J. Milk Food Technol. Vol. 38, No. 8, Pages 453-456 (August, 1975)

Comparative Validity of Members of the Total Coliform and Fecal Coliform Groups for Indicating the Presence of *Salmonella* in the Eastern Oyster, *Crassostrea virginica*

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(Received for publication December 11, 1974)

ABSTRACT

During a 24-month survey, 539 samples each of the Eastern oyster, *Crassostrea virginica*, and the overlying water were collected to determine the relation of most probable number (MPN) of the total and fecal coliform groups in shellfish and water to the presence of *Salmonella* in the shellfish themselves. Occurrence of *Salmonella* in the shellfish more closely paralleled a progressive increase in the fecal coliform MPN as compared to the total coliform MPN in the water and shellfish meat. The percentage of *Salmonella*-positive shellfish samples was somewhat higher in oysters harvested from waters conforming to the present bacteriological approved growing area standard of $\leq$70 total coliforms per 100 ml water as compared to these same waters meeting a recently proposed fecal coliform standard of $\leq$14 organisms per 100 ml. In no instance was *Salmonella* detected in oysters from growing areas officially approved for harvesting on the basis of both a bacteriological and sanitary survey. Of a variety of enrichment broths and plating media used for recovery of *Salmonella* from oysters, direct enrichment in tetrathionate broth with added brilliant green followed by streaking on bismuth sulfite agar was the most productive combination of media for recovering a large variety of *Salmonella* serotypes.

The routine analysis of shellfish-growing waters for the presence of pathogens remains an impractical task for state and federal officials charged with guaranteeing the quality of shellfish. The concept of utilizing coliforms to infer the presence of pathogens is based on work of Excherich (*5*) who, in 1885, identified *Bacillus coli* as being part of the natural intestinal microflora in warm-blooded animals. During the 90 years that followed, published reports appeared both supporting (*9, 10, 13*) and criticizing (*6, 7, 11*) the use of coliforms to infer the presence of such pathogens as *Salmonella, Shigella, Vibrio, Pasteurella, Leptospira,* and enteroviruses in fresh waters.

The National Shellfish Sanitation Program (NSSP), a cooperative organization of representatives from the shellfish-producing states, private industry, and the Food and Drug Administration (FDA), has traditionally utilized the total coliform group to assess the bacteriological quality and safety of shellfish-growing waters. At the 1974 NSSP National Workshop (Proceedings of the Eighth NSSP Workshop, *in press*) a proposal was made to substitute a fecal coliform standard for the total coliform standard. Anticipating such a proposal, it was decided to determine the comparative validity of members of the total coliform and fecal coliform groups for indicating the presence of *Salmonella* in shellfish. Results of this survey are detailed in this paper.

MATERIAL AND METHODS

Sampling areas and collection of samples

The Eastern oyster, *Crassostrea virginica*, was chosen for this study because more is known and has been published about the bacteriology and physiology of this species of shellfish (*8*), it is the most economically important shellfish species in this country, and it is readily available. Water and shellfish samples were collected by the Northeast Technical Services Unit (NETSU) located at Davisville, Rhode Island, and the Gulf Coast Technical Services Unit (GCTSU) located at Dauphin Island, Alabama, both of which are field facilities of FDA's Shellfish Sanitation Branch. Each field unit routinely collected up to eight shellfish samples monthly from an area approved for harvesting and a similar number of samples from an adjacent, conditionally approved, restricted, or prohibited area. These sampling areas were all classified in accordance with the specification of the NSSP Manual of Operations (*17*). From each shellfish sampling site, a sample of surface water immediately overlying the shellfish bed was collected in sterile glass containers and determinations of salinity (conductometric method), temperature, and content of total coliforms and fecal coliforms were made. Water samples were iced at or below 10 C during transportation to the laboratory in accordance with American Public Health Association (APHA) recommendations (*1*). In no instance did more than 30 h elapse between collection and initiation of analysis of the water samples.

Rather than restricting the survey to a limited number of sampling sites, it was decided that sampling from a varied and extensive geographical area would be more compatible with the objectives of this study. NETSU was responsible for collecting samples from estuaries in Rhode Island, Connecticut, New York, New Jersey, and Maryland, whereas GCTSU collected samples from South Carolina, Florida, Alabama, Mississippi, and Louisiana. Water depth over the actual sampling areas ranged from 1 to 40 ft. Salinities ranged from <1.0 to 30.0 parts per thousand, and water temperatures ranged from 0.8 to 18.8 C during November-April and from 16.6 to 30.5 C during May-October. The survey was initiated on July 1, 1972, and ended on June 30, 1974.

From each sampling site, enough shellstock was collected to yield a sample of 500 g of shellfish meat and liquor. Additionally, a surface water sample of 150 to 200 ml was collected at each sampling site.

Coliform analysis

Before being shucked, the shellstock was scrubbed thoroughly to remove loose material and debris from the shell surface. Shellfish meats were tared into sterile 1-pint polyethylene jars. To effect an even distribution of the analytical work load, all water samples and those shellfish samples from approved growing areas were analyzed by the field units, and all shellfish samples from non-approved areas were sent to the Division of Microbiology laboratories in Washington, D.C. Polyethylene jars of shellfish samples were placed in insulated mailing containers (Polyfoam Packers Corp., Chicago, Ill.), packed with Polar Ice refrigerant (Divajex, Santa Ana, Calif.), and mailed air express. Preliminary experiments demonstrated the capacity of this system to maintain the shellfish at 4 to 7 C for 24 h.

Two hundred grams of the shellfish sample were used for determining the total coliform and fecal coliform content and the remaining 300 g were reserved for the analysis of *Salmonella*. The most probable number (MPN) technique was used to quantitate levels of total coliforms and fecal coliforms in the shellfish meats and waters. Dilutions of water samples were directly inoculated into lauryl sulfate tryptose (LST) broth (Difco) and 200-g oyster samples were each blended in a high-speed blender with an equal weight of phosphate buffer dilution water for 60-120 sec at approximately 14,000 rev/min. Appropriate dilutions were made of the oyster homogenate and inoculated into LST broth. Tubes of LST broth, inoculated with dilutions of the water or shellfish samples, were incubated at 35 ± 0.5 C and at 24- and 48-h intervals. Sterile applicator sticks were used to make transfers from gassing tubes of LST broth to tubes containing brilliant green lactose bile (BGLB) broth (Difco). Cultures producing gas in BGLB broth after 48 h of incubation at 35 C were classified as total coliforms. Fecal coliform confirmation was made by transferring all tubes of LST broth prositive for gas after 24 to 18 h of incubation at 35 ± 0.5 C to EC medium. The EC medium was incubated in a water bath at 44.5 ± 0.2 C for 24 h. All cultures producing gas in EC medium under these conditions were classified as fecal coliforms.

Salmonella analysis

Both the lactose pre-enrichment and direct selective enrichment procedures of the *Bacteriological Analytical Manual* (BAM) (*18*) were used in the *Salmonella* analysis of the oysters. The 300-g reserves of the shellfish samples were divided into three 100-g portions, each of which was blended in a high-speed blender for 60-120 sec at 14,000 rev/min with 150 ml of 35 C-tempered lactose, selenite cystine (SC), or tetrathionate (TT) broth containing 10 mg of brilliant green dye per liter. The homogenates were poured into flasks containing the remainder (750 ml) of the 900 ml of the respective broths. After adjusting the pH of the broth mixtures to 6.8 ± 0.2, flasks were incubated in a walk-in incubator at 35 C. After 24 h of incubation, 100 ml of lactose pre-enrichment mixture was subcultured to flasks containing 900 ml of fresh, 35 C-tempered SC or TT broth. After 24 and 48 h of incubation, the contents of the flasks containing the selective enrichment broths were streaked with a 3-mm loop to plates of brilliant green, bismuth sulfite (BS) (Difco), and *Salmonella-Shigella* agar. All plates were incubated and examined after a 24-h incubation period. The BS agar plates were examined after 24 h, but were incubated an additional 24 h at which time they were re-examined. When present, at least two colonies suspicious for *Salmonella* were picked per plate in tandem to triple sugar iron agar and lysine iron agar. Cultures giving reactions typical for *Salmonella* were screened biochemically (*4*). The somatic and serological grouping, as described by Edwards (*3*), was followed by definitive serotyping (*4*).

RESULTS AND DISCUSSION

The relationship between the levels of total coliforms and fecal coliforms in water to the presence of *Salmonella* in the shellfish harvested from these waters was established (Table 1). The present growing-area standard allows the harvesting of shellfish from waters having a total coliform MPN of ≤70 organisms per 100 ml of water (*17*). The proposed standard would allow ≤14 fecal coliform organisms per 100 ml of approved shellfish-growing water (Proceedings of the Eighth NSSP Workshop, *in press*). Except for these two categories, the categories of water MPN in Table 1 are arbitrary. In the shellfish harvested from waters within allowable limits of total coliform and fecal coliform levels, 7.5 and 2.4%, respectively, of the samples contained *Salmonella*. There was no level of fecal coliforms in the water below which *Salmonella* could not be recovered from the shellfish meats. One *Salmonella*-positive shellfish sample was harvested from water having a total coliform and fecal coliform MPN of 11 and ≤1.8, respectively. However, all *Salmonella*-positive shellfish samples were obtained from waters which did not meet approved growing-area criteria. Waters which may be intermittently polluted will at times meet the bacteriological standards but fail to meet the sanitary survey criteria needed to be

TABLE 1. *Correlation of* Salmonella *in oysters to the total coliform and fecal coliform densities of the overlying waters*

Indicator organism	Indicator organism density in overlying waters per 100 ml	Number of oyster samples examined	*Salmonella*-Positive	
			Number	Percent
Total coliform	0-70	199	15	7.5
	71-200	105	13	12.4
	201-1,000	144	15	10.4
	>1,000	91	17	18.7
	Total	539	60	
	Overall average			11.1
Fecal coliform	0-14	255	6	2.4
	15-70	118	6	5.1
	71-200	59	13	22.0
	201-1,000	66	18	27.3
	>1,000	41	17	41.5
	Total	539	60	
	Overall average			11.1

classified as an approved shellfish-growing area. Demonstration of increased recovery of *Salmonella* from shellfish harvested from waters with a progressive increase in total coliform MPN was not apparent. With the fecal coliform group, however, there was an increase in the number of *Salmonella*-positive oyster samples with a progressive increase in the MPN of the overlying waters.

In addition to a growing-area standard, a market quality standard is used to indicate the degree of process and handling abuse of the product after harvesting. Shellfish having a fecal coliform MNP of ≤230 per 100 g and a total plate count not in excess of 500,000 organisms/g of shellfish meat are considered to be of acceptable quality at the wholesale market (*17*). With the exception of ths category of fecal coliform MPN, the range of MPN categories shown in Table 2 is arbitrary.

TABLE 2. *Correlation of* Salmonella *in oysters to the total coliform and fecal coliform densities in oysters*

Indicator organism	Indicator organism density in oyster meats per 100 g	Number of oyster samples examined	*Salmonella*-Positive	
			Number	Percent
Total coliform	0-100	57	0	0
	101-1,000	119	1	0.8
	1,001-10,000	121	5	4.1
	10,001-100,000	113	26	23.0
	>100,000	129	28	21.7
	Total	539	60	
	Overall average			11.1
Fecal coliform	0-230	268	5	1.9
	231-1,000	84	10	11.9
	1,001-10,000	97	19	19.6
	>10,000	90	26	28.9
	Total	539	60	
	Overall average			11.1

The isolation of *Salmonella* from the shellfish meat was not consistent with the level of total coliforms in the shellfish meat. However, a direct relationship was demonstrated in the shellfish meats between the presence of *Salmonella* and levels of fecal coliforms.

The relative efficacy of the enrichment broths and plating media in the recovery of *Salmonella* from oysters is shown in Table 3. Most of the procedures in the BAM

TABLE 3. Salmonella*-positive oyster samples listed by pre-enrichment, selective enrichment, and plating media*

Pre-enrichment and selective enrichment media	Number of samples positive			
	Brilliant green	Bismuth sulfite	*Salmonella-Shigella*	Total
Lactose pre-enriched, selenite cystine	11 (18.3)[a]	15 (25.0)	7 (11.7)	33
Lactose pre-enriched, tetrathionate	21 (35.0)	39 (65.0)	18 (30.0)	78
Selenite cystine	18 (30.0)	21 (35.0)	9 (15.0)	48
Tetrathionate	29 (48.3)	50 (83.3)	21 (35.0)	100
Total	79	125	55	259

[a]Numbers in parentheses percentage of the total number of 60 *Salmonella*-positive oyster samples.

(*18*) used in the analysis of foods for the presence of *Salmonella* require pre-enrichment of the sample in lactose broth, a practice based on the finding of North (*15*). Hence, the practice of pre-enriching most processed foods in the analysis for *Salmonella* has been accepted as a standard procedure by FDA. In a raw product such as oysters, lactose-fermenting organisms may be expected to successfully compete and over-grow the *Salmonella* organisms in certain instances. To maximize the possibility of *Salmonella* recovery from oysters, both the lactose pre-enrichment and the direct selective enrichment procedures were used. The most productive combination of enrichment broths and plating media was direct enrichment in TT broth followed by streaking on plates of BS agar. Of the 60 samples positive for *Salmonella*, 50 (83.3%) were positive by this combination.

TABLE 4. *Relative frequency of isolation of* Salmonella *serotypes from oysters*

Serotype	Somatic group	Number of samples positive for this serotype
S. derby	B	21
S. infantis	C_1	19
S. newport	C_2	12
S. hartford	C_1	4
S. heidelberg	B	3
S. manhattan	C_2	3
S. binza	E_2	2
S. montevideo	C_1	2
S. muenchen	C_2	2
S. typhimurium	B	2
S. worthington	G	2
S. gaminara	I	1
S. give	E_1	1
S. kentucky	C_2	1
S. mississippi	G	1
S. rubislaw	F	1
S. sachsenwald	R	1

Table 4 shows the relative frequency of isolation of 17 *Salmonella* serotypes from oysters. Although a large variety of serotypes was isolated, *Salmonella derby, Salmonella infantis,* and *Salmonella newport* accounted for most of the isolates. No member of the closely-related genus, *Arizona,* was isolated.

Salmonella was chosen as representative of pathogenic flora since the methodology for its detection is established and relatively simple. Other pathogens such as *Vibrio parahaemolyticus* and enteroviruses may constitute a part of the pathogen load in shellfish and should, therefore, lead to caution in the interpretation of data from this study. Results of this survey demonstrate the greater validity of the fecal coliform group as compared to the total coliform group for indicating the presence of 17 serotypes of *Salmonella* in the Eastern oyster, *Crassostrea virginica.* The percentage of *Salmonella*-positive shellfish samples was somewhat higher in oysters harvested from waters conforming with the present approved growing-area standard of ≤70 organisms per 100 ml of water as compared to these same waters meeting the recently proposed fecal coliform standard of ≤14 organisms per 100 ml (Table 1). Furthermore, a more evident correlation was seen between the presence of *Salmonella* in shellfish and the levels of fecal coliform in these shellfish compared to the

levels of total coliforms (Table 2). The findings of this study would support the use of the fecal coliform standard for judging the safety of shellfish-growing waters. The continued introduction of improved techniques for detecting bacterial pathogens (*2, 12, 14, 16*) as well as development of practical methods for detecting viral pathogens in sea water may ultimately make the routine search for the specific pathogens themselves a distinct possibility.

ACKNOWLEDGMENTS

The authors thank Captain Jim Verber, Chief, Northeast Technical Services Unit, and Dr. Al Story, Chief, Gulf Coast Technical Services Unit, and their staffs for collection of samples. Appreciation is expressed to Aida Romero and Paul Poelma, Division of Microbiology, for serotyping of *Salmonella* isolates. Special thanks also are expressed to Dan Hunt, Shellfish Sanitation Branch, for helpful suggestions and for co-ordination of the activities of the field units.

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Erratum

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This paper appeared on pages 453-456 of the August (No. 8) issue of Volume 38 (1975) of the *Journal of Milk and Food Technology*. On page 454, beginning at the fifth sentence of the fourth paragraph it should read: Tubes of LST broth, inoculated with dilutions of the water or shellfish samples, were incubated at 35 ± 0.5 C, and at 24- and 48-h intervals, sterile applicator sticks were used to make transfers from gassing tubes of LST broth to tubes containing brilliant green lactose bile (BGLB) broth (Difco). Cultures producing gas in BGLB broth after 48 h of incubation at 35 C were classified as total coliforms. Fecal coliform confirmation was made by transferring all tubes of LST broth positive for gas after 24 to 48 h of incubation at 35 ± 0.5 C to EC medium.

Erratum

Behavior of Selected Food-Borne Pathogens in Raw Ground Beef

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This paper appeared on pages 449-452 of the August, 1975 (No. 8, Vol. 38) issue of the *Journal of Milk and Food Technology*. Table 2E. on page 450 should be entitled S. AUREUS rather than ENTEROCOCCI.