinating in polyvalent O and in Spicer-Edwards H antisera are classified as salmonellae. Subcultures are sent to the State Public Health Laboratories for final serotyping. For confirmatory purposes only, the following biochemical tests are conducted on positive cultures: urease, lysine decarboxylase, indole and dulcitol, and lactose broths.

On the basis of our experience, it is suggested that the control laboratory, unequipped for complete antigenic analysis, can confidently classify an organism as a member of the genus *Salmonella* on the basis of the serological tests described above. Certainly, omission of H agglutination tests may lead to serious errors in classification.

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